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

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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. Computer analyses of the HA proteins identified several regions that might be possible adhesion domains (ADs) responsible for cell-cell and/or cell-surface binding. We cloned 6 *Xf* HA fragments that may contain potential ADs into protein expression vectors and to date have prepared antibodies against 1 AD protein fragment that is conserved in both HxfA and B. Recombinant proteins from the other 5 ADs are being purified and prepared for injection. Western blot analyses of using *Xf* proteins extracted from *Xf* cells grown in liquid culture showed a very faint reaction with an *Xf* protein of approximately 220kd but this result needs to be confirmed using a higher quality antibody. Recent discoveries in the Bruening lab using phage technology indicate that HA are more abundant in cells grown on solid medium compared to liquid medium and we are now repeating our Western analyses using cells grown on solid medium. Once *Xf* HA cell-cell binding domains are identified they will be expressed in transgenic tobacco and grapevines where we hope the proteins 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 (200-300kD) 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* (Guilhabert and Kirkpatrick 2005). When inoculated into grapevines the mutant cells showed hypