Interim Progress Report for CDFA Agreement Number 12-0116-SA

Title of Project
Blocking *Xylella fastidiosa* transmission

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Introduction

The goal of this project is to develop a technology to block the vector transmission of *Xylella fastidiosa* to plants. The approach focuses on the disruption of *X. fastidiosa*-vector interactions, so that the transmission of *X. fastidiosa* from one plant to another is affected. Our work has demonstrated that this approach is feasible and that we can disrupt sharpshooter transmission of *X. fastidiosa* to grapevines using generic molecules to block bacterial access to insect receptors (Killiny et al. 2012). The specific goal of this proposal is to identify and test *X. fastidiosa* candidate proteins that can be used as specific transmission-blocking molecules, so that this strategy can be tested under field conditions in the near future. This report describes results achieved since October 2012. First, we summarize our efforts made up to now to search for proteins implicated in the transmission of *X. fastidiosa* including work conducted on the identification of chitin-binding proteins and the characterization of a chitinase mutant. Second, we also have used several *X. fastidiosa* recombinant proteins or peptides as transmission-blocking molecules to disrupt vector transmission under greenhouse conditions. Results obtained with six of these constructs are summarized.

List of Objectives – as in the approved research proposal
Objective 1. Continue efforts to identify additional targets implicated in *X. fastidiosa* transmission by insects.
Objective 2. Test specific and efficient molecules to disrupt vector transmission.

Description of activities

OBJECTIVE 1
Proteomics-based identification of transmission-blocking proteins
Previous work done in the laboratory highlighted the importance of chitin in *X. fastidiosa* initial adhesion to sharpshooter vectors (Killiny et al. 2009a). In order to identify *X. fastidiosa* proteins able to interact with chitin, we used a proteomics-based approach. Briefly, total *X. fastidiosa* proteins have been extracted from cells grown on XFM-chitin, which induce phenotypic and gene expression changes in *X. fastidiosa*, including increased adhesiveness (Killiny and Almeida 2009a, b, Killiny et al. 2010). Extracts have been incubated in a colloidal chitin column, and proteins specifically bound to that substrate were eluted then separated on SDS-PAGE. This approach allowed us to identify one candidate named PD1764. Others proteins have been visualized on SDS-PAGE but remained unidentified. One of the main limitations of previous attempts concerning protein identification using mass spectrometry was the low concentration of recovered proteins (below the detection threshold). In order to circumvent this limitation, another approach is currently being used that takes advantage of magnetic chitin beads (New England Biolabs) instead of chitin columns for retention and selection of chitin-binding proteins. The main advantage of this approach is that instead of recovering high volume protein fractions, which require protein concentration, washes and specific elutions are done with much smaller volumes. This alternate approach is currently ongoing in the laboratory and results will be soon available.

