Blocking the Transmission of a Noncirculative Vector-Borne Plant Pathogenic Bacterium

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The successful control of insect-borne plant pathogens is often difficult to achieve due to the ecologically complex interactions among pathogens, vectors, and host plants. Disease management often relies on pesticides and other approaches that have limited long-term sustainability. To add a new tool to control vector-borne diseases, we attempted to block the transmission of a bacterial insect-transmitted pathogen, the bacterium *Xylella fastidiosa*, by disrupting bacteria–insect vector interactions. *X. fastidiosa* is known to attach to and colonize the cuticular surface of the mouthparts of vectors; a set of recombinant peptides was generated and the chemical affinities of these peptides to chitin and related carbohydrates was assayed in vitro. Two candidates, the *X. fastidiosa* hypothetical protein PD1764 and an N-terminal region of the hemagglutinin-like protein B (HxfB) showed affinity for these substrates. These proteins were provided to vectors via an artificial diet system in which insects acquire *X. fastidiosa*, followed by an inoculation access period on plants under greenhouse conditions. Both PD1764 and HxfAD1-3 significantly blocked transmission. Furthermore, bacterial populations within insects over a 10-day period demonstrated that these peptides inhibited cell adhesion to vectors but not bacterial multiplication, indicating that the mode of action of these peptides is restricted to limiting cell adhesion to insects, likely via competition for adhesion sites. These results open a new venue in the search for sustainable disease-control strategies that are pathogen specific and may have limited nontarget effects.

Vector-borne plant pathogens are among the most important threats to agriculture. Despite their economic importance, management of such diseases relies heavily on pesticide applications, primarily due to a poor understanding of their biology and how these pathogens are transmitted from plant to plant. Insect-borne pathogens have a complex lifestyle requiring the successive colonization of or interactions with two independent hosts, the plant and the vector. Numerous strategies developed to control these pathogens have independently targeted the insect or plant hosts, but the impact of these diseases continues to be significant. Alternatively, more selective approaches must be developed. A novel strategy that has been recently pursued, primarily with viral pathogens, is the disruption of vector–pathogen molecular

interactions (Whitfield and Rotenberg 2015). Although the specific approaches that have been used to disrupt intricate vector–virus interactions vary, the general concept is based on providing insects with an excess of virus-encoded proteins or peptides required for interactions so that virions are outcompeted and cannot bind to putative receptors; in other words, insect receptors are masked so that virions are not retained (e.g., Guoying et al. 1999; Montero-Astúa et al. 2014). There is one example that used a proof-of-concept approach to demonstrate that transmission of a plant-pathogenic bacterium by an insect vector could be disrupted (Killiny et al. 2012). Here, we expand on that work by demonstrating that a bacterial pathogen-derived protein efficiently blocks vector transmission to host plants.

Xylella fastidiosa, the etiological agent of Pierce’s disease of grapevines, is a xylem-limited bacterium that causes disease in many other crops, including almond, citrus, coffee, and plum (Hill and Purcell 1997). Early epidemiological data indicated the involvement of insects in *X. fastidiosa* spread, leading to the identification of xylem sap-sucking sharpshooter leafhoppers (Hemiptera, Cicadellidae) and spittlebugs (Hemiptera, Aphrophoridae) as vectors (Severin 1949, 1950). Unlike other important vector-borne plant-pathogenic bacteria (phytoplasmas, spiroplasmas, liberibacters), *X. fastidiosa* colonizes the cuticular foregut of vectors in a noncirculative propagative manner (Almeida et al. 2005). In other words, the bacterium forms a biofilm on the cuticular surface of the foregut of vectors and is persistent unless the insect molts (Almeida and Purcell 2003; Purcell and Finlay 1979). Within the foregut, *X. fastidiosa* specifically colonizes the precibarium and cibarium, which are distal in relation to the stylets (Almeida and Purcell 2006; Brlansky et al. 1983; Purcell et al. 1979). Research on *X. fastidiosa*–vector interactions has focused on only one model system so far (a subspecies *fastidiosa* strain that causes Pierce’s disease of grapevine and its efficient vector *Graphocephala atropunctata*, the blue-green sharpshooter). It is expected that knowledge from this system would be applicable to all other *X. fastidiosa*–vector combinations, as there is no evidence of vector–pathogen specificity and all insect vectors are considered to be potential vectors of different *X. fastidiosa* genotypes (Almeida et al. 2005).

A mechanistic view of *X. fastidiosa*–vector interactions is being developed by focusing on the bacterium rather than the insect host. The chemical composition of the foregut regions colonized by *X. fastidiosa* remains unknown, but it has been shown that chitin and *N*-acetylglucosamine (GlnNAc) serve as proxies for *X. fastidiosa*–vector interaction (Killiny and Almeida 2009a). *Xylella fastidiosa* has an intricate lifestyle requiring adaptation to its two hosts, the plant and the insect vector. To achieve this particular life cycle, this bacterium has a diffusible signaling factor (DSF)-based cell-to-cell signaling system, which

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regulates both plant and insect colonization. Within plants, at low cell densities, *X. fastidiosa* spreads through the xylem network of its host while repressing the expression of adhesins; at high cell densities, the expression of fimbrial or afimbrial adhesins is up regulated while genes associated with within-plant movement are repressed, effectively limiting plant colonization but increasing cell-surface stickiness (Newman et al. 2004). These adhesins, however, are required for successful attachment to insect hosts and are up regulated when DSF accumulates in an environment with plant polysaccharides (Killiny and Almeida 2009b). Mutant strains of afimbrial hemagglutinin-like proteins (HxfA and HxfB) are deficient in both cell adhesion in vitro and to insect in vivo (Killiny and Almeida 2009a); while chaperone-usher pili (*fimA*, pilus rod-forming protein [Zaini et al. 2015]) are also important for vector colonization (Killiny and Almeida 2014). Although *X. fastidiosa* has a functional chitinase (ChiA), the role of this enzyme on insect colonization remains to be determined (Killiny et al. 2010). Similarly, other studies have shown that mutant strains with disrupted cell-to-cell signaling communication are affected in biofilm formation in vitro, with equivalent phenotypes in insect vectors (Almeida et al. 2012; Chatterjee et al. 2008b). In other words, *X. fastidiosa* vector colonization involves a trade-off between colonization of plant and insect hosts (Chatterjee et al. 2008a). Altogether, a model for *X. fastidiosa* early colonization stages of vectors is emerging, in which afimbrial and fimbrial adhesins, upregulated when bacterial populations reach high cell densities within plants, allow cells to bind to the cuticle of leafhoppers and initiate biofilm formation (Retchless et al. 2014). It should, however, be mentioned that *X. fastidiosa* is persistent in vectors and late colonization processes have been poorly studied, despite the fact that available evidence indicates that different proteins are important for the various stages of biofilm formation and insect colonization (Killiny and Almeida 2014).

