Progress Report for CDFA Agreement Number 10-0275

Title of Project
Blocking *Xylella fastidiosa* transmission

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Time Period Covered by the Report
April 2012- June 2012

Introduction
The goal of this project is to develop a technology to block the vector transmission of *Xylella fastidiosa* to plants. In order to understand how interactions between *X. fastidiosa* and its insect vectors can be disrupted, experiments conducted in this project previously showed that carbohydrate-binding proteins are essential for colonization of insect vectors, likely functioning as adhesins during the first contact between insect and pathogen. Based on that work, we demonstrated that blocking of *X. fastidiosa* transmission using generic molecules is a feasible approach to control disease spread (Killiny et al. 2012).

This report resumes our efforts made to identify new transmission-blocking proteins, we report on two new candidates for the moment. It also focuses on the preparation of these candidate proteins, both new and already identified, in order to test them as competitors for *X. fastidiosa* transmission to plants.

List of Objectives – as in the approved research proposal

Objective 1. Molecular characterization of the X. fastidiosa-vector interface
Objective 2. Identification of new transmission-blocking chitin-binding proteins

Objective 1 was completed in a timely manner, some of the results have been published and a few papers are now being drafted. Essentially, we showed that:

i) *X. fastidiosa* utilizes chitin as a carbon source,
ii) tested 20 mutants in relation to their transmissibility - greenhouse assays,
iii) performed mRNA-seq studies to analyze gene expression patterns to better understand *X. fastidiosa* colonization of vectors and to identify new targets to block transmission,
iv) showed that protein-carbohydrate interactions are very important for *X. fastidiosa* colonization of vectors and that disrupting these interactions blocks transmission of the pathogen to grapevines under greenhouse conditions.

Efforts focusing on Objective 2 are described in this report.

Description of activities

Objective 1. Molecular characterization of the X. fastidiosa-vector interface
As mentioned above, activities associated with Objective 1 have been previously reported, and some of that work has been published in peer-reviewed journals. We refer the reader to those sources. However, if the reader would like to have a detailed description about those efforts the Principal Investigator (Almeida) would be happy to provide that information via phone or email.

Objective 2. Identification of new transmission-blocking chitin-binding proteins
The specific goal of this Objective was to identify *X. fastidiosa* novel chitin-binding proteins using a proteomics approach.

Description of the experiment leading to the detection of ten proteins bands, specifically eluted from a chitin column, has been detailed in the previous report covering period December 2011-March 2012. Unfortunately, due to incompatibilities between proteins concentrations after trypsinization and the limit of detection of the mass spectrometer used for proteins identification (UC Davis facility), subsequent analyses enabled us to only identify two new candidates out of the ten bands. Efforts are currently being made to increase protein concentration after trypsinization to circumvent this limitation. This will be done by increasing initial amount of total *X. fastidiosa* proteins used to load the chitin column or by pulling together several specific elutions obtained from identical independent experiments. Nevertheless, mass spectrometry analyses allow us to identify two proteins in proteins bands located at 42kDa and 30kDa (see previous report for picture of the proteins bands), named respectively MopB (PD1709) and PD1764 based on the genome of the *X. fastidiosa* Temecula strain.

First, MopB is the major outer membrane protein of *X. fastidiosa*, distributed over the surface of the bacteria (Bruening et al., 2005). As a major outer membrane protein, MopB is abundant in the *X. fastidiosa* proteome compare to others proteins. Identification of abundant proteins at this point requires additional verification in order to confirm the specificity of its affinity to chitin.

The other candidate, PD1764, is a hypothetical protein conserved in several in *X. fastidiosa*. BlastP analyses showed a high conservation rate with a LysM containing-domain protein, present in several species of *Xanthomonas* (99% identity). LysM (for lysine motif, Pfam PF01476) domains are known for their role in plant immune responses where they can serve as receptors for the recognition of common microbe associated molecular patterns (MAMPs) (for a review, Gust et al., 2012). Interestingly, those MAMPs are generally composed of N-acetylglucosamine (GlcNAc)-containing molecules and LysM motifs have been shown to recognize and bind to numerous of those compounds, such as chitin of pathogenic fungi (Ohnuma et al., 2008). More recently, the LysM motif *Lactococcus lactis* was shown to bind to fungal chitin cell wall material (Visweswaran et al., 2012). Altogether, PD1764 is a very interesting candidate as a chitin-binding protein. Moreover, this finding validates our method for detection of potential chitin-binding proteins, and we will continue our efforts to identify the other protein bands previously reported. To our knowledge, this is the first example of a *X. fastidiosa* protein containing an already described chitin-binding domain.