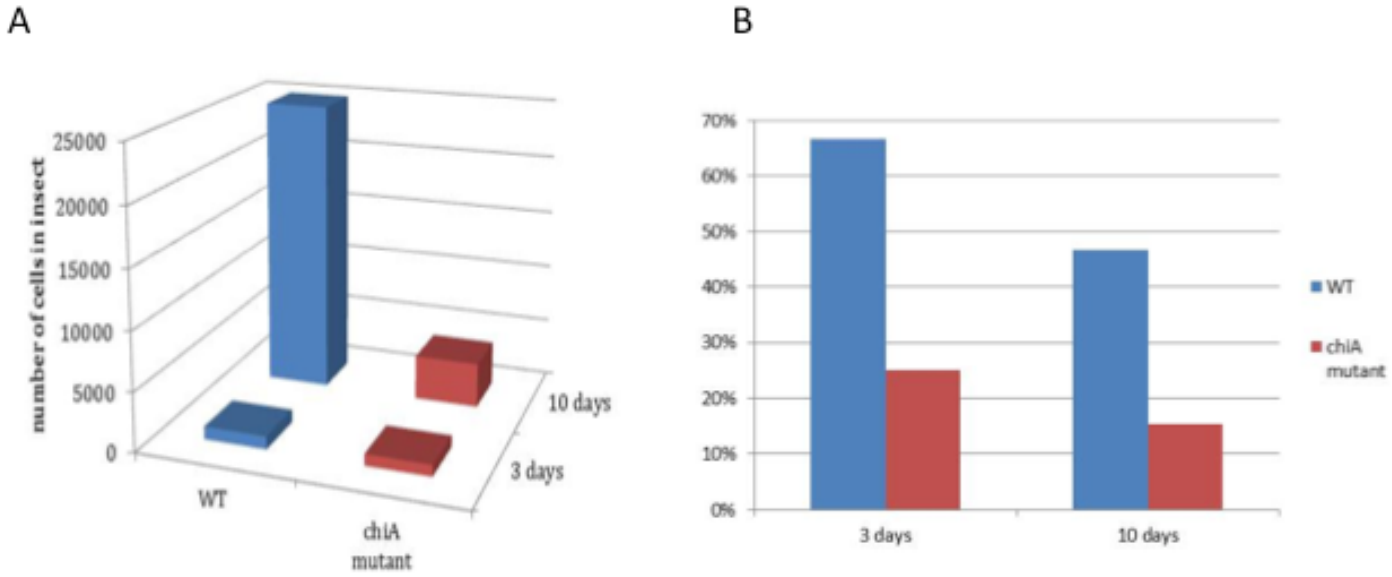
Results obtained using PD1764 (see Objective 2), the candidate identified with this approach, are very encouraging and indicate that the pipeline should lead to the identification of additional highly interesting candidates.

Chitinase and its associated chitin-binding domain

Previous work on *X. fastidiosa*-insect interactions highlighted the importance of chitin in *X. fastidiosa* colonization of vectors (Killiny et al. 2010). The observation that *X. fastidiosa* possesses a functional chitinase (ChiA) and all the machinery required to assimilate it led us to further test if *X. fastidiosa* i) could use chitin as a carbon source for successful colonization of vectors and ii) could use ChiA as an adhesin to bind to vectors. Results previously obtained showed that a *X. fastidiosa* chitinase mutant (*chiA* mutant) is not able to grow with chitin as a sole carbon source in comparison to the wild type strain. These results confirmed our hypothesis that *X. fastidiosa* has a functional chitinase allowing it to assimilate chitin as a carbon source. Then, we conducted experiments to assess the ability of this *chiA* mutant to colonize its insect vectors. Briefly, insects acquired the *chiA* mutant or wild type cells from this artificial diet system, with cell suspension between two Parafilm layers for 4 hours. Insects (n=12) were removed from these sachets and transferred to basil plants for cells to colonize insects. Three and 10 days after acquisition, insects were recovered from basil plants and tested with quantitative PCR (qPCR) to quantify bacterial population in the vectors' foregut (see panel A below). No differences were obtained after 3 days post-acquisition. But the *chiA* mutant is affected in its vector colonization, i.e. a significant reduction in bacterial population was observed for the *chiA* mutant compared to the control, at 10 days post-acquisition. We suggest that the *chiA* mutant is deficient in insect colonization because it cannot use chitin as a carbon source.

chiA mutant is affected in its transmission by insect vectors

To address the importance of *chiA* on *X. fastidiosa* vector transmission, we used the same protocol previously described to assess *chiA* mutant vector colonization. Three and 10 days after acquisition, instead of testing vectors using qPCR (data show in A panel), insects were individually transferred to healthy grapes to test their ability to transmit *X. fastidiosa* to another plant (B panel).

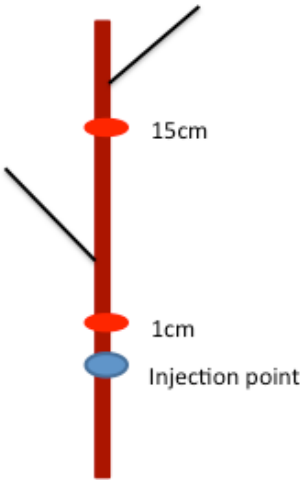


At 3 days post-acquisition, the transmission rate for the *chiA* mutant was significantly lower than the control. Transmission rate for *chiA* mutant was only 25% (3 out 12 plants tested positives) so a reduction of 41% in comparison to the wild type (8/12, 66%). At 10 days post-acquisition, even if the overall transmission rates are lower than those obtained at 3 days post-acquisition, transmission rate for the *chiA* mutant (2/13, 15%) is also significantly reduced in comparison to the control (7/15, 47%). This latter case should be linked to the different abilities of the *chiA* mutant and the wild-type to colonize the insect. Ten days post-acquisition, the bacterial population colonizing insects is almost 10 times greater for the wild type than for the *chiA* mutant. This