At the cell level, adhesion to and colonization of vectors is thought to be a relatively rare event, with many more cells ingested by insects (Rashed et al. 2011) than the number of observable colonization events on the foregut (Almeida and Purcell 2006). This discrepancy is hypothesized to be a consequence of the dynamic nature of fluid flow through the mouthparts, which, among other things, has been estimated to occur at speeds higher than 8 cm per second (Purcell et al. 1979). Biochemical assays have shown that *X. fastidiosa* surface proteins bind to chitin and other polysaccharides; furthermore, competition assays demonstrated that cell adhesion to vector wings, which are expected to be chemically similar to the cuticular surface of the foregut, decreased with increasing concentrations of GlnNAc (Killiny and Almeida 2009a). These interactions were recently exploited with the goal of blocking *X. fastidiosa* transmission by vectors (Killiny et al. 2012). Lectins and carbohydrates were used to saturate putative receptors on the cuticular surface of the foregut of insects or on the surface of *X. fastidiosa*, respectively; in both cases, vector transmission to plants was reduced, suggesting that disrupting vector-pathogen molecular interactions could lead to a new strategy to limit disease prevalence.

While such interactions may yield highly specific transmission-blocking management strategies, their efficacy must first be determined prior to any nontarget testing. Therefore, we proceeded with experiments aimed at determining if *X. fastidiosa* chitin-binding proteins could reduce vector transmission of this bacterium. Ideally these proteins would be highly adapted to binding to chitin and the cuticle of vectors, consequently having high affinity and specificity for these substrates, reducing potential indirect impacts of their large-scale use in agricultural settings. We used a biochemical approach based on the affinity of *X. fastidiosa* proteins for chitin and other insect-related polysaccharides to identify a protein (PD1764) that binds to

GlnNAc. In addition to the approach described above, we pursued a targeted alternative based on the assumption that *X. fastidiosa* proteins previously identified as important for insect colonization could also block transmission to plants. The results obtained show that *X. fastidiosa* can be used as a model to study the adhesion of bacterial plant pathogens transmitted by insect vectors and that blocking pathogen-vector interactions is a potential tool to limit the spread of economically important vector-borne diseases.

RESULTS

Identification of PD1764, a chitin-binding protein.

We used chromatography columns with colloidal chitin to identify *X. fastidiosa* chitin-binding proteins. Several proteins, ranging in molecular mass from 16 to 95 kDa, were identified in cells grown on XFM medium supplemented with colloidal chitin (Killiny et al. 2010), a medium known to induce *X. fastidiosa* adhesion. As a control, XFM medium alone was used and did not appear to induce the expression of chitin-binding proteins (Supplementary Fig. S1). A protein of approximately 42 kDa was analyzed by mass spectrometry and was identified as PD1764 based on the genome of the *X. fastidiosa* Temecula strain. PD1764 and another abundant protein (major outer membrane protein B) obtained were identified, but only PD1764 was selected for further assays, due to its amino acid (aa) sequence. In silico analyses predicted that the molecular mass of PD1764 was 43.2 kDa (392 aa), with an isoelectric point of 6.16; PD1764 had a signal peptide of 30 aa. PD1764 is annotated as a hypothetical protein present in several genomes of *X. fastidiosa*, a BlastP search showed it to be conserved (>98% sequence similarity) within the species. PD1764 protein was, on average, 60% identical to the *Xanthomonas* spp. and *Stenotrophomonas* spp. orthologs (ranging from 62% for *Xanthomonas vesicatoria* to 59% for *Stenotrophomonas maltophilia*). Identity to other bacteria was less than 40% (*Rhodonobacter* spp.) and 30% (*Pseudomonas* spp.); in fact, most of the sequence similarity found for PD1764 corresponded to an approximately 40-aa region identified as a peptidoglycan-binding motif named LysM (Pfam PF01476).

The PD1764 LysM domain

is responsible for chitin-binding activity.

In an attempt to identify *X. fastidiosa* chitin-binding proteins, we generated a series of recombinant peptides (Fig. 1), two expressing different domains of the hemagglutinin-like protein HxfB (HxfAD1-3 [1,168 aa] and HxfAD4 [1,026 aa] [Voegel et al. 2010]), one for the full-length chaperone-usher pilus subunit FimA (182 aa) and one for the *X. fastidiosa* chitinase (ChiA, 352 aa [Killiny et al. 2010]). These proteins were included in the study because of the availability of data on their role in *X. fastidiosa*-vector interactions (Killiny and Almeida 2009a, 2014, Killiny et al. 2010). Finally, two different constructs for PD1764, including or not the LysM motif (respectively named PD1764 [361 aa] and PD1764ΔLysM [304 aa]), were generated. Binding of those six *X. fastidiosa* candidates to four polysaccharides (chitin, colloidal chitin, chitosan, and cellulose) was tested (Fig. 2A). Binding affinity differed significantly among *X. fastidiosa* proteins and polysaccharides ($n = 6$; peptides: $F_{6,139} = 335.24$, $P < 0.0001$; polysaccharides: $F_{3,139} = 207.33$, $P < 0.0001$; peptide-polysaccharide interaction: $F_{18,139} = 71.37$, $P < 0.0001$). PD1764 and HxfAD1-3 interacted with chitin and colloidal chitin, resulting in the lowest unbound protein concentrations remaining. Binding affinity of PD1764 for chitin, colloidal chitin, and chitosan was significantly greater than affinities of bovine serum albumin (BSA), FimA, ChiA, HxfAD4, and PD1764ΔLysM (contrast:

$Z = -33.72$, $P < 0.0001$). Likewise, binding affinity of HxfAD1-3 for the three substrates was significantly greater than affinities of BSA, FimA, ChiA, HxfAD4, and PD1764 Δ LysM (contrast: $Z = -34.20$, $P < 0.0001$). While ChiA and HxfAD4 also significantly

interacted with chitin, colloidal chitin, and chitosan, their affinities to these substrates were relatively minor. No significant binding was observed to any of the polysaccharides for PD1764 Δ LysM, FimA, or BSA.

Fig. 1. Diagram of recombinant peptide constructs used in this study based on **A**, PD1764, **B**, HxfB, or **C**, additional *Xylella fastidiosa* proteins involved in transmission by vectors (ChiA or FimA). Numbers indicate amino acid positions on each protein sequence. Black diamonds represent the histidine tag fused to each construct (this tag is removed before any experiments performed in this study). The dashed rectangle represents the signal peptide identified between amino acid 1 and 30 in PD1764 and excluded in each PD1764-derived construct.

Fig. 2. *Xylella fastidiosa* proteins involved in vector colonization have different affinities to polysaccharides. **A**, Mean $\pm$ standard error of the mean concentration ($\mu\text{g/ml}$) of *X. fastidiosa* proteins remaining unbound in solution (or binding affinity) to four different polysaccharides or **B**, to chitin measured over a 4-h period. In **A**, asterisks above bars indicate a significant difference in binding affinity compared with bovine serum albumin (BSA) within the same substrate treatment. Supplementary Table S2 provides results of pair-wise comparisons for all treatments. In **B**, lower-case letters in the legend indicate results from multiple comparisons using Tukey's honestly significant difference test; proteins with the same letter were not significantly different in their binding affinity.

To determine the dynamics of these interactions, a similar assay was performed over time with chitin as a substrate (Fig. 2B). PD1764 and HxfAD1-3 quickly bound to chitin and were significantly different from the other proteins in their binding affinity over time ($n = 9$, $F_{6, 54.84} = 97.58$, $P < 0.0001$). No significant interactions were detected for the other proteins. The equilibrium dissociation constants (K_d) and binding capacities (B_{max}) obtained for chitin-binding by PD1764 and HxfAD1-3 were K_d of 1.34 and 3.24 μM , and B_{max} of 3.67 and 5.44 $\mu\text{mol/g}$, respectively. These values are in the same range as K_d and B_{max} values reported for other carbohydrate-binding proteins and isolated carbohydrate-binding domains (Goldstein et al. 1993; Li et al. 2003; Vaaje-Kolstad et al. 2005).

