BLOCKING *XYLELLA FASTIDIOSA* TRANSMISSION

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ABSTRACT
This report describes work performed since October 2010. First, we summarize results on the transmission efficiency of 20 *Xylella fastidiosa* (*Xf)* mutants. Many mutants were deficient in vector transmission, but none resulted in complete abolishment of transmission. These results support previous results and indicate that *Xf* colonization of vectors is a complex process. Second, we present data on the interference of *Xf* vector transmission by disrupting cell-surface molecular interactions. Three groups of molecules were used to block transmission, i) lectins that would bind to receptors on the vector's foregut, ii) carbohydrates that would bind to adhesins on *Xf*'s surface, and iii) antibodies that bound to various *Xf* cell surface components. All these approaches resulted in significant blockage of vector transmission. These results not only provide confirmation of the nature of *Xf*-vector interactions, but also show that targeting specific interactions at the molecular level is a feasible approach to control disease spread.

LAYPERSON SUMMARY
Pierce's disease (PD) of grapevines and other *Xylella fastidiosa (Xf)* diseases are the outcome of complex interactions among plants, sharpshooters and bacterial pathogen. To move between hosts *Xf* must be transmitted by sharpshooter vectors, and although this represents an essential component of PD (i.e. no transmission no disease), it is also its most poorly studied aspect. Research on *Xf*-vector interactions will not only lead to novel approaches to limit pathogen transmission, but will also lead to new biological insights that may assist in the development of other control strategies. Here we report on the transmission efficiency of several *Xf* mutants and on efforts to block transmission. Results indicate that vector colonization is a complex process, as several mutants were impacted in vector transmission, but none resulted in zero transmission. One of the most important findings is that *Xf* transmission can be disrupted by blocking adhesins on the cell surface that interact with vectors, and receptors on the cuticle of vectors that cells use to initiate sharpshooter colonization. In other words, disrupting vector transmission of *Xf* is feasible and would lead to control strategies that are not aimed at killing vector or pathogen, but suppressing disease spread in a sustainable and environmentally sound manner.

INTRODUCTION
This report is divided into two sections: i) testing of several mutants for their transmissibility by vectors, and ii) work to show that transmission can be disrupted by the use of specific molecules that affect *Xylella fastidiosa (Xf)*-vector interactions. Although both sections address molecular aspects of *Xf* transmission, we provide a brief introduction to each, as the specific goals of these studies are different. Final results are pending for aspects of the study using mutants, while those are final for the transmission blocking work reported here; the latter is also followed by a detailed discussion. Sections of this report are redundant with previously submitted reports (unpublished) as there is an overlap in the reporting period (specially the work with mutants).

OBJECTIVES
1. Molecular characterization of the *Xf*-vector interface
2. Identification of new transmission-blocking chitin-binding proteins.

The long-term goal of this work is to develop tools to block *Xf* transmission by sharpshooter vectors, which we show here to be a feasible disease control strategy.

RESULTS AND DISCUSSION
In a previous report (2010 Proceedings) we report on initial findings part of this project. Essentially, we found that *Xf* has a functional chitin utilization machinery and is capable of utilization chitin as a carbon source. That work has been published and will not be discussed here (Killiny, N., Prado, S.S. and Almeida, R.P.P. 2010. Chitin utilization by the insect-transmitted

bacterium *Xylella fastidiosa*. Applied and Environmental Microbiology 76: 6134-6140). Here we focus on unpublished data. We have also submitted several libraries for RNA-Seq using the Illumina platform, those are currently pending in the UC Berkeley sequencing facility. The goal of those is to compare media that induce *Xf* transmission with those that do not, hopefully allowing us to identify novel transmission blocking candidate genes.

Transmission of *Xf* mutants

So far the role of very few genes on vector transmission of *Xf* is understood. To address this important knowledge gap we tested the transmission efficiency of 20 *Xf* mutants. We used a protocol developed by our group based on an artificial diet system that eliminates the need of using infected plants as a pathogen source to insects (Killiny and Almeida 2009b), which prohibits adequate analyses of vector-pathogen interactions because many mutants are deficient in plant colonization. We note that these mutants were generated by other research groups and kindly provided to us for this study.