Even if the identification of a definitive list of transmission blocking peptides is delayed due to the technical issue for protein identification, we already have started making constructs to express proteins/peptides we already know to be involved in transmission so that testing of candidates can be initiated this summer, as previously planned.

Preparation of candidate proteins and peptides

In addition to the search for novel chitin-binding proteins described above, a targeted alternative based on the assumption that *X. fastidiosa* proteins we have identified as important for sharpshooter colonization could be used to block transmission to plants (i.e. Hxfs, XadA, ChiA and FimA) is also being conducted. Based on the work described in this report, protein PD1764 has been added to this list.

Work-flow for preparation of recombinant proteins: example of His6-ChiA

As an example, figure 1 (below) illustrate all significant steps conducted since the last report for one of the recombinant proteins/peptides potentially involved in *X. fastidiosa* transmission, His6-ChiA.

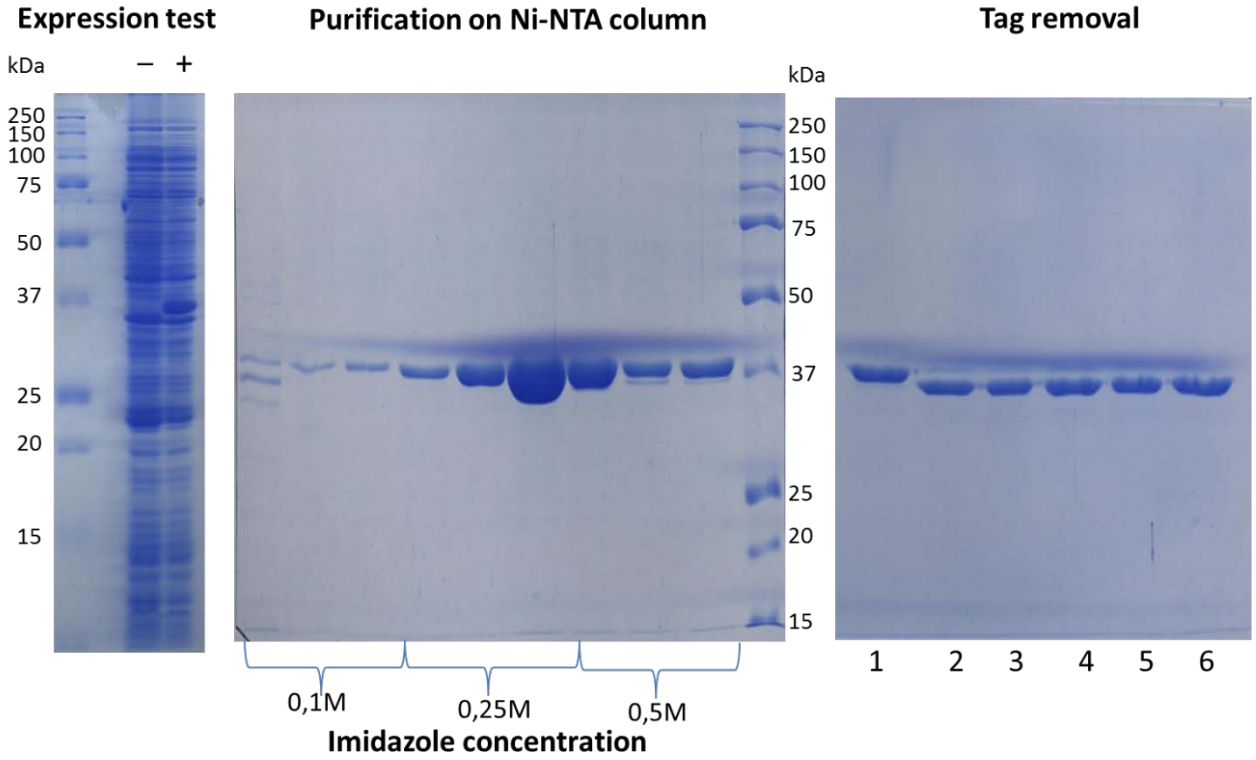

Figure1:

Expression test: Induction (+) with 1mM IPTG during 4h at 28°C of His6-ChiA cloned into pET28b expression vector (Novagen) transformed into *E.coli* Rosetta strain. Expression of the recombinant protein is clearly visible at 35kDa compared to negative control without induction (-) with IPTG.