Bacterial populations and recombinant peptide concentration in artificial diet impact vector transmission rate.

Because our goal was to test if chitin-binding proteins would outcompete *X. fastidiosa* and not permit cells to bind to retention

sites on the cuticular surface of the mouthparts of vectors, two assays were performed to optimize experimental conditions. First, we determined the number of cells per milliliter to be delivered to insects prior to transmission rates reaching an asymptote, indicating excess numbers of bacterial cells in the diet that would not simulate natural conditions, in which it has been proposed that very few cells actually colonize vectors (Retchless et al. 2014). *X. fastidiosa* concentrations used in this test were ranging from 10^2 to 10^9 cells per milliliter and were either grown into a medium capable of inducing *X. fastidiosa* transmission (XFM-Gal) and another without the inducer (XFM) as a control. The relationship between transmission rate and number of cells in diets was not linear (Fig. 3A); 10^6 cells per milliliter was the first concentration of cells with detectable transmission (5/34); high transmission rates (>75%) were detected for the three highest populations used (10^8 , 5×10^8 , and 10^9 cells per milliliter). On the other hand, when *X. fastidiosa* cells were grown on control medium in the absence of an inducer (XFM), low transmission rates were detected only in the two highest concentrations used (1/19 and 3/21, respectively, for 5×10^8 and 10^9 cells per milliliter). Based on criteria mentioned above, approximately 10^8 cells per milliliter grown in XFM-Gal were used for the subsequent experiments.

To determine the concentration of transmission-blocking molecules suitable in experimental assays, we focused our attention on two parameters, the presence of an acute toxicity effect to insect vectors that acquired those molecules and the effect of various concentrations on *X. fastidiosa* transmission. We selected two candidates for this assay, PD1764 and ChiA, based on their differential behaviors in our binding assays. Insects were allowed to feed for 4 h on an artificial diet system containing 10^8 cells per milliliter and various concentrations of PD1764 and ChiA, ranging from 10 to 250 μM . Then insects were either i) transferred to basil plants to test their mortality rate over 4 days or ii) transferred to healthy grapevines for a 24-h inoculation access period (Fig. 3B). No difference in insect survivorship was evident for either protein at any concentration. No clear difference in insect behavior on plants (location, flying, feeding) was observed. The same five concentrations were used to determine their effect on *X. fastidiosa* transmission. In the case of ChiA, no significant decrease of transmission rate was obtained with 10 and 25 μM of proteins. A slight but significant reduction of transmission ($P < 0.05$) was obtained with the three higher concentrations used (50, 100, and 250 μM). However, no dose-dependent reductions were observed between these concentrations; the maximal reduction of transmission (22% decrease) was obtained with 100 μM of ChiA (53% transmission versus 75% for the control). More interestingly, PD1764 showed a complete disruption of *X. fastidiosa* transmission by its vectors when 100 μM of proteins was used ($P < 0.001$). No significant reduction of transmission was obtained with the lower concentrations tested.

PD1764 and HxfAD1-3 reduced *X. fastidiosa* vector transmission efficiency.

The effects of six candidate peptides on vector transmission of *X. fastidiosa* were tested. A control without any blocking agent was also included to normalize the results along with an additional BSA control to test the effect of nonspecific proteins. No change in transmission relative to control was detected when 100 μM of BSA, FimA, HxfAD-4 domain, and PD1764 ΔLysM domain were added into the diet medium (Fig. 4A). However, PD1764 and HxfAD1-3 had a significant effect on *X. fastidiosa* transmission. While ChiA also significantly inhibited transmission, the rate reduction was smaller compared with PD1764 and HxfAD1-3.

Fig. 3. A, Vector transmission efficiency with *Xylella fastidiosa* acquisition via artificial diets, increments in the number of live bacterial cells led to higher transmission of the pathogen to plants. Numbers in parentheses indicate the total number of individuals that fed on diets with cells originating from XFM-Gal medium (squares) to the number of individuals that fed on XFM medium (triangles). **B,** Effect of transmission-blocking peptide concentration on transmission efficiency.

Transmission-blocking proteins reduced the size of *X. fastidiosa* populations retained by vectors.

The size of *X. fastidiosa* populations found in vectors differed significantly among the recombinant peptides tested ($n = 5$, $F_{3, 48} = 41.13$, $P < 0.0001$) and among days since acquisition ($n = 5$, $F_{2, 48} = 102.27$, $P < 0.0001$). Bacterial populations were consistently below the detection threshold at 1 day post-acquisition regardless of peptide tested. Interestingly, *X. fastidiosa* cells were not detected when vectors were provided PD1764 at 100 μ M even at 10 days post-acquisition (Fig. 4B). Bacterial population growth was not particularly affected with HxfAD1-3 (100 μ M) and PD1764 50- μ M treatments. We interpret these results as evidence that PD1764 blocked the adhesion of *X. fastidiosa* cells to vectors only at 100 μ M, while at 50 μ M it may have had an impact on reducing the number of cells initially adhering to the cuticle of vectors (which was not possible to estimate, as those were below the detection threshold). Similarly, these results suggest that the role of PD1764 and HxfAD1-3 is limited to early stages of cell adhesion to vectors, not impacting cell multiplication or other physiological processes.

PD1764 has no essential role on *X. fastidiosa* transmission.

To determine if PD1764 played a role in vivo during vector transmission, we generated a *pd1764* knockout strain (discussed below). This mutant strain was tested for insect transmission, following a standard protocol with cell acquisition from artificial diets, followed by insect inoculation into susceptible plants. No significant difference (χ^2 test; $df = 1$, $\chi^2 = 0.52$, $P = 0.47$) was found for its transmission rate (10 of 23 positive plants) in comparison with the wild-type control (Temecula strain; 18 of 26 positive plants).

DISCUSSION

The management of vector-transmitted plant diseases is often challenging, due to the ecological complexity of these systems, and frequently relies heavily on pesticides. We pursued the possibility of blocking *X. fastidiosa* vector transmission as an alternative approach to limit the spread of this pathogen by exploiting the fact that this bacterium uses surface proteins to adhere to and colonize the cuticular foregut of insect vectors (Killiny and Almeida 2009a, 2014). A previous study demonstrated that lectins (i.e., wheat germ agglutinin) reduced vector transmission of *X. fastidiosa* to plants when offered to insects

together with bacterial cells (Killiny et al. 2012). Likewise, the use of *X. fastidiosa*-derived peptides, originating from proteins involved in insect colonization, could yield highly specific technologies to lower transmission rates, if competition for adhesion sites on the foregut of vectors was similarly affected as with lectins. Following this rationale, we proceeded to execute a series of experiments with previously identified transmission-blocking candidate peptides as well as with the identification of a novel *X. fastidiosa* protein with chitin-binding activity.