difference could easily explain the reduction of the transmission for chiA mutant due to a lower number of inoculation events or a lower numbers of bacteria inoculated during each inoculation event. The explanation is less straightforward at 3 days post-inoculation. Results for insect colonization, where no difference were shown for the *chiA* mutant and the wild type strain, cannot explain difference in transmission obtained here. One possible explanation is that, due to the absence of ChiA, attachment of cells is still possible (as shown by the same number of cells colonizing the vector) but somehow less functional in relation to transmission. This result argues in favor of a probable implication of ChiA in *X. fastidiosa* attachment process to the insect foregut even if ChiA should not be involved in the first steps of this adhesion.

chiA mutant is affected in its capacity to colonize grapevines

Thanks to our artificial diet system, no *X. fastidiosa* acquisition through plants by insects was required to characterize the *chiA* mutant in relation to vector transmission. Therefore, its capacity to colonize plants still had to be determined. To address that question we mechanically inoculated grapevines (n=12) with the *chiA* mutant or wild type cells as previously described (Almeida et al. 2001). Two months after inoculation, we monitored symptom development and cultured plants at two sites to assess the colonization of *X. fastidiosa* strains in plant. This figure summarizes the experiment.



	WT	chiA mutant
Symptoms	Yes (12/12)	No (0/12)
1cm	Yes (5/5)	Yes (5/5)
15cm	Yes (5/5)	No (0/5)

Surprisingly, none of the plants infected with the *chiA* mutant developed symptoms. In comparison, plants infected with the wild type strain developed characteristic symptoms of *X. fastidiosa* infection. In addition, cultures obtained from test plants 1cm above the inoculation site allowed us to recover both strains in their respective plants, meaning that the *chiA* mutant was able to survive *in planta* and the absence of symptoms previously described was not due to the death of the mutant. However, at 15cm above the inoculation site only the wild type strain was recovered.

Altogether, these results show that the *chiA* mutant, even if capable of surviving in grapevines, is not able to move over long distances, and is therefore not capable of inducing Pierce's disease symptoms. This was unexpected because no role for ChiA in plants has been suggested; additional experiments are needed to define what is its function. Complementation of the *chiA* mutant is currently ongoing in the laboratory following Chatterjee et al. (2010). This complemented strain will be used to confirm properties tested up to this point, especially to restore its ability to be transmitted by insects and to successfully colonize plants.

OBJECTIVE 2. Test specific and efficient molecules to disrupt vector transmission.

