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

Title of Project
Blocking *Xylella fastidiosa* transmission

Principal Investigator
Rodrigo Almeida, Associate Professor
Dept. Environmental Science, Policy and Management
University of California, Berkeley
Berkeley, CA, 94720

Time Period Covered by the Report
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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 April 2013. In the previous report we summarized our efforts 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. We also showed that one of these proteins has a peptide that completely abolished *X. fastidiosa* transmission by sharpshooters in greenhouse experiments. In this interim report we focus on novel results obtained in the last three months.

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

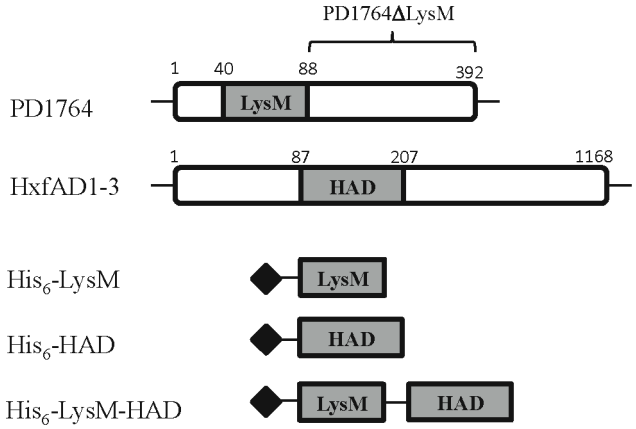
OBJECTIVE 1

i) Construction of additional transmission blocking peptides

We took advantage of the recent results obtained with PD1764 and the hemagglutinin-like protein HxfB (HxfAD1-3; PD1792) as transmission-blocking molecules to continue our research for smaller optimized peptides. This is of great importance because utilization of small peptides could greatly enhance the blocking efficiency of our molecules but also be more adequate for a

transgenic system in the field. Based on *in silico* analyses, we designed 3 additional peptides corresponding to the sequences of domains potentially having the blocking activity.

Figure 1 represents the location of the first two domains, LysM and HAD, on each protein but also the construction of a fused peptide including both domains.



The region of PD1764 involved in transmission disruption has been restricted to the first 88 amino acids (aa) on the N-terminal of the protein (no significant effect with PD1764ΔLysM, see previous report). 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. For the HxfB protein, a region of 1,168aa (HxfAD1-3) has been implicated in the reduction of transmission (see previous report for transmission results). 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 haemagglutinin such as the FHA of *Bordetella pertussis* and plays a role in adhesion to host cells (Kajava et al. 2001). Lastly, a peptide sharing both domains (LysM+HAD) was constructed by mutagenesis PCR (Heckman and Pease, 2007). Domains are separated by a spacer containing a 4-aminoacid sequence (GSTA) between the two domains to avoid any conformational problems during protein expression. Cloning strategy has been the same for all the above mentioned constructions. PCR fragments have been digested by NdeI/HindIII and cloned into pET28a plasmid (Novagen) into the same sites. Expression and purification of these His_6-tagged peptides is ongoing and each of these transmission blocking peptides will be tested in transmission experiments this summer as previously done with full-length proteins (see previous report). We expect that the utilization of these optimized peptides will allow us to reduce the concentration of molecules required to block the transmission of *X. fastidiosa*. This is highly expected for the chimera because of the combined effect of each peptide.

ii) Construction of a PD1764 mutant

Even though it is clear that PD1764 blocks *X. fastidiosa* transmission, it is possible that this protein may have a biological role not associated with transmission. To confirm the possible role in *X. fastidiosa* transmission, we disrupted the pd1764 gene in *X. fastidiosa* by double crossing-over following standard protocols used in our laboratory (Kung and Almeida, 2011). Briefly, sequences located upstream and downstream of the pd1764 gene were cloned on each side of the sequence of the kanamycin gene, and then this construction was cloned into the pGEM-T vector

background (Newman and al. 2004). Once obtained, this construction has been used to transform the *X. fastidiosa* Temecula strain. Disruption of the pd1764 gene and presence of the kanamycin gene in *X. fastidiosa* chromosome were both confirmed by PCR (data not shown). This mutant will be tested this summer for plant colonization but also, and more importantly, for vector transmission to plants (Killiny and Almeida 2009b). The ability of mutants to successfully colonize the insect foregut will also be verified using scanning electron microscopy (Almeida and Purcell 2006). Importantly, this will not change in any way the importance of PD1764 in our transmission blocking concept (i.e. it blocks transmission in greenhouse conditions), but it will help us determine its biological role.