Four proteins, namely PD1764, HxfB, ChiA, and FimA, were targeted in this study. One, PD1764, was identified in this study using a pipeline targeting *X. fastidiosa* chitin-binding proteins, whereas the other three, ChiA, FimA, and HxfB, have been previously studied in relation to *X. fastidiosa* transmission by its insect vector. The biochemical assays performed confirmed expectations based on previous work or homology-based functional expectations. ChiA and FimA had no affinity for chitin or chitin-like molecules; ChiA is a functional chitinase potentially involved in vector transmission that lacks a chitin-binding domain (Killiny et al. 2010), whereas FimA is the main shaft-forming protein in this chaperone-usher type pilus, which is complemented by an adhesin at its tip, recognizing host molecular patterns (Zaini et al. 2015). So, even if a *X. fastidiosa* *fimA* mutant strain is deficient in vector colonization (Killiny and Almeida 2014), it is very unlikely that FimA could directly interact with chitin due to its spatial organization during *X. fastidiosa* pilus formation. On the other hand, PD1764 and HxfB showed some chitin-binding properties, potentially interesting for our search for *X. fastidiosa* transmission blocking derivatives. The function of PD1764 in *X. fastidiosa* biology remains unknown at this moment, it has a LysM domain involved in chitin-binding, but a knockout strain was not affected in vector transmission, while HxfB (hemagglutinin-like protein) has been shown to be important for efficient cell early attachment to insect vectors (Killiny and Almeida 2009a). HxfB and PD1764 had high yet different affinity profiles. HxfB bound to chitin, chitosan, and colloidal chitin with similar rates, while PD1764 had less affinity toward chitosan. Neither bound to cellulose.

It is important to note that two constructs were used for both proteins. HxfB has over 3,000 aa; therefore, the domains previously identified in this protein were split into two peptides, namely HxfAD1-3 and HxfAD4 (Voegel et al. 2010). Chitin-binding activity was only observed with HxfAD1-3. Interestingly,

Fig. 4. *Xylella fastidiosa* vector transmission and colonization were affected by chitin-binding proteins. **A**, Vector transmission efficiency to grapevines following acquisition of *X. fastidiosa* and various peptides and bovine serum albumin (BSA) from artificial diets (* and *** indicate P values < 0.05 and < 0.001 , respectively, in relation to the control treatment). **B**, Mean $\pm$ standard error of the mean populations of *X. fastidiosa* in insect vector heads at days 1, 3 (gray bars), and 10 (white bars). Treatments with the same letter were not significantly different according to Tukey's honestly significant difference ($\alpha = 0.05$). All populations were below the detection threshold at 1 day after acquisition (n.d. = not detectable).

the HxfB protein possesses two RGD (Arg-Gly-Asp) cell attachment motifs known to allow cell adhesion with integrin receptors (Ruoslahti 1996). The first RGD motif is located on HxfAD4, which had no affinity for chitin. It is, therefore, unlikely that such a motif plays a role in vector transmission, helping the bacteria to bind to host cells. The second RGD motif is located in the C-terminal part of the HxfB protein, which is lost after protein maturation, reinforcing the previous hypothesis. HxfAD1-3 had no RGD motifs on its sequence, but in silico analysis revealed the presence of a hemagglutination activity domain (HAD) (Voegel et al. 2010). This domain is located between aa 87 and 207 of the AD1-3 domain. In *Bordetella pertussis*, HAD sequences are shared by secreted or surface-located proteins and are known to act as major virulence attachment factors. They generally function as adhesins that allow adhesion to cells of the respiratory epithelium (Inatsuka et al. 2005). This HAD domain located on the HxfB could be potentially involved in some carbohydrate-binding properties, and studies are actually ongoing to explore that possibility. In the case of PD1764, the identification of a LysM domain led us to generate a full-length PD1764 peptide and a version without LysM, due to its known conserved function as a GlcNAc-binding domain (Visweswaran et al. 2012). As predicted, loss of LysM led to loss in PD1764 chitin-binding activity; recombinant expression of LysM alone was attempted but not successful. PD1764 is annotated as a hypothetical protein that is present in other *X. fastidiosa* genomes. LysM (for lysin motif, Pfam PF01476) domains are well-known for their role in plant immune responses, in which they may serve as receptors for the recognition of common microbe-associated molecular patterns (MAMPs) (Gust et al. 2012). Interestingly, MAMPs recognized by LysM are generally composed of GlcNAc-containing molecules, such as chitin on the surface of pathogenic fungi (Ohnuma et al. 2008). More recently, it was found that the LysM motif of bacteria *Lactococcus lactis* was able to bind to fungal chitin cell-wall material (Visweswaran et al. 2012). Because PD1764 has no role in *X. fastidiosa*-vector interactions (*pd1764* mutant strain was not impacted on vector transmission), we propose that the LysM domain anchors this protein on the peptidoglycan layer and that other domains may be exposed on the outer membrane (we found no evidence of transmembrane domains [data not shown]).

Although in vitro assays do not accurately represent complex interactions between *X. fastidiosa* and vectors, it has been demonstrated that *X. fastidiosa* attachment to chitin as well as insect hind wings serve as proxies to bacterial adhesion to insect foreguts (Baccari et al. 2014; Killiny and Almeida 2014). Results obtained in this study corroborate these earlier findings in two ways. First, peptides with strong affinity for chitin also yielded significantly lower vector transmission of *X. fastidiosa* to plants; PD1764 as well as HxfAD1-3 reduced *X. fastidiosa* transmission efficiency. Interestingly, ChiA also lowered pathogen transmission but to a much lower extent than the other two peptides. It is possible that ChiA had some activity on the cuticular surface of insects, disrupting protein-carbohydrate molecular interactions and, therefore, early cell adhesion to vectors. It is also possible that ChiA, which was provided in excess compared with normal amounts likely encountered by cells within insects, interacted with chitin-binding proteins secreted or on the surface of *X. fastidiosa*, also leading to lower rates of cell-insect interactions. The role of ChiA in these interactions as well as insect colonization remains to be determined. On the other hand, we propose that the transmission-blocking activity of both PD1764 and HxfAD1-3 are the result of competition for putative receptors (or cuticular surface space) between the peptides and *X. fastidiosa* cells, in which cells are acquired but cannot bind to insects because required molecular patterns are masked by these peptides.

In fact, data on bacterial colonization of the foregut of vectors over time support this hypothesis. Populations of *X. fastidiosa* in HxfAD1-3-fed insects increased over time, following the same patterns observed in control vectors; the same was observed for PD1764 at 50 μ M. These results indicate that vector colonization was not affected but early attachment was limited, leading to lower population size during the timeframe measured in this study. In the case of PD1764 at 100 μ M, cells were not able to adhere to insects, and no *X. fastidiosa* was detected even at 10 days post-acquisition. These results suggest that the effect of these peptides in blocking transmission is based on initial bacteria-vector interactions and that there are no impacts on cells if they are already bound or able to bind to the cuticle. If this mode of action is correct, then the time after acquisition during which pathogen acquisition remains affected should ultimately also be determined, as it would likely impact the usefulness of this approach to control pathogen spread. This observation has potentially relevant long-term consequences, as the blocking of *X. fastidiosa* transmission may be done with peptides with similar but distinct affinities (e.g., PD1764 and HxfAD1-3), which may have complementary as well as redundant activity in this context. In this scenario, it is possible that using a range of transmission-blocking peptides would reduce the speed with which the bacterium would overcome this type of plant resistance, which does not directly act on insect vector or bacterial pathogen.