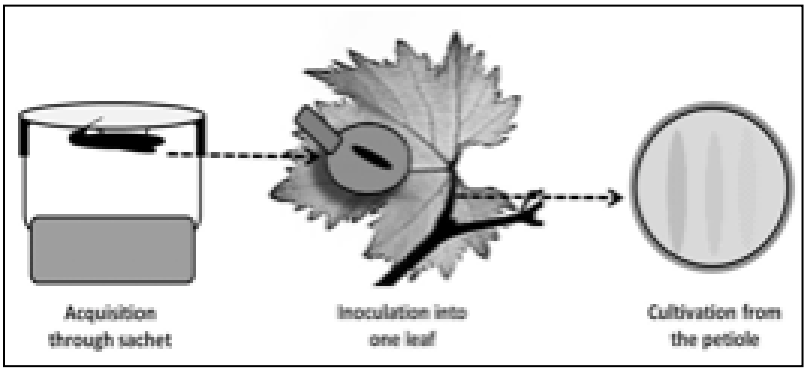

Figure 1. Experimental design to test *Xf* mutants for vector transmission.

Briefly, our experimental design was aimed at determining if strains were transmitted by vectors without considering plant-pathogen interactions (or minimizing its importance). Insects acquired cells from an artificial diet system for a few hours, then were transferred to a leaf on a healthy plant for inoculation (**Figure 1**). This leaf was tested two weeks after inoculation for *Xf*. We used plants for inoculation, but those were tested two weeks after insect access to reduce potential problems with mutants that cannot colonize plants. This approach allowed us to show that mutants that are deficient in plant colonization are transmissible by vectors. We were successful in obtaining positive inoculation events into artificial diets as well (Rashed et al. 2011), but we believe that vector probing behavior in plants and diets are reasonably different and could affect inoculation efficiency. Thus, the approach used looks at initial cell adhesion and colonization of vectors, followed by inoculation into plants, requiring short term survival/multiplication of cells, which is expected even from mutants that do not colonize grapevines. We have also quantified bacterial populations within sharpshooters with qPCR (Killiny and Almeida 2009a) after acquisition and one week later to gain insights on retention of the various strains. Data have been collected but are still being analyzed.

Because of the large number of mutants tested, different experiments (n=8) were performed and each one had its own wild type control (**Figure 2**). Transmission rates for the wild type ranged from 80 to 92%. The figure below summarizes our results. For comparative purposes, transmission rates of the wild type control in independent experiments were all normalized (to 100%), allowing a comparison among the various mutants. Statistical analyses need to be performed to compare the treatments tested, but for the purpose of discussion we may consider mutants with less than ~40% transmission as being deficient in this essential trait to *Xf*'s biology.

Mutants tested so far can be arbitrarily divided into four categories: adhesins, gene regulation, AT-1 transporters, and gum- and pectinase-deficient mutants. We will briefly discuss our results; a more complete discussion depends on the retention data we are yet to analyze. Among adhesins, all those tested affected transmission, except for *pilB*, which is part of type IV pilus involved in *Xf* twitching motility. Gum-deficient mutants were expected to be unable of biofilm formation within vectors, thus their lower transmission rates is not unexpected. We have previously shown that pectin degradation by the polygalacturonase (PglA) is required for vector transmission of *Xf* cells grown on the XFM-pectin medium used to induce vector transmissibility (Killiny and Almeida 2009). Thus, we did not expect much if any transmission of the *pglA* mutant. Regulatory genes involved in cell-cell signaling and within-cell signaling have been demonstrated to be deficient in transmission, results obtained here were similar to those with whole plants, for example, *rpfC* is affected in transmission, but less than *rpfF* (Newman et al. 2004, Chatterjee et al. 2008, 2010). Our results were within expectations, as much of *Xf*'s biology is affected by cell-cell signaling and within cell gene regulation. The AT-1 autotransporters tested were not affected as much, although the double mutant *xatA/B* was transmitted only 40% of the time. However, results with the complemented mutant *xatA* show that this protein has some role in transmission, as complementation revert transmission back to wild type level.