Preparation of candidate proteins and peptides of their respective binding sites

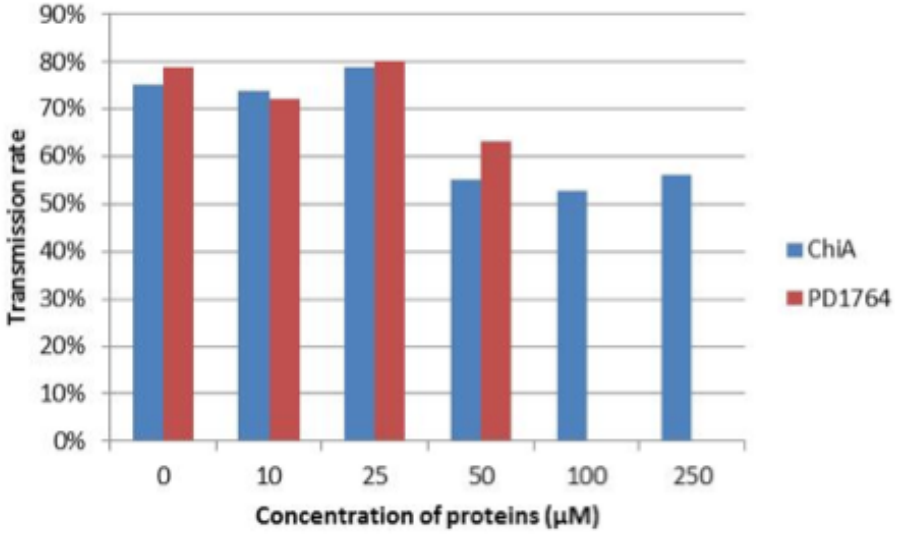
In order to test specific *X. fastidiosa* proteins as transmission-blocking molecules we expressed different proteins or domains as recombinant proteins. Several *X. fastidiosa* adhesins have been targeted based on previous works showing their involvement in vector colonization and transmission. In that way, different constructs were realized; one for the full-length type I pilus FimA (Killiny et al. 2012), two expressing different domains of the hemagglutinin-like protein HxfB (PD1798, Voegel et al. 2010), one for *X. fastidiosa* chitinase (ChiA; Killiny et al. 2010 and Objective 1 of this report) and finally, two different constructs for the candidate that we identified using our proteomic-based pipeline (PD1764) expressing or not an adhesion domain, called LysM domain, which has already been implicated in binding to chitin (Visweswaran et al. 2012). To our knowledge, this is the first example of a *X. fastidiosa* protein containing an already described chitin-binding domain. All these 6 constructs were expressed as N-terminal His_6-fusion peptides using *E. coli* Rosetta strain and purified on Ni-NTA columns. Before using them the His_6-tag was removed.

Transmission experiments using transmission-blocking molecules

Determination of protein concentration to use in our transmission-blocking assays

These experiments were conducted to accurately determine what concentration of transmission-blocking molecules should be used in our experiments so that a significant disruption of *X. fastidiosa* transmission occurred. ChiA and PD1764 recombinant proteins, two of our more promising candidates, were separately provided to insect vectors together with *X. fastidiosa*. Briefly, insects were allowed to acquire *X. fastidiosa* and proteins from an artificial diet system for 4 hours, and were then transferred to healthy grapevines for a 24h-inoculation access period. The *X. fastidiosa* suspension was adjusted to 10^8 cells/ml (previously described in previous progress report covering June 2012-October 2012 period) and different concentrations of ChiA and PD1764, ranging from 0 to 250µM, were mixed with bacteria cells.

protein concentration (µM)	Number of plants tested positives	
	ChiA	**PD1764**
0	15/20	15/19
10	14/19	13/18
25	15/19	16/20
50	11/20	12/19
100	10/19	0/19
250	9/16	0/16



Concerning ChiA, none of the concentrations tested resulted in a complete disruption of the transmission of *X. fastidiosa.* First, no significant decrease of transmission was obtained with 10 and 25µM of proteins. A slight but significant reduction of the transmission ($P<0.05$) was obtained with the three higher concentrations used (50, 100 and 250µM). However, no dose-dependent reductions were observed between these concentrations; the maximal reduction of transmission (22% of decrease) was obtained with 100µM of ChiA (53% of transmission versus 75% for the control). On the other hand, PD1764 showed a complete disruption of the transmission of *X. fastidiosa* by its vectors when at least a concentration of 100µM of proteins is present ($P<0.001$). No significant reduction of transmission was obtained with the lower concentrations tested. Again, no dose-dependent blocking could be observed with the concentrations used. Same experiments with proteins concentrations ranging from 50 to 100µM could allow us to better define if a dose-dependent response exists or not.