X. fastidiosa transmission is the result of molecular interactions between pathogen and vector that may be disrupted under laboratory and greenhouse conditions. This study demonstrated for the first time that pathogen-derived transmission-blocking peptides disrupt these interactions, effectively preventing vector transmission of *X. fastidiosa* to plants. Importantly, additional molecules and, in particular, protein domains (i.e., LysM on PD1764 and HAD on HxfB) should be identifiable, given our recent biological observations. Those peptides, because of their smaller sizes, are expected to be more promising for subsequent applications for disease management. In addition, results obtained with PD1764 confirm the feasibility of our approach and validate the pipeline to identify new transmission-blocking proteins. Other *X. fastidiosa* candidates that have affinity for chitin but which we have failed to identify in this study could represent additional targets with high potential.

MATERIALS AND METHODS

Strains, plasmids, and media.

The strain *Xylella fastidiosa* subsp. *fastidiosa* Temecula (Van Sluys et al. 2003) was used in this study. *X. fastidiosa* cells were propagated on periwinkle wilt medium with Gelrite (Hill and Purcell 1995) or on *X. fastidiosa* medium (XFM [Killiny and Almeida 2009b]) at 28°C. For transmission experiments and identification of chitin-binding proteins, *X. fastidiosa* cells were propagated, respectively, on XFM solid medium supplemented with galacturonic acid (XFM-Gal, 0.01% wt/vol) or colloidal chitin (XFM-chitin, 0.1g/liter wt/vol) as inducers of the transmission process (Killiny and Almeida 2009b, Killiny et al. 2010). When appropriate, kanamycin and chloramphenicol were added to a final concentration of 30 and 10 μ g/ml, respectively.

The PD1764 mutant strain was obtained following Kung and Almeida (2011). Briefly, sequences located upstream and downstream of *pd1764* were cloned into the pGEM-5zf(+) vector backbone (Promega) flanking a kanamycin-resistance cassette. This plasmid was propagated in *Escherichia coli* EAM1, which expresses a *X. fastidiosa* methylase that increases the recombination efficiency of plasmids (Matsumoto and Igo 2010). The *pd1764* mutant was obtained by transforming strain Temecula, taking advantage of the fact that *X. fastidiosa* is naturally

competent (Kung and Almeida 2011). Disruption of *pd1764* and the presence of the kanamycin-resistance cassette were both confirmed by polymerase chain reaction (PCR) (data not shown).

For expression of the recombinant His₆-tagged proteins used as transmission-blocking molecules, pET28b(+) vector (Novagen) was used for all constructs generated in this study. Constructs used for the expression of peptides HxfAD1-3 and HxfAD4 were obtained from Voegel et al. (2010) (provided by B. Kirkpatrick) and used pET30b(+) (Novagen). All constructs were propagated in *E. coli* BL21λDE3 Rosetta strain (Novagen). Cultures were grown in Luria Bertani (LB) medium supplemented with 30 µg/ml of kanamycin at 37°C under shaking conditions.

His₆-tag was excised from all these constructions for all interaction or transmission experiments.

Identification of candidate

X. fastidiosa chitin-binding protein.

The expression of chitin-binding proteins in *X. fastidiosa* was induced by culturing cells on XFM-chitin medium. One week after plating, cells were collected into resuspension buffer (2 mM KH₂PO₄, 8 mM Na₂HPO₄, 2 mM KCl [pH 7.4]) containing 1 mM phenylmethanesulfonylfluoride (PMSF) and were stored at -20°C. Several batches of cells were prepared and combined to minimize potential variability in growth conditions or other factors. Disruption of the cells was initiated with three freeze-thaw cycles (15 min at -20°C and 15 min at room temperature) followed by sonication (Sonic Dismembrator; Fischer Scientific) (pulse mode 70% maximal power, 4°C; 1 min of sonication and 1 min on ice, alternately, three times). The solution was then centrifuged for 1 min at 5,000 × g in order to remove cellular debris, and the final protein concentration in the supernatant was determined by the Bradford assay, according to manufacturer instructions (Thermo Scientific). Proteins were not further purified, to avoid removing potential proteins of interest, and aliquots of the supernatant (20 µg) were stored at -20°C. In order to trap *X. fastidiosa* chitin-binding proteins, custom chitin columns were prepared. Briefly, chitin extracted from crab shells (Sigma) was homogenized into the same resuspension buffer previously described for *X. fastidiosa* proteins at a final concentration of 1mg/ml. This chitin preparation was loaded on an empty disposable PD-10 column (GE Healthcare) until a 1-ml retention volume was obtained; then, it was washed with 10 volumes of resuspension buffer. A total of 500 µg of *X. fastidiosa* protein was loaded onto chitin columns and nonspecifically bound proteins were washed away with an additional 10 ml of resuspension buffer. *Xylella fastidiosa* proteins bound to chitin were gradually eluted with a 0 to 2 M α-D methylpyrannoside gradient acting as a specific competitor (total volume, 20 ml). All the eluted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie gel staining. To test the specificity of the proteins eluted from the column, the same experiment was conducted with *X. fastidiosa* proteins grown on XFM medium not supplemented with colloidal chitin. Fractions eluted from this control column were also subjected to SDS-PAGE, and protein profiles were compared after gels were silver-stained, according to manufacturer instructions (PierceSilver stain for mass spectrometry). Protein bands, corresponding to *X. fastidiosa* proteins bound to chitin, were excised from the gel, and were trypsinized and sent to the Proteomic Facility (University of California, Davis) and were analyzed by liquid-chromatography tandem mass spectrometry. Sequences obtained were then compared with the *X. fastidiosa* subsp. *fastidiosa* Temecula1 genome.

Expression and purification of recombinant proteins.

In addition to PD1764, identified via the above-described pipeline, other candidate transmission-blocking peptides were

recombinantly expressed in *E. coli*, based on previous work identifying proteins involved in *X. fastidiosa*-vector interactions (Killiny and Almeida 2009a, 2014). Those include a chitinase (ChiA [Killiny et al. 2010]), a chaperone-usher pilus rod-forming protein (FimA), and two regions of a hemmagglutinin-like protein (HxfB). The HxfB protein (PD1792) was previously divided into domains AD1-3 and AD-4, due to its size (Voegel et al. 2010). Primers for loci amplifications ahead of cloning are listed in Supplementary Table S1. Amplified products were inserted into a plasmid for amplification (pGEM-T Easy, Promega) and the construct was used to transform *E. coli* DH10B; at that point, sequencing of inserts was performed. The gene of interest was then transferred into an expression vector (pET28a(+) Novagen), and 1 µg of the recombinant plasmid was used to transform *E. coli* DH10B for amplification. The expression vector (1 µg) was used to transform *E. coli* BL21λDE3 Rosetta strain (Novagen) for protein expression. For each construction, one transformant was cultivated in 500 ml of LB medium with kanamycin (30 µg/ml) until late log phase (optical density at 600 nm of 0.6). The recombinant peptides were produced, at 28°C in culture, by adding 0.1 mM isopropyl-β-D-thiogalactoside for 3 h. Bacterial cells were centrifuged at 7,000 × g for 10 min at 4°C, and the pellet was resuspended in 10 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 0.3 M NaCl, 25 mM MgSO₄, 2.5 mM MnCl₂, 1 mM PMSF, and 100 unit of DNase I per milliliter). After 10 min of incubation at room temperature, lysozyme was added at 0.2 mg/ml for 10 min. The mixture was lysed by sonication (Sonic Dismembrator, Fischer Scientific) (pulse mode 50% maximal power, 4°C; 1 min of sonication and 1 min on ice alternatively) until a clear lysate was obtained. The insoluble proteins were removed by subsequent centrifugation at 13,000 × g at 4°C for 10 min. The recombinant peptides were further purified by affinity chromatography, using Ni²⁺-nitrilotriacetic acid prepacked columns (Qiagen) according to the manufacturer's instructions. His₆-tag was removed by the action of a protease (thrombin) that cleaves the histidine tag from the peptide at a specific cleavage sequence present on the expression vector. Thrombin was added to the protein suspension (5 NIH units per milligram of protein) at room temperature for 2 h. Action of the protease was stopped by addition of 1 mM of PMSF. Protein expression and purification were confirmed with polyacrylamide gel electrophoresis based on band sizes (data not shown). Concentration of purified peptides was then assessed using the Bradford procedure, and solutions were aliquoted and stored at -20°C until needed.