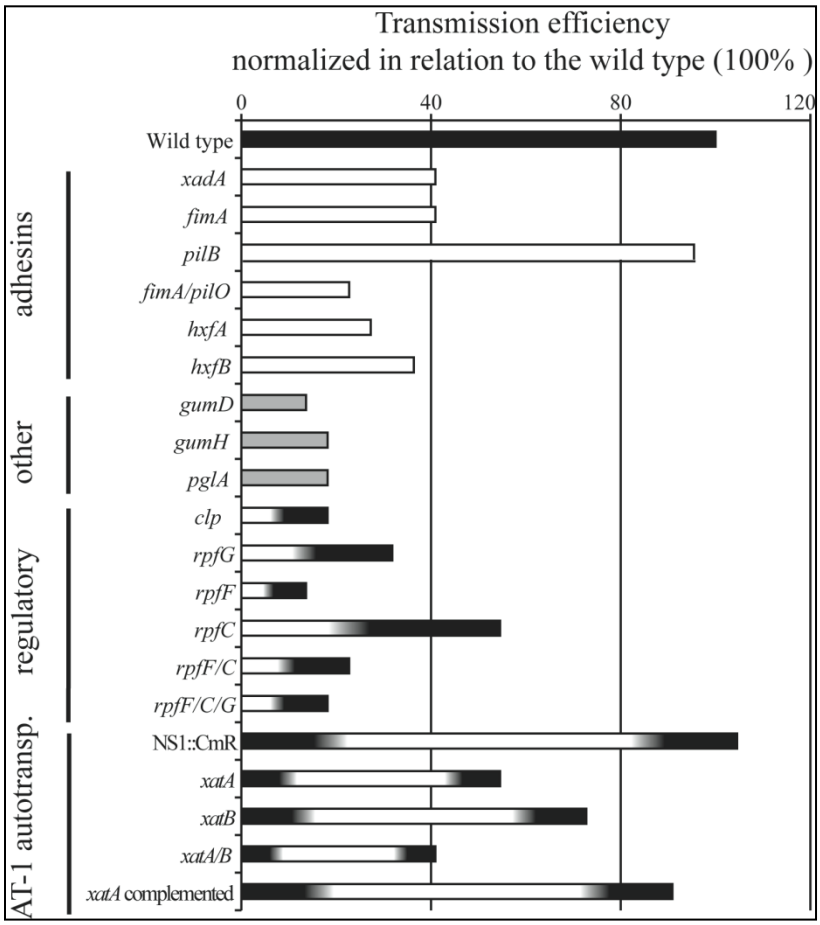

Figure 2. Transmission efficiency of *Xf* mutants in relation to the wild type.

A few general points need to be addressed. First, it is now evident that the *in vitro* system developed to study transmission independently of plants mimics experiments with plants adequately. For example, general trends obtained with transmission of the following mutants with plants were reproduced with the artificial diet system: *hxfA*, *hxfB*, *rpfF*, *rpfC*. This is important as a majority of mutants, such as *gumD* and *pilB*, cannot be tested using plants, but this protocol allows them to be studied in relation to vector transmission. Second, all mutants were transmissible to some degree in this study. This indicates that transmission is complex and not dependent on a single factor, such as only one adhesin. The fact that multiple factors are important for vector colonization is not surprising, but the results identified novel targets to block transmission. It should be mentioned that we believe that cells provided to insects are 'super sticky' and offered at higher densities than if acquisition occurred from plants, potentially inflating the proportion of transmitting individuals. In other words, transmission rates could be lower if plant-to-plant assays were performed.

Blocking of *Xf* transmission

The surface colonized by *Xf* in insects is not well characterized, but the nature of cell-vector interactions has been demonstrated to depend on carbohydrate-protein interactions (Killiny and Almeida 2009a). Cell surface proteins mediated *Xf* attachment to various substrates, including leafhopper foregut extracts and hindwings. In addition, adhesion decreased when certain carbohydrates were added to suspensions in adhesion assays, indicating that carbohydrate-binding proteins on the cell surface are substrate specific and that saturation of these proteins affects adhesion. Much like a biofilm, however, *Xf* colonization of vectors is likely a complex multi-step process, in which different factors are important for each step of biofilm formation, from initial cell adhesion to colony maturation (Almeida and Purcell 2006, Killiny and Almeida 2009a). Work on *Xf*-vector interactions has focused primarily on the early stages of biofilm formation, i.e. initial cell adhesion. Because of the protein-carbohydrate nature of the *Xf*-vector interface, it should be possible to disrupt transmission by saturating carbohydrate-binding proteins on cell surfaces; alternatively, lectins (carbohydrate-binding proteins) could be used to mask carbohydrate coated surfaces on the foregut of leafhoppers. In addition, *Xf*-derived antibodies could also reduce transmission if they were to bind to proteins on the cell surface that are involved in vector adhesion. We performed a series of experiments testing different approaches to block leafhopper transmission of *Xf* to plants. The experimental design used here was similar to tests with mutants, except that molecules (lectins, carbohydrates or antibodies) were all mixed together with cells in the diet. We will focus on results instead of methods.