Altogether, these results indicate that 100µM of proteins is an ideal concentration to use in our experimental transmission system. At this given concentration, PD1764 completely blocked *X. fastidiosa* transmission by its insect vectors. These results also confirm the feasibility of our approach, and validate the proteomics pipeline to identify new transmission-blocking proteins. This result is highly encouraging because this is the first time that one treatment focusing on disrupting *X. fastidiosa*-vector interactions completely blocked the transmission of *X. fastidiosa.*

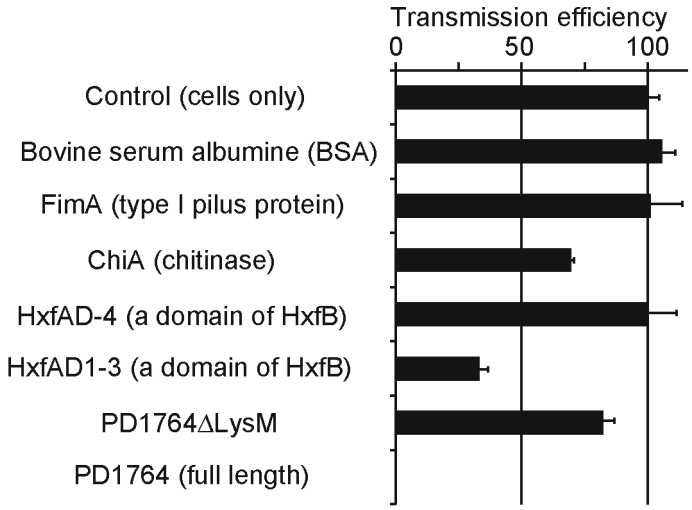
Effect of others X. fastidiosa transmission-blocking molecules constructed

The experiment described above was repeated with all *X. fastidiosa* proteins expressed as recombinant proteins using a concentration of 100µM of proteins. For each treatment, 15 insects were used and the experiment was repeated twice. Results are summarized in Figure 4 below.

According to these results, we found that most candidates did not reduce transmission efficiency. This is particularly true for ChiA, FimA and HxfAD-4 domain. However, in addition to the blocking effect previously observed for PD1764, one candidate (HxfAD1-3) had a significant effect on transmission. This construct expresses the 1,168 amino acids (aa) N-terminal part of the hemagglutinin-like protein HxfB. Interestingly,

according to SMART (http://smart.embl-heidelberg.de/), this region contains a 120 aa domain called haemagglutination activity domain (HAD; Voegel et al. 2010), which has been suggested to be a carbohydrate-dependant haemagglutination activity site. It has been found in a number of adhesins or filamentous haemagglutinins such as the FHA of *Bordetella pertussis* and plays a role in adhesion to host cells (Kajava et al. 2001). Concerning PD1764, the second construct tested here named PD1764ΔLysM, in which the LysM domain is absent, showed no significant effect in reducing *X. fastidiosa* transmission. Thus, the region of PD1764 involved in transmission disruption could be restricted to the first 89aa on the N-terminal of the protein. Within this region, the LysM domain, which is known to have chitin-binding activity (Vieweswaran et al. 2012), was identified between aa 41 and 89. Identification of domains on HxfAD1-3 and PD1764 (respectively named HAD and LysM) involved in blocking *X. fastidiosa* transmission by insects will lead to the construction of shorter transmission-blocking peptides. This is of great importance because utilization of small peptides could greatly enhance the efficiency of our transgenic system in the field, as the medium-term goal of this system is to develop transgenic grapevines expressing these transmission-blocking molecules constitutively.

Fig. 4. Transmission-blocking efficiency of tested peptides. The putative domain of HxfB (D1-3) and protein PD1764 blocked transmission in relation to controls. The LysM domain of PD1764 is required for blocking activity. All treatments included bacterial cells and peptides (100 µM), except for the control. Replicates were combined and all treatments normalized (control =100%).



CONCLUSIONS

This project is proceeding very well. First, we showed that the chitinase mutant is affected in its transmission by vectors but also, and more surprisingly, in its ability to colonize plants. Tests will be done with a complemented strain to confirm its interesting behavior. Based on these results, ChiA is unlikely involved in *X. fastidiosa* adhesion to insect foregut but is probably involved is transmission, in addition to its role in insect colonization using chitin as a carbon source. More importantly, we also succeed to test several *X. fastidiosa* proteins as transmission-blocking molecules. Very promising results were obtained with HxfAD1-3 but especially with PD1764, the candidate identified using our proteomic-based approach, which completely disrupted the transmission of *X. fastidiosa*. *In silico* analyses also identified two domains (HAD and LysM) that could be highly promising peptides to continue our search for optimized transmission-blocking molecules.

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