ISOLATION, CHARACTERIZATION AND GENETIC MANIPULATION OF *XYLELLA FASTIDIOSA* HEMAGGLUTININ GENES

Project Leader:
Bruce Kirkpatrick
Dept. of Plant Pathology
University of California
Davis, CA 95616
bckirkpatrick@ucdavis.edu

Cooperators:
Tanja Voegel
Dept. of Plant Pathology
University of California
Davis, CA 95616
tmvoegel@ucdavis.edu

Michele Igo
Section of Microbiology
University of California
Davis, CA 95616
mmigo@ucdavis.edu

George Bruening
Dept. of Plant Pathology
University of California
Davis, CA 95616
gebruening@ucdavis.edu

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ABSTRACT

Xylella fastidiosa (*Xf*) possesses genes for hemagglutinins (HAs), large adhesion proteins involved in cell-cell aggregation and biofilm formation. Mutations in either one of the functional HAs, HxfA (PD2118) or HxfB (PD1792), result in hypervirulent strains that move faster and cause more severe disease in grapevines. We divided *Xf* HAs into smaller domains and generated antibodies against 3 possible adhesion domains (ADs) responsible for cell-cell and/or cell-surface binding. The abs were used in Western blot analysis and determined that HxfB was approximately 220kDa while HxfA was slightly larger. Western blot analysis showed that both HAs are secreted into the culture supernatant as well as being found in the bacterial outer membrane. Monomeric Fab fragments against AD1-3 and AD4 of HxfB reduced cell-cell aggregation when added to *Xf* cells growing in liquid culture.

Xf HxfB will be expressed in transgenic tobacco and grapevines where we hope the protein will act as a "molecular glue" to aggregate insect-inoculated *Xf* cells, retard their ability to systemically colonize plants and potentially provide a unique form of resistance against PD.

INTRODUCTION

Xylella fastidiosa (Xf) hemagglutinins (HAs) are large secreted proteins that play important roles in mediating cell-cell contact and plant pathogenicity. Mutations were made in both *Xf* HA genes, HxfA (PD2118) and HxfB (PD1792), by transposon mutagenesis and the resulting mutants did not form aggregates in liquid culture and they had reduced biofilm formation *in vitro* and *in planta* (1). When inoculated into grapevines the mutant cells showed hypervirulence and more rapid colonization of xylem vessels (1). The premise of this research is to determine whether over-expressing *Xf* HA adhesion domains in the xylem by transformation of grapevines, the HA will act as a "molecular glue" to clump *Xf* cells and retard their ability to systemically colonize grapevine and cause Pierce's disease (PD).

Because of the large size of the HA genes (10kb), it is difficult to transform grapevines with the whole HA gene. Therefore we are trying to identify the active adhesion domains (ADs) responsible for cell-cell aggregation by dividing the HA genes into several smaller fragments that should contain the cell-cell AD. Recombinant proteins derived from these fragments were expressed in *E. coli*, purified and injected into rabbits to produce AD specific antisera. The resulting antisera was used in ELISA, Western blot analysis, immunolocalization studies and cell-cell clumping experiments to determine which of the HA fragment(s) contain functional ADs that could later be transformed into plants.

OBJECTIVES

1. **a.** Use antibodies we have prepared against a conserved, putative binding domain (AD2) that is present in both *Xf* hemagglutinins (HA) to determine the native size and location of *Xf* HA in cultured *Xf* cells and PD-affected grapevines.
   b. Determine if these antibodies (Fab fragments) can prevent cell-cell clumping in liquid *Xf* cultures.
   c. Prepare an affinity column using HA domain antibodies and isolate native *Xf* HAs from culture cells. Establish the identity of affinity purified, putative HAs by N-terminal sequencing.
   d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells.
   e. Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies. Determine if HxfA and B specific antibodies can block cell-cell clumping of *Xf* grown in liquid medium.
2. **a**. PCR-amplify, clone and express as fusion proteins, additional hypothetical adhesion domains of HxfA and B.
   b. Prepare rabbit polyclonal antibodies against each Hxf A/B domain fusion protein.
   c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.
3. **a.** Transform tobacco, an experimental host of *Xf* and an easily transformable plant, with *Xf* HA binding domains. Use antibodies prepared in Objective 2 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.
   b. Mechanically inoculate HA-transgenic tobacco with wild type (wt) *Xf* cells. Compare disease progression and severity in transgenic tobacco with non-protected controls.

RESULTS

Objective 1a. Determination of native size and location of *Xf* HA in *Xf* cultured cells and grapevines by Western blot analysis using AD2 antibodies. Western Blot analysis with AD2 abs using a variety of protein samples showed a faint band of ~220kDa corresponding to HA. But the signal was very weak and there were non-specific interactions between other *Xf* cellular proteins and AD2 abs (2). Objective 1a was repeated using higher quality abs (see objective 2b).