Lectins affect vector survivorship. We detected a significant among-treatment variation in insect survivorship (log rank X_6^2 = 165.63, $P < 0.001$) with lentil lectin (LL) and peanut lectin (PL) being the two treatments inflicting the highest mortality rate across all three concentrations over time. Neither concentration nor treatment caused insect mortality within the first 48 hours of exposure. Following this verification of survivorship within experimental time-frame for transmission experiments (4 hours of exposure followed by at 12-hour IAP on healthy grapevines) we proceeded to the competition assay with lectins using the 0.1% concentration. This intermediate concentration was selected to reduce potential effects of higher concentrations on insect feeding, meanwhile assuring the presence of a minimum number of competitor molecules in suspensions for the competition experiments.

Lectins reduce transmission efficiency. The probability of a successful transmission event was significantly affected by lectin treatments (Wald X_6^2 = 44.84, $P < 0.001$, **Figure 3**). Compared to the sharpshooters that fed in the control diet, insects belonging to all lectin treatments showed a significant reduction in transmission rate; PL (Wald X_1^2 = 4.93, P == 0.026), LL (Wald X_1^2 = 5.15, P = 0.023), wheat germ agglutinin (WGA) (Wald X_1^2 = 23.67, $P < 0.001$), and concanavalin A (CoA) (Wald X_1^2 = 11.71, $P < 0.001$). *Xf* transmission rate by leafhoppers fed on diets with either BSA or OV was not different from that of the control (BSA: Wald X_1^2 = 0.01, P = 0.975; OV: Wald X_1^2 = 1.49, P = 0.221). Transmission rate of the insects treated with WGA was significantly lower than all other treatments except for CoA. Insects treated with CoA also had lower transmission rates than both OV and BSA treatments, but not compared to peanut and lentil lectins.