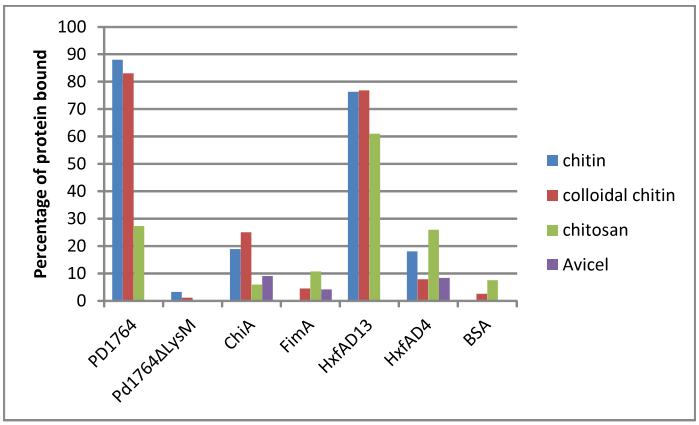
iii) Complementation of the chitinase mutant

The interesting results, obtained while studying the behavior of the chitinase (*chiA*) mutant during plant and insect colonization (see previous report for details), forced us to complement this mutant in order to confirm them. Complementation of the *chiA* mutant was done following Kung and Almeida (2011). Briefly, the entire *chiA* sequence, along with its own promoter, was cloned into a pAX1-Cm plasmid from Matsumoto et al., and then *X. fastidiosa* Temecula strain was transformed with this construct. The insertion of *chiA* in the non-coding region NS1 (neutral site 1) of *X. fastidiosa* chromosome was verified by PCR (data not shown). Fifteen plants have already been inoculated with the complemented *chiA* mutant, in parallel to the wild-type Temecula strain and the *chiA* mutant. Symptoms development will be surveyed over the summer to determine if the *chiA* mutant colonizes grapevines in greenhouse conditions. Experiments to confirm the restored capacities for the complemented *chiA* mutant to use chitin as a carbon source (Killiny et al. 2010) and to fully colonize insects during transmission process using electron microscopy are also ongoing.

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

i) Binding of transmission blocking molecules to different polysaccharides

We used different candidate peptides, previously tested in our transmission-blocking experiment, in a binding assay to assess or confirm their ability to bind to chitin or other related insect polysaccharides. Binding of seven *X. fastidiosa* candidates (PD1764, PD1764ΔLysM, FimA, ChiA, HxfAD1-3, HxfAD4 and BSA) has been tested on four different polysaccharides (chitin, colloidal chitin, chitosan and Avicel). Briefly, 1mg of each insoluble polysaccharide was incubated with 100µg of each of the 7 candidates in a 1-ml final reaction volume. Incubation was conducted at room temperature for 1h under shaking conditions (60rpm; rotary shaker with vertical orbital motion). Reaction mixtures were centrifuged at low-speed and supernatants were used to determine concentration of proteins not bound to polysaccharides. In order to normalize our results, the same binding assay was conducted for each candidate in absence of polysaccharides as the protein concentration measurable when no interactions occur in the system. Figure 2 below shows results obtained.

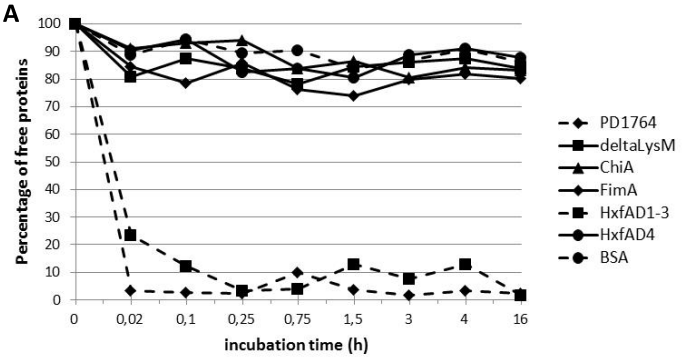


PD1764 and HxdAD1-3 strongly interacted with chitin (88% and 76% respectively) and colloidal chitin (83% and 77% respectively). This is in accordance with previous results showing that PD1764 could be specifically trapped on a chitin column whereas Hxfs proteins act as adhesins important for binding on insect cells (Killiny et al. 2009). No interactions were found with PD1764ΔLysM nor HxfAD4 reinforcing the suggested role for LysM and HAD domains respectively in chitin interaction. Interestingly, these two candidates were the ones having an effect on the disruption of *X. fastidiosa* leading to the conclusion that reduction of the transmission rate previously observed is a direct consequence of the interaction of transmission-blocking molecules on insect putative receptors.