Objective 1b. Determine if AD2-antibodies can facilitate clumping of *Xf*. Due to the low quality of AD2 abs, there was no difference in *Xf* cultures treated with AD2 abs versus cultures treated with preimmune serum (2). Objective 1b was repeated using higher quality abs (see objective 2c).

Objective 1c. prepare affinity column using HA domain antibodies to isolate native HAs from culture cells. Since HAs are secreted into the medium (see objective 2b), concentrated culture supernatant was used to isolate native HAs. Antibodies against AD1-3 and AD4 (see objective 2a) were bound to a Pierce protein A column and 1l of culture supernatant was concentrated and passed through the column according to the manufacturer's instructions. Neither denatured nor native supernatant protein samples interacted with the abs bound to the column and no HAs could be eluted from the column. Additional immunocapture strategies are being evaluated.

Objective 1d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells. In order to determine if HA fusion proteins can bind to *Xf* cells, ELISA plates have been coated with AD1-3 or AD4 fusion protein (see objective 2) and incubated with Wt, HxfA and HxfB mutant cells. Results of this one experiment, which needs to be repeated, suggests the denatured HA AD fusion proteins did not bind to and trap *Xf* cells. This suggests that the whole HA protein may be needed for binding to cells or that only cell bound HA has effective binding properties or that AD1-3 and AD4 did not contain the cell binding domain.

Objective 2a. Identification, cloning and expression of additional ADs for antibody generation

It has been shown for FHA, the filamentous hemagglutinin of *Bordetella pertussis* (3) that the active HA domains are located at the N-terminal half of the protein and that C-terminal deletions have no effect of the HA activity or secretion of the protein.

The secretion domain (TPS-domain) was identified at the N-terminal end of HxfA and HxfB (1) and RGD (Arg-Gly-Asp) sites at position 2780 in HxfA and at positions 1805 and 3103 in HxfB were found (Figure1). The RGD site in FHA of *Bordetella pertussis* mediates binding of *B. pertussis* to lung epithelial cells (4).

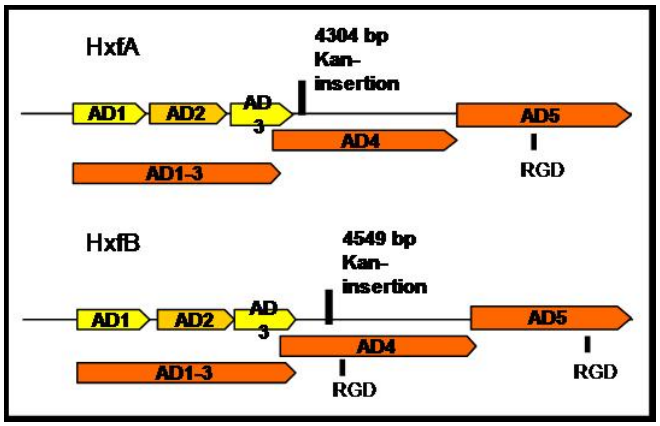

Figure 1. Identification of putative *Xf* HA adhesion domains (ADs) based on data base analysis. Antibodies against AD1-3 and AD4 of HxfB were generated and used in Western blot analysis.

Data base analyses led us to divide HxfA and HxfB into 3 domains named AD1-3, AD4 and AD5, each for HxfA and HxfB (Figure 1). All 6 ADs were cloned into the protein expression plasmid pet30b, the identity and integrity of the amplified fragments was verified by sequencing and the constructs were transformed into *E.-coli*. AD1-3 and AD4 of HxfB were expressed to high levels and the integrity of the affinity purified proteins was verified by sequencing.

Objective 2b. Prepare rabbit polyclonal antibodies against each HxfA/B domain fusion protein. Purified AD1-3 and AD4 antigen were each injected into two rabbits and sera obtained. In contrast to AD2 (objective 1), indirect ELISA showed that high titer abs were obtained in all injected rabbits.

Determine the native size and location of *Xf* HA in *Xf* cultured cells using AD1-3 and AD4 antibodies. Outer membrane proteins isolated from Wt, HxfA and HxfB mutants by sucrose gradients (protocol of Michele Igo) revealed that HxfA and HxfB are inserted into the outer membrane of *Xf*. Both HAs are ~220kDa with HxfA being slightly larger (Figure 2). In Wt samples a doublet of bands is detectable since the AD4 antibodies can detect both HxfA and HxfB. Supernatants of *Xf* cultures concentrated by PEG and extracted using phenol/methanol also revealed bands of 220kDa (Figure 2). HxfA is again slightly larger than HxfB. This shows that HAs are not only inserted into the outer membrane, but also are secreted into the culture medium.

To evaluate if HAs are also secreted into the xylem of infected grapevines, xylem sap was obtained by using a pressure bomb (5). The proteins were purified as described for supernatant proteins and subjected to Western blot analysis. No bands corresponding to *Xf* HAs could be detected in either Wt or HA mutant cells although other xylem proteins were detectable on a coomassie blue stained control gel.

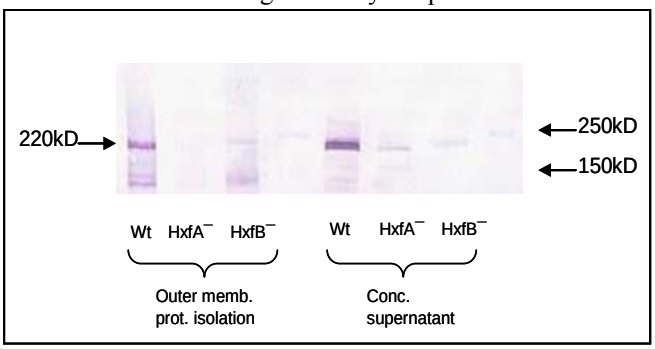

Figure 2. Western Blot analysis using AD4 abs shows that HAs are both inserted into the *Xf* outer membrane and secreted into liquid medium. HxfA is approximately 220kDa and slightly larger then HxfB.

Objective 2c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium. Incubation of *Xf* cells with AD1-3 and AD4 abs resulted in an increase of clumping due to cross linking of the cells. To identify the ADs on the HA protein that are responsible for cell-cell aggregation, monomeric Fab fragments were generated from AD1-3 and AD4 IgG. IgG was purified using protein A column chromatography and digested with papain. The Fab fragments were purified by protein A column chromatography and eluted from Tris/HCl gels. Monomeric Fab fragment, which contains the HA antigen recognition site was used in the clumping experiments. We would expect to see a decrease in clumping in the *Xf* cultures treated with Fab fragment if the abs were made against a possible HA cell-cell binding domain. We observed a decrease in clumping after inoculation of various amounts of both Fab fragments which suggests that the cell binding domains reside in AD1-3 and AD4 (Figure 3).

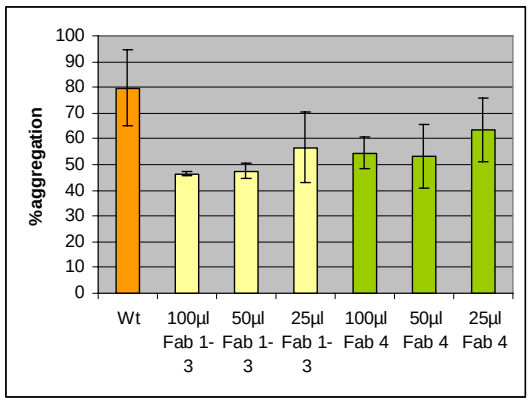

Figure 3. Blocking of cell-cell aggregation by adding of Fab fragments against AD1-3 and AD4 suggests that cell-cell binding domains are contained in AD1-3 and AD4.

Objectives 3 and 4 Our data suggests that large portions of HA protein will be needed for efficient clumping of *Xf* cells. Therefore we will try to transform plant hosts (tobacco and grapevines) with a DNA fragment containing both AD1-3 and AD4. Arrangements with the Plant Transformation facility on the UC Davis campus have been made and plasmids needed for transformation have been obtained from the Dandekar lab. These plasmids will be used to fuse the pear signal peptide pGIP to the HA fragment which will direct the HA fusion protein into the xylem using a method called splicing by overlap extension. This method was successfully used by the Dandekar lab to transform grapevines (6).

Additional work not included in original project objectives: HAs contain an N-terminal secretion domain for secretion mediated by bacterial typeV secretion (two-partner secretion pathway, TPS). In silico analysis identified a putative β-barrel forming secretion partner of HAs that is inserted in the outer membrane (PD1933) and we created knock-out mutants (insertion and deletion) of PD1933. These mutants were grown and supernatant proteins were isolated as described in objective 2b. The samples were used in Western blot analysis for detection of secreted HAs. No HAs were detected in the supernatant of mutant cells, whereas HA proteins were found in wt cells. This indicates that HAs in *Xf* are secreted via the TPS pathway and that PD1933 represents their secretion partner.

We are also in process to generate a HxfA/HxfB double mutant with a chloramphenicol cassette obtained from Ayumi Matsumoto (Michele Igo).

CONCLUSIONS

By generating high quality abs we could show that *Xf* HAs are processed upon or during secretion because the predicted size of the ~10kb HA gene product (365 kDa) was not be detected, instead proteins of approximately 220kDa were identified. We also showed that *Xf* HAs are inserted into the bacterial outer membrane as well as secreted into liquid medium. Since the isolation of native HAs using HA abs in affinity column chromatography was not initially successful, we will now use FPLC to isolate native HAs for additional experiments. Native HAs will be sequenced to identify the processing/cleavage site. The cell clumping experiments using Fab fragments suggest that cell-cell binding domain(s) reside in AD1-3 and AD4. Therefore a portion of the HA gene containing AD1-3 and AD4 will be expressed in tobacco and grapevine that will be subsequently challenged with *Xf* to determine if these proteins can bind to and retard the systemic movement of *Xf* in plant hosts.

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