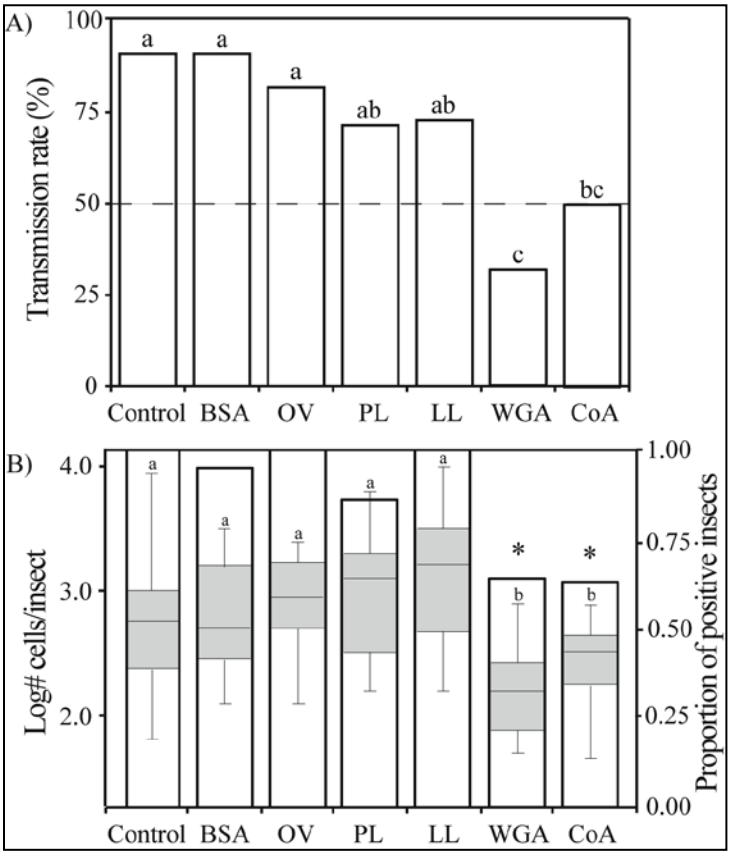

Figure 3. Lectins affect vector transmission of *Xylella fastidiosa* to plants. A) Lectins, BSA and OV were provided to insects through diet solutions containing *Xf* cells; control contained cells only. Treatments with different letters on bars indicate statistically different treatments. B) *Xf* retention and populations within vectors after the inoculation access period no plants. Y-axis on the right represents the proportion of insects that were positive for *Xf* (empty bars); WGA and CoA were the only treatments statistically different from the control (marked with asterisks). Y-axis on the left shows bacterial populations within insects as measure by quantitative PCR; boxes show the interquartile range including 50% of results, mid-horizontal line represents the median; different letters on top of error bars represent statistically significant different treatments. BSA – bovine serun albumine, OV – ovalbumine, PL – peanut lectin, LL – lentil lectin, WGA – wheat germ agglutinin and CoA – concanavalin A.

There was a significant variation in acquisition rates among the insects treated with different lectins (X_6^2 = 27.70, $P < 0.001$), which was measured by quantitative PCR after the 12-hour IAP on plants. The proportion of leafhoppers that acquired the

pathogen in WGA and CoA treatments was significantly lower than that of the control (Ps < 0.003). Acquisition rate for the other treatments was not statistically different from the control (Ps > 0.232). These differences are likely not associated with feeding deterrence due to the presence of lectins in the diet, as LL and PL were the lectins that were toxic to leafhoppers, not WGA or CoA. Similarly to the acquisition rates, the number of bacterial cells acquired and retained by the experimental insects varied significantly among-treatments (ANOVA: $F_{6, 126} = 7.71$, $P < 0.001$). Further pairwise comparisons indicated the number of cells recovered from the insect heads in WGA treatment was significantly lower than all other treatments (Tukey, Ps < 0.043), with the exception from CoA (Tukey, $P = 0.54$).

Carbohydrates with GlcNAc units affect transmission rate. Overall, carbohydrate groups with an acetyl amine group significantly reduced *Xf* transmission rate by vectors (**Figure 4**). For the insects treated with glucose, galactose or mannose, all lacking GlcNAc, the rate of successful transmission events was not statistically different from the control (binary logistic regression: Ps > 0.60). However, transmission rates varied significantly between vectors treated with the carbohydrates possessing GlcNAc and the control (Ps < 0.001). Transmission rates were not different among vectors treated with glucose, galactose, and mannose ($X_2^2 = 2.61$, $P = 0.12$). Likewise, no difference in transmission rates was detected among insects treated with GlcNAc, $GlcNAc_2$and $GlcNAc_3$ ($X_2^2 = 1.73$, $P = 0.42$).

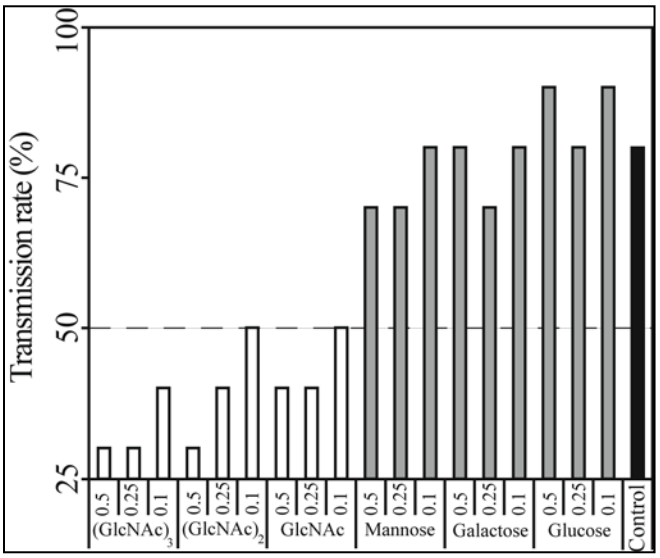

Figure 4. Carbohydrates with N-linked residues decrease *Xylella fastidiosa* vector transmission efficiency to plants. Three concentrations (0.1, 0.25 and 0.5M) were used for each saccharide tested in this competition assay, no differences among concentrations for any treatment were statistically significant; the control only included *Xf* cells. Carbohydrates with an *N*-acetylglucosamine (GlcNAc) residue were statistically equivalent to each other (white bars), but all were different from glucose, mannose and galactose, which also did not differ from each other (grey bars).