Purification on Ni-NTA column: His6-ChiA protein is loaded on top of a Ni-NTA column. After several washes (data not shown), elution proteins is made with increasing concentrations of

imidazole, a specific competitor of histidine residues for nickel. Bands of purified His6-ChiA protein are visible at 35kDa without any impurities.

Tag removal: After a desalting step in order to eliminate imidazole, the His6-tag is removed by the action of a protease called thrombin, which specifically cleaves between the histidine tag and the protein. Thrombin was added to the protein suspension (5units NIH per mg of protein) at RT. Action of the protease was stopped by addition of 1mM of PMSF. Lane1, His6-ChiA (no treatment); lane2, 30minutes of thrombin action; lane3, 1h; lane4, 2h; lane5, 3h; lane6, 4h. Shift in the molecular weight between lane1 (control) and other lanes indicates removal of the histidine tag. Detagged ChiA could be seen for the first treatment (30minutes) but some traces of tagged proteins could be detected. 2h of treatment seems to be the first satisfactory condition (no tagged protein detected and relatively short protease action time avoiding unspecific cleavage and protein degradation. This successful histidine tag removal also indicates that the protein is correctly folded.

Table 1 below summarizes proteins targeted in this study and steps done and to be done in order to use these molecules as competitors for disrupting *X. fastidiosa* transmission by vectors.

proteins / steps	chiA	FimA	XadA	HxfB (Voegel et al.)			PD1764		
				AD1-3	AD4	AD5	full-lengh	without LysM domain	LysM domain only
PCR amplification	✓	✓	✓	✓	✓	✓	✓	✓	✓
cloning into pET28b	✓	✓	✓	✓	✓	✓	✓	✓	✓
sequence verification	✓	✓	⚠	✓	✓	✓	✓	✓	✓
Expression test	✓	✓		✓	✓	✓	✓	✓	✓
Purification	✓	✓		✓	✓	✓	✓	✓	⚠
Tag excision	✓	✓		⚠	⚠	⚠	✓	✓	

Table1: Work progress for preparation of recombinant proteins and peptides required for disruption of transmission

Except for XadA for which we encountered cloning difficulties, we are glad that preparations of all others proteins are going well and candidates are, or will be really soon, ready to be tested as transmission-blocking molecules in our *in vivo* artificial diet system (Killiny and Almeida 2009b) in the near future.

Preliminary experiments, testing which concentration of proteins optimal to disrupt *X. fastidiosa* transmission, have been performed and results are pending. Such experiments have been conducted with PD1764 full-lengh, ChiA and FimA using 5 different concentrations (250mM, 100mM, 50mM, 25mM and 10mM). In parallel, survival of insect acquiring these same

concentrations of recombinants proteins is also being tested under greenhouse conditions. Once results are known transmission experiments using the optimal concentration of proteins (considering disruption and survival rates) will be conducted for all proteins.

Publications and presentations related to the funded project

Peer-reviewed publications

Almeida, R.P.P., Killiny, N., Newman, K.L., Chatterjee, S., Ionescu, M. and Lindow, S.E. 2012. Contribution of *rpfB* to cell-cell signal synthesis, virulence, and vector transmission of *Xylella fastidiosa*. Molecular Plant-Microbe Interactions 25: 453-462.

Killiny, N., Rashed, A. and Almeida, R.P.P. 2012. Disrupting the transmission of a vector-borne plant pathogen. Applied and Environmental Microbiology 78: 638-643.

Chatterjee, S., Killiny, N., Almeida, R.P.P. and Lindow, S.E. 2010. Role of cyclic di-GMP in *Xylella fastidiosa* biofilm formation, plant virulence and insect transmission. Molecular Plant-Microbe Interactions 23: 1356-1363.

Killiny, N., Prado, S.S. and Almeida, R.P.P. 2010. Chitin utilization by the insect-transmitted bacterium *Xylella fastidiosa*. Applied and Environmental Microbiology 76: 6134-6140.