Interaction of *X. fastidiosa* proteins with different carbohydrates.

We assessed the binding activity of six peptides (PD1764, PD1764ΔLysM, FimA, ChiA, HxfAD1-3, and HxfAD4) to bind to chitin and other polysaccharides. Four different polysaccharides (α-chitin from shrimp shells [Sigma], freshly prepared colloidal chitin (Hsu and Lockwood 1975), chitosan [Sigma], and crystalline cellulose [Avicel; Sigma]) were used as substrates. BSA was included in our test as a negative control. The substrates were suspended into a resuspension buffer (2 mM KH₂PO₄, 8 mM Na₂HPO₄, 2 mM KCl, pH 7.0) to yield 20-mg/ml stock suspensions that were continuously stirred in order to obtain uniform concentrations when pipetting. Each polysaccharide (1 mg) was incubated with 100 µg of each of the seven proteins (six candidates and BSA) in a 1-ml final reaction volume. Incubation was conducted at room temperature for 1 h under shaking conditions (60 rpm; rotary shaker with vertical orbital motion). Reaction mixtures were centrifuged at 14,500 × g for 3 min. The Bradford assay was used to determine protein concentration in the supernatant (i.e., proteins not bound to substrate). The assay was performed in two complete blocks with

three replicates in each block ($n = 6$). In order to normalize our results, the same binding assay was conducted for each candidate in the absence of polysaccharides, and we calculated the protein concentration measurable with no interactions with a substrate.

The same experiment was modified to determine the effect of time on binding dynamics (0, 0.02, 0.1, 0.25, 0.75, 1.5, 3, and 4 h). The two *X. fastidiosa* proteins (PD1764 and HxfAD1-3) with significant chitin-binding activity were further analyzed by using a second binding assay meant to estimate equilibrium K_d and $B_{\max}$. For those assays, a 500- μ l solution with a known protein concentration (determined by Bradford method) was diluted to various concentrations. Before the addition of chitin, protein concentrations were measured again, thus creating individual standard curves. Subsequently, chitin was added, bringing the reaction volume to 1 ml and the chitin concentration to 0.5 mg/ml; protein concentrations used were 10, 20, 50, 100, 150, 200, and 300 μ g/ml. After the addition of chitin, solutions were mixed by vertical rotation (60 rpm) at room temperature for up to 4 h. Subsequently, samples were treated as previously described. All assays were performed in three complete blocks with three replicates in each block ($n = 9$), each independent block including a blank (buffer + 0.5 mg/ml chitin) and a negative control to correct for nonspecific binding (buffer + BSA). The equilibrium K_d (μ M), and substrate $B_{\max}$ (μ mol/g), were determined by fitting the binding isotherms to the one-site binding equation in which P stands for protein: $[P_{\text{bound}}] = B_{\max} [P_{\text{free}}]/K_d + [P_{\text{free}}]$, by nonlinear regression.

Insect transmission and blocking assays.

We collected adults of the leafhopper vector *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae) on different host plants (mainly California wild grapes) in Berkeley, California. Insects were kept on sweet basil (*Ocimum basilicum*) under greenhouse conditions and were transferred to new basil plants as needed. We used second-generation adults to perform the transmission tests; *X. fastidiosa* is not transovarially or transstadially transmitted (Freitag 1951; Purcell and Finlay 1979), therefore we expected none of the individuals used for experiments to be infected, even if any of the field-collected insects carried *X. fastidiosa*. To estimate *X. fastidiosa* vector transmission efficiency, we slightly modified a previously described protocol (Killiny et al. 2012).

Prior to testing candidate transmission-blocking molecules with an artificial diet system used for the delivery of cells (Killiny and Almeida 2009b), we tested the impact of cell numbers in suspension on the transmission rate. Our goal was to select an optimal number of cells per milliliter that led to an experimentally measurable transmission rate without reaching a saturation point, as providing too many cells could lead to potential experimental artifacts, as previously addressed by Killiny and Almeida (2014). Insects were allowed to acquire *X. fastidiosa* on diet solutions containing different densities of *X. fastidiosa*; treatments ranged from 10^2 to 10^9 cells per milliliter. After a 4-h acquisition access period, insects were individually transferred to a single leaf on an uninfected grapevine (*Vitis vinifera*) and were provided an inoculation access period of 24 h. One month later, the presence of *X. fastidiosa* in plants was determined by culturing the petiole from leaves on which insects were placed (Hill and Purcell 1995). This assay was performed in two blocks, with 20 and 12 plants for each bacterial density per block, depending on insect survivorship (combined numbers of both replicates is reported in Figure 3).

Acute toxicity effects on insects acquiring different concentrations of recombinant proteins was also tested under greenhouse conditions. Briefly, insects were allowed to feed on a diet solution containing 10^8 *X. fastidiosa* cells per milliliter, complemented with 250, 100, 50, 25, or 10 mM of recombinant

protein for 4 h. These experiments were conducted for PD1764 and ChiA. Insects were immediately transferred to basil plants and their survival was assessed every day over a 4-day period. During these 4 days, no insect death was observed for any of the two proteins or any of the five concentrations. Twenty-five insects were used for each peptide concentration. Before testing candidate transmission-blocking peptides, we also performed a test to determine what concentrations of transmission-blocking peptides were suitable to use in our blocking assays. To do so, we chose a candidate (PD1764) that interacted with polysaccharides in our binding assays and another one that did not (ChiA). Protocols were identical as described above, with acquisition of cells and proteins from an artificial diet system, followed by a 24-h inoculation access period on grapevines with one insect per grapevine ($n = 15$ plants).

Once protocols were optimized, the six *X. fastidiosa*-derived candidate peptides were tested; BSA was included as a control. A positive control (no transmission-blocking molecule) was used to normalize transmission rates. Each assay was performed in two complete blocks with 15 plants each for each peptide, with one insect vector per grapevine, as described above. A third block using 12 additional plants and including only the internal control, PD1764, and PD1764 Δ LysM was done to confirm previous results. Due to a few missing data, this resulted in the following sample sizes: control = 42, BSA = 29, ChiA = 29, FimA = 25, HxfAD1-3 = 27, HxfAD4 = 28, PD1764 = 38, and PD1764 Δ LysM = 39.