Antibody-mediated blocking of cell surface reduces transmission rate. There was a significant variation in transmission efficiencies among vectors treated with different antibodies (Wald $X_7^2 = 31.22$, $P < 0.001$; **Figure 5**). With the exception of PilA2 ($P = 0.068$) and PilC ($P = 0.111$), the two type IV pilus antibodies, all of the remaining antibody treatments significantly reduced transmission rate (Ps < 0.007). A separate Chi-square analysis revealed no variation in transmission rates of insects treated with antibodies against gum, whole cell, and the three different afimbrial adhesins ($X_4^2 = 4.53$, $P = 0.34$) .

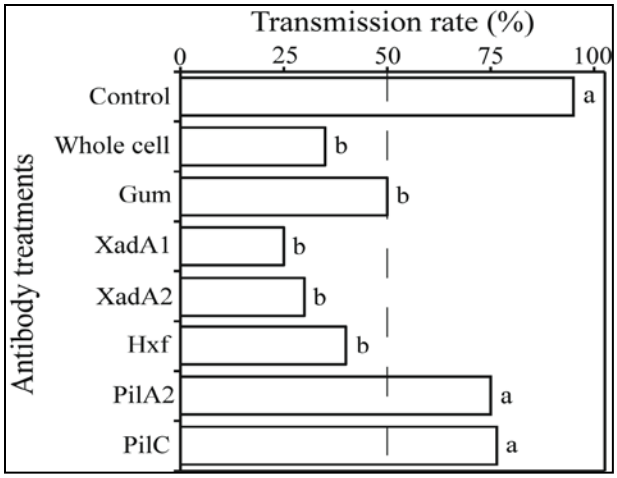

Figure 5. Cell surface antibodies reduce *Xylella fastidiosa* transmission to plants. Various antibodies were added to the diet solution containing *Xf*; different letters on bars indicate statistically different treatments. Antibodies prepared against whole cell, afimbrial adhesins (XadA1, XadA2, Hxf) and gum (EPS) all reduced transmission, while those against type IV pilus proteins did not.

Altogether, we tested three distinct approaches to determine if *Xf* transmission efficiency could be reduced by competitor molecules, based on the fact that carbohydrate-protein interactions appear to be essential in the pathogen-vector interface. Competition for receptors on the vector's foregut was performed using lectins with affinity for GlcNAc, which resulted in reduced transmission rates. Likewise, saturation of carbohydrate-binding proteins on the cell surface with GlcNAc-containing sugars reduced the rate of successful transmission events. Lastly, the use of various antibodies had a similar effect. Thus, various approaches significantly reduced vector transmission of *Xf*; however, no treatment completely abolished transmission. Similar results were observed with circulative viruses. Due to the biological complexity of biofilm establishment and development, it was expected that no individual treatment would result in total blockage of transmission.

Although two of the tested lectins, PL and LL, reduced insect survivorship down the road, this affect did not appear in the first 48 hours of the assay. However, it is possible that insect behavior was affected after acquisition of these molecules, resulting in the small but significant decrease in *Xf* transmission for those two treatments (PL and LL) in relation to the controls, as they were not expected to have much affinity for carbohydrates on the foregut surface. This possibility is highlighted by the fact that the number of cells in the foregut of vectors after the transmission experiment was not different from that of the control nor was the proportion of infective insects. In other words, vector colonization did not appear to be affected by PL or LL, and we interpret the small differences in transmission as being a consequence of changes in vector behavior. CoA and WGA, on the other hand, were lectins that resulted in significant decreases in bacterial populations in insects, a smaller proportion of positive individuals, and a decrease in transmission efficiency; neither was detrimental to insect survivorship. Although the number of vector colonization events is unknown when the artificial diet system employed here is used for pathogen acquisition, we expect that multiple colonization events occurred as insects were exposed to high densities of adhesive cells. Moreover, acquisition through artificial system occurs in a less turbulent environment compared to acquisition from plants. We interpret the results as a significant reduction in *Xf* initial adhesion to vectors, leading to fewer colonization events, as observed by a smaller proportion of positive insects. However, the smaller population in insects would be due to the fact that fewer cells were able to colonize the foregut, not an effect on those that successfully colonized that surface.