In addition, HxfAD1-3 also interacts with chitosan (61% of proteins bound). The difference between chitin (or colloidal chitin) and chitosan polysaccharides is the presence of an acetyl group ($COCH_3$) on the main chitin subunit. Based on this result, the acetyl residue seems to play an important role in the binding of PD1764 on chitin-related polysaccharides whereas it doesn't seem to be a requirement for HxfAD1-3 interaction with such molecules. This is of interest because it could mean that these two peptides recognize different domains or different receptors.

In parallel, the same experiment as described above was conducted but interactions between different candidates and chitin was measured over the time (time course interaction). Results are presented in Figure 3A. Interestingly, more than 95% and 75% of interactions between chitin and PD1764 and HxfAD1-3 respectively occurred in the first minute of the binding assay. In addition, interactions are stable in our conditions for at least 16h. No significant interaction was detected for the other candidates whatever the time considered. This result suggest that both proteins have affinity for chitin which seems to be a requirement *in vivo* where interactions between *X. fastidiosa* proteins and insect receptors occur in a highly turbulent environment due to the simultaneous ingestion of xylem sap by the insect when feeding. This suggestion was confirmed in a third experiment in which dissociation constant (Kd, ligand concentration that binds to half the receptor sites at equilibrium) and Bmax (ligand concentration to saturate maximum number of binding sites) were calculated (Figure 3B).

A



B

Protein	K_d (µM)	B_{max} (µM of protein/g)
PD1764	1,34±0,27	3,67±0,21
HxfAD1-3	3,24±0,38	5,44±0,72

ii) Construction of transgenic plants expressing transmission-blocking molecules

The last goal of our project is to develop a technology using transgenic grapevines expressing transmission-blocking molecules directly in xylem. We decided to develop 3 different constructions based on the results obtained with PD1764. Sequences of the full-length protein but also PD1764ΔLysM and LysM domain were synthetized by GenScript after optimization of their sequences for their production in grapevines and introduction of a xylem-specific promoter. Each sequence was cloned into a plasmid carrying a 35S promoter and a translational enhancer specifically designed for strong protein expression. The plasmid is then introduced into an *Agrobacterium tumefaciens* strain to create a functional plant transformation system. This is ongoing (currently transforming *A. tumefaciens*) and the construct will be sent to the Ralph M. Parsons Plant Transformation Facility at University of California, Davis.

In parallel, plants expressing peptides HxfAD1-3 and HxfAD4 (used in blocking experiments, see previous report) have been produced by Bruce Kirkpatrick (UC Davis). Those plants are currently being tested as part of a collaborative project. This is of great interest because HxfAD1-3 was responsible for a partial but significant decrease in the transmission of *X. fastidiosa*. Early testing of these plants will also help us identify and troubleshoot potential experimental problems with the experimental design used for the identification of transmission-blocking peptides.

CONCLUSIONS

This project is proceeding very well. The most immediate result was the identification of PD1764 as a protein that blocked *X. fastidiosa* transmission to plants under greenhouse conditions. That work was described in the previous report. Here we follow up on what was done since that. Mainly, these last three months were spent on different mutants/constructs that will be used during the summer period in greenhouse tests. Importantly, the construction of 3 optimized transmission-blocking molecules is of great interest as they may improve the efficiency of our strategy.

The confirmation of the specific interaction of LysM and HAD domains with chitin related polysaccharides also confirm that the blocking effect seen when using these peptides as transmission-blocking molecules is due to the direct interaction of these domains to putative insect receptors. In addition, differences in their ability to bind to these different polysaccharides (chitosan, N-acetyl group) suggest that these domains could recognized different receptors on the insect cuticle and be complementary rather than redundant. This is promising for our test using both domains fused together as an optimized transmission-blocking construct.

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