In parallel, quantitative (q)PCR was used to determine bacterial populations in the heads of insects. Briefly, after feeding *Graphocephala atropunctata* insects on *X. fastidiosa* suspensions (as described above), supplemented or not with different concentrations of PD1764 (50 and 100 μ M) or HxfAD1-3 (100 μ M) for a 4-h acquisition period, we confined insects on basil for various times post-acquisition (1, 3, and 10 days); we randomly selected insects from colonies, extracted the DNA from their whole heads, and used qPCR to quantify the number of *X. fastidiosa* cells within those individuals. SYBR green technology was used and amplification was performed with a Fast ABI 7500 real-time PCR system (Applied Biosystems) following Francis et al. (2006) and Killiny and Almeida (2009a). Five insects were tested for each condition and genomic DNA of *X. fastidiosa* Temecula was used for a standard curve.

Statistical analyses.

First, we tested for interactions among *X. fastidiosa* peptide and various polysaccharides using a randomized complete block two-way factorial mixed-effects analysis of variance (ANOVA). The experiment was fully repeated twice with three replicates in each and we treated the experiment repetitions as a random block effect. Peptide and polysaccharide factors were fixed effects. Second, we analyzed the time-series experiment as a randomized complete block repeated-measures ANOVA. Here, the experiment was fully repeated three times with three replicates in each, and we treated the experiment repetitions as a random block effect. Time was also considered a random effect, while peptide was considered a fixed effect. Peptide concentration (response variable) was square root-transformed to meet assumptions of linear models. To test for differences among specific interactions, we performed two contrasts among peptide interactions with three of the polysaccharides (chitin, colloidal chitin, and chitosan): i) HxfAD1-3 versus BSA, FimA, ChiA, HxfAD4, and PD1764 Δ LysM and ii) PD1764 versus BSA, FimA, ChiA, HxfAD4, and PD1764 Δ LysM. For the repeated-measures ANOVA, we used Satterthwaite's approximation for degrees of freedom (df) and Nelder-Mead optimization to improve convergence on the maximum likelihood estimate.

In the vector-transmission experiments, we hypothesized that *X. fastidiosa* populations in vectors would be impacted by recombinant proteins provided in artificial diets together with bacterial cells and that this would, in turn, influence the probability of vector transmission. First, we tested for differences in vector transmission using bacteria populations in vectors with a two-way fixed-effects ANOVA, in which peptide and day measured were factors. Each peptide-day treatment combination was replicated five times. Bacterial population was log-transformed to meet linear model assumptions. Second, we tested for differences in probability of transmission using logistic regression. The experiment was fully repeated twice with 15 replicates in each and we treated the experiment repetitions as a random block effect. Because no transmission occurred for some proteins—resulting in quasi-complete separation of factor levels—we used logistic regression with Firth’s bias correction to estimate all factor-level parameters (Firth 1993). We used Tukey’s honestly significant difference test for all pair-wise comparisons among treatments. All analyses were conducted in R 3.2.0 (R Core Team 2014). We ran mixed-effects models with the lme4 package (Bates et al. 2014), used the multcomp package for pair-wise comparisons and contrasts (Hothorn et al. 2008), and used the logistf package for Firth-corrected logistic regression (Heinze et al. 2013).

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LITERATURE CITED

- Almeida, R. P. P., Blua, M. J., Lopes, J. R. S., and Purcell, A. H. 2005. Vector transmission of *Xylella fastidiosa*: Applying fundamental knowledge to generate disease management strategies. *Ann. Entomol. Soc. Am.* 98:775-786.
- Almeida, R. P. P., Killiny, N., Newman, K. L., Chatterjee, S., Ionescu, M., and Lindow, S. E. 2012. Contribution of rpfB to cell-to-cell signal synthesis, virulence, and vector transmission of *Xylella fastidiosa*. *Mol. Plant-Microbe Interact* 25:453-462.
- Almeida, R. P. P., and Purcell, A. H. 2003. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata* (Hemiptera: Cicadellidae). *J. Econ. Entomol.* 96:264-271.
- Almeida, R. P. P., and Purcell, A. H. 2006. Patterns of *Xylella fastidiosa* colonization on the precubarium of sharpshooter vectors relative to transmission to plants. *Ann. Entomol. Soc. Am.* 99:884-890.
- Baccari, C., Killiny, N., Ionescu, M., Almeida, R. P. P., and Lindow, S. E. 2014. Diffusible signal factor-repressed extracellular traits enable attachment of *Xylella fastidiosa* to insect vectors and transmission. *Phytopathology* 104:27-33.
- Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R. H. B., Singmann, H., and Dai, B. 2014. Package “lme4.” <https://cran.r-project.org/web/packages/lme4/lme4.pdf>. The R Foundation, Vienna.
- Bransky, R. H., Timmer, L. W., French, W. J., and McCoy, R. E. 1983. Colonization of sharpshooter vectors, *Oncometopia nigricans* and *Homalodisca coagulata*, by xylem-limited bacteria. *Phytopathology* 73:530-535.
- Chatterjee, S., Newman, K. L., and Lindow, S. E. 2008a. Cell-to-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. *Mol. Plant-Microbe Interact* 21:1309-1315.
- Chatterjee, S., Wistrom, C., and Lindow, S. E. 2008b. A cell-cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. *Proc. Natl. Acad. Sci. U.S.A.* 105:2670-2675.
- Firth, D. 1993. Bias reduction of maximum likelihood estimates. *Biometrika* 80:27-38.
- Francis, M., Lin, H., Rosa, J. C.-L., Doddapaneni, H., and Civerolo, E. L. 2006. Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. *Eur. J. Plant Pathol.* 115:203-213.
- Freitag, J. H. 1951. Host range of Pierce’s disease virus of grapes as determined by insect transmission. *Phytopathology* 41:920-934.
- Goldstein, M. A., Takagi, M., Hashida, S., Shoseyov, O., Doi, R. H., and Segel, I. H. 1993. Characterization of the cellulose-binding domain of the *Clostridium cellulovorans* cellulose-binding protein A. *J. Bacteriol.* 175:5762-5768.
- Guoying, Z., Xiongbin, L., Huijuan, L., Juanli, L., Shengxiang, C., and Zuxun, G. 1999. Rice ragged stunt oryza virus: Role of the viral spike protein in transmission by the insect vector. *Ann. Appl. Biol.* 135:573-578.
- Gust, A. A., Willmann, R., Desaki, Y., Grabherr, H. M., and Nürnberger, T. 2012. Plant LysM proteins: Modules mediating symbiosis and immunity. *Trends Plant Sci.* 17:495-502.
- Heinze, G., Ploner, M., Dunkler, D., and Southworth, H. 2013. logistf: Firth’s bias reduced logistic regression. R package version 1.21. <http://CRAN.R-project.org/package=logistf>. The R Foundation, Vienna.
- Hill, B. L., and Purcell, A. H. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. *Phytopathology* 85:209-212.
- Hill, B. L., and Purcell, A. H. 1997. Populations of *Xylella fastidiosa* in plants required for transmission by an efficient vector. *Phytopathology* 87:1197-1201.
- Hothorn, T., Bretz, F., and Westfall, P. 2008. Simultaneous inference in general parametric models. *Biom. J.* 50:346-363.
- Hsu, S. C., and Lockwood, J. L. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Appl. Microbiol.* 29:422-426.
- Inatsuka, C. S., Julio, S. M., and Cotter, P. A. 2005. *Bordetella* filamentous hemagglutinin plays a critical role in immunomodulation, suggesting a mechanism for host specificity. *Proc. Natl. Acad. Sci. U.S.A.* 102:18578-18583.
- Killiny, N., and Almeida, R. P. P. 2009a. *Xylella fastidiosa* afimbrial adhesins mediate cell transmission to plants by leafhopper vectors. *Appl. Environ. Microbiol.* 75:521-528.
- Killiny, N., and Almeida, R. P. P. 2009b. Host structural carbohydrate induces vector transmission of a bacterial plant pathogen. *Proc. Natl. Acad. Sci. U.S.A.* 106:22416-22420.
- Killiny, N., and Almeida, R. P. P. 2014. Factors affecting the initial adhesion and retention of the plant pathogen *Xylella fastidiosa* in the foregut of an insect vector. *Appl. Environ. Microbiol.* 80:420-426.
- Killiny, N., Prado, S. S., and Almeida, R. P. P. 2010. Chitin utilization by the insect-transmitted bacterium *Xylella fastidiosa*. *Appl. Environ. Microbiol.* 76:6134-6140.
- Killiny, N., Rashed, A., and Almeida, R. P. P. 2012. Disrupting the transmission of a vector-borne plant pathogen. *Appl. Environ. Microbiol.* 78:638-643.
- Kung, S. H., and Almeida, R. P. P. 2011. Natural competence and recombination in the plant pathogen *Xylella fastidiosa*. *Appl. Environ. Microbiol.* 77:5278-5284.
- Li, Z., Li, C., Yang, K., Wang, L., Yin, C., Gong, Y., and Pang, Y. 2003. Characterization of a chitin-binding protein GP37 of *Spodoptera litura* multicapsid nucleopolyhedrovirus. *Virus Res.* 96:113-122.
- Matsumoto, A., and Igo, M. M. 2010. Species-specific type II restriction-modification system of *Xylella fastidiosa* temecula1. *Appl. Environ. Microbiol.* 76:4092-4095.
- Montero-Astúa, M., Rotenberg, D., Leach-Kieffaber, A., Schneweis, B. A., Park, S., Park, J. K., German, T. L., and Whitfield, A. E. 2014. Disruption of vector transmission by a plant-expressed viral glycoprotein. *Mol. Plant-Microbe Interact* 27:296-304.
- Newman, K. L., Almeida, R. P. P., Purcell, A. H., and Lindow, S. E. 2004. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. *Proc. Natl. Acad. Sci. U.S.A.* 101:1737-1742.
- Ohnuma, T., Onaga, S., Murata, K., Taira, T., and Katoh, E. 2008. LysM domains from *Pteris ryukyuensis* chitinase-A: A stability study and characterization of the chitin-binding site. *J. Biol. Chem.* 283:5178-5187.
- Purcell, A. H., and Finlay, A. H. 1979. Evidence for noncirculative transmission of Pierce’s disease bacterium by sharpshooter leafhoppers. *Phytopathology* 69:393-395.
- Purcell, A. H., Finlay, A. H., and McLean, D. L. 1979. Pierce’s Disease bacterium: Mechanism of transmission by leafhopper vectors. *Science* 206:839-841.
- R Core Team. 2014. R: A language and environment for statistical computing. <http://www.R-project.org/>. The R Foundation, Vienna.
- Rashed, A., Daugherty, M. P., and Almeida, R. P. P. 2011. Grapevine genotype susceptibility to *Xylella fastidiosa* does not predict vector transmission success. *Environ. Entomol.* 40:1192-1199.
- Retchless, A. C., Labrousseau, F., Shapiro, L., Stenger, D. C., Lindow, S. E., and Almeida, R. P. P. 2014. Genomic insights into *Xylella fastidiosa* interactions with plant and insect hosts. Pages 177-202 in: *Genomics of*