Pathogen adhesion to carbohydrates functioning as host receptors for attachment are common. For example, a streptococcal adhesin that recognizes and binds to a galactosyl-α 1-4-galactose-containing glycoconjugates host receptor has been described recently. Likewise, *Vibrio cholerae* surface proteins mediate cell attachment to chitin and the surface of copepods. In addition, the presence of chitin may induce the expression of proteins with strong affinity for chitin and chito-oligomers. Thus, lectin-carbohydrate interactions mediate the binding of several pathogens to their hosts; interfering with these interactions may lead to development of novel disease control strategies. *Xf* colonizes carbohydrate-rich surfaces in both plants and insects; therefore, it was not surprising that it adheres to such molecules. We have previously shown that cell adhesion to insect hindwings is reduced with the addition of GlcNAc, indicating that *Xf* carbohydrate-binding proteins are saturated by the presence of this monosaccharide. These *in vitro* findings were confirmed and successfully applied in multiple biological assays in the present study. GlcNAc-containing sugars significantly reduced transmission efficiency to plants, whereas mannose, galactose and glucose had no effect. The fact that the carbohydrates containing an acetyl amino group appeared to saturate adhesins on the cell surface indicates that the cuticular surface of leafhopper vectors is rich in

these molecules. The recent finding that *Xf* has a functional chitinase is further indication that GlcNAc is important for vector colonization.

Although assays based on competitor molecules as those with lectins and carbohydrates provide general information about the nature of *Xf*-vector interactions, they do not address the role of specific components of the cell's surface that may be required for vector colonization. Targeting specific genes via knockout mutants is one approach to identify factors required for transmission, such as work performed with the afimbrial adhesins HxfA and HxfB. An alternative approach is to use antibodies against the same targets, where masking of Hxfs, for example, would reduce transmission if they were involved in that process. Indeed, we found that antibodies generated against whole cells, gum, and afimbrial adhesins (XadA1, XadA2 and Hxf) all reduced vector transmission of *Xf* to plants. HxfA and HxfB have been shown to be involved in transmission, but no data are available for the other adhesins or gum. We propose that XadA1 and XadA2 are also involved in vector transmission. It is possible that gum is involved in initial attachment to vectors, it is also plausible that gum is evenly distributed on the cell surface and that these antibodies may have resulted in inadvertent masking of adhesins actually involved in this vector early colonization. This interpretation does not mean that gum is not required for successful vector colonization, but that it may not be as important for initial adhesion. Lastly, PilC is involved in type IV pilus assembly and appears to be bound to the cell membrane, therefore it does not appear to be located on the cell surface and should not affect cell attachment. PilA2 is a rod-forming unit and was observed both in cell membranes and outside cells, presumably on pili. The fact that the PilA2 antibody did not reduce transmission efficiency is suggestive that type IV pili are not involved in the initial colonization of vectors. Twitching motility is mediated by type IV pili in *Xf*, and is involved in cell movement within plants. As mentioned previously, because the experimental design may result in higher transmission rates than if plant-to-plant experiments were used, we would also expect that the efficiency of blocking molecules would be higher (i.e. lower transmission) if conditions for vector colonization had not been artificially optimized.

CONCLUSIONS

We report on the role of 20 *Xf* genes in vector transmission using an artificial diet system. Many mutants were impacted on transmission, although none was not transmissible. This may be a consequence of the fact that cells used were highly adhesive and provided in large number to insect vectors. Overall, results suggest that transmission is a complex process mediated by various factors, and that the experimental approach allows for meaningful comparisons in relation to the wild type. Proof-of-concept experiments were performed to determine if it was possible to disrupt *Xf* transmission by blocking pathogen-vector interactions. Results showed that carbohydrates can saturate adhesins on the cell surface so attachment to vectors is reduced, and that lectins can coat receptors on the vector's foregut so that cell can not bind to and colonize sharpshooters. These data show that blocking transmission is a feasible concept and represents a novel mechanism to suppress PD spread in vineyards. Current efforts are aimed at testing *Xf*-derived targets (i.e. adhesins that bind to vectors) because those would be ideal for field trials, as they are specific to this system and evolved to bind to sharpshooters.

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