We also expect to publish a few more manuscripts based on work already done testing several *X. fastidiosa* mutants for their transmissibility. Transcriptomics work (mRNA-seq) yielded high quality data that are now being analyzed, a couple of manuscripts are expected from that work as well. We are not including reports at the PD/GWSS symposium on this list as those are required by this funding program and archived by CDFA.

Presentations

Talk: Host switching in a vector-borne plant pathogen. Essig Museum talk series, University of California Berkeley. March 2012.

Talk: Ecology of *Xylella fastidiosa* diseases. International Congress for Bacterial Diseases of Stone Fruits and Nuts. Joint Meeting European Cost 873 and ISHS. Zurich, Switzerland. February 2012.

Talk: New insights on the vector transmission of *Xylella fastidiosa*. Pierce's Disease Research Symposium, Sacramento, CA, sponsored by CDFA. December 2011.

Talk: Regulatory networks and the leafhopper transmission of the bacterium *Xylella fastidiosa*. Annual Meeting of the Entomological Society of America, Reno, NV. November 2011

Talk: Regulation of host switching and transmission in *Xylella fastidiosa*. Hemipteran-Plant Interactions Symposium, Piracicaba, Brazil. July 2011.

Talk: Vector transmission of *Xylella fastidiosa*. Departmental seminar, Department of Entomology, sponsored by University of California, Riverside. March 2011.

Talk: Blocking the transmission of a leafhopper-borne bacterial pathogen. Facing the Challenges of Vector-Borne Diseases in the 21st Century Symposium, Center for Disease Vector Research, UC Riverside. Riverside, CA. March 2010.

Research Relevance Statement

Current efforts to control Pierce's disease focus on either reducing pathogen populations after plant infection or reducing vector populations. This project focuses on vector-pathogen interactions and how to disrupt them, effectively leading to reduced *X. fastidiosa* spread in vineyards. Initial work characterizing these interactions lead to the hypothesis that *X. fastidiosa*

chitin-binding adhesins were responsible for adhesion to vectors. This was tested using lectins and carbohydrates, when we showed that transmission can be blocked *in vivo* (already published). Current efforts are aimed at identifying and testing chitin-binding proteins/peptides for their *in vivo* activities. This research will contribute towards finding solutions to Pierce's disease by exploiting an essential component of this disease system, i.e. vector transmission, which represents an untapped source of novel control strategies.

Layperson Summary of Project Accomplishments

This project had two goals. First, to characterize *X. fastidiosa*-vector interactions so that knowledge about how the pathogen colonizes sharpshooters was improved. This work was successfully performed, resulting in proof-of-concept experiments under greenhouse conditions that clearly demonstrate the feasibility of blocking sharpshooter transmission of *X. fastidiosa* to grapevines. The second goal, to identify specific transmission-blocking candidate proteins is now is its final phase (i.e. sequencing and identification itself) and we have started to express and purify already available candidates for *in vivo* testing this summer. Altogether, we expect a final list of candidates that have been already tested *in vitro* and *in vivo* in the greenhouse to be available by the end of 2013, when planning for larger experiments would be initiated.

Status of Funds

We were granted a no-cost extension to finish ongoing experiments with those funds, a limited amount of funds was still available. Those have already been allocated and we expect that a final report will be submitted once the funds have been spent.

Summary and Status of Intellectual Property

None expected so far, but PIPRA has been contacted in this regard and will be consulted if IP issues become relevant.

Literature Cited

Bruening G, Civerolo EL, Lee Y, Buzayan JM, Feldstein PA, Re E. 2005. A major outer membrane protein of *Xylella fastidiosa* induces chlorosis in *Chenopodium quinoa*. Phytopathology 95: S14.

Gust AA, Willmann R, Desaki Y, Grabherr HM, Nürnberger T. 2012. Plant LysM proteins: modules mediating symbiosis and immunity. Trends in Plant Science: in press.

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Visweswaran GRR, Dijkstra BW, Kok J. 2011. Murein and pseudomurein cell wall binding domains of bacteria and archaea—a comparative view. Appl Microbiol Biotechnol. 92: 921–928.

Voegel TM, Warren JG, Matsumoto A, Igo MM, Kirkpatrick BC. 2010. Localization and characterization of *Xylella fastidiosa* haemagglutinin adhesins. Microbiol. 156:2172-2179.