- Plant-Associated Bacteria. D. C. Gross, A. Lichens-Park, and K. Chittaranjan, eds. Springer, Berlin.
- Ruoslahti, E. 1996. RGD and other recognition sequences for integrins. *Annu. Rev. Cell Dev. Biol.* 12:697-715.
- Severin, H. 1949. Transmission of the virus of Pierce's disease of grapevines by leafhoppers. *Hilgardia* 19:190-206.
- Severin, H. 1950. Spittle-insect vectors of Pierce's disease virus II. Life history and virus transmission. *Hilgardia* 19:357-382.
- Vaaje-Kolstad, G., Houston, D. R., Riemen, A. H. K., Eijssink, V. G. H., and van Aalten, D. M. F. 2005. Crystal structure and binding properties of the *Serratia marcescens* chitin-binding protein CBP21. *J. Biol. Chem.* 280:11313-11319.
- Van Sluys, M. A., de Oliveira, M. C., Monteiro-Vitorello, C. B., Miyaki, C. Y., Furlan, L. R., Camargo, L. E. A., da Silva, A. C. R., Moon, D. H., Takita, M. A., Lemos, E. G. M., Machado, M. A., Ferro, M. I. T., da Silva, F. R., Goldman, M. H. S., Goldman, G. H., Lemos, M. V. F., El-Dorry, H., Tsai, S. M., Carrer, H., Carraro, D. M., de Oliveira, R. C., Nunes, L. R., Siqueira, W. J., Coutinho, L. L., Kimura, E. T., Ferro, E. S., Harakava, R., Kuramae, E. E., Marino, C. L., Giglioti, E., Abreu, I. L., Alves, L. M. C., do Amaral, A. M., Baia, G. S., Blanco, S. R., Brito, M. S., Cannavan, F. S., Celestino, A. V., da Cunha, A. F., Fenille, R. C., Ferro, J. A., Formighieri, E. F., Kishi, L. T., Leoni, S. G., Oliveira, A. R., Rosa, V. E., Jr., Sasaki, F. T., Sena, J. A. D., de Souza, A. A., Truffi, D., Tsukumo, F., Yanai, G. M., Zaros, L. G., Civerolo, E. L., Simpson, A. J. G., Almeida, N. F., Jr., Setubal, J. C., and Kitajima, J. P. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *J. Bacteriol.* 185:1018-1026.
- Visweswaran, G. R. R., Dijkstra, B. W., and Kok, J. 2012. A genetically engineered protein domain binding to bacterial murein, archaeal pseudomurein, and fungal chitin cell wall material. *Appl. Microbiol. Biotechnol.* 96:729-737.
- Voegel, T. M., Warren, J. G., Matsumoto, A., Igo, M. M., and Kirkpatrick, B. C. 2010. Localization and characterization of *Xylella fastidiosa* haemagglutinin adhesins. *Microbiology* 156:2172-2179.
- Whitfield, A. E., and Rotenberg, D. 2015. Disruption of insect transmission of plant viruses. *Curr. Opin. Insect Sci.* 8:79-87.
- Zaini, P. A., Burdman, S., Igo, M. M., Parker, J. K., and De la Fuente, L. 2015. Fimbrial and afimbrial adhesins involved in bacterial attachment to surfaces. *Virulence Mechanisms of Plant Pathogenic Bacteria*. N. Wang, L. De La Fuente, J. Jones, J. Ham, F. White, G. Sundin, S. Hogenhaut, and C. Roper, eds. American Phytopathological Society, St. Paul, MN, U.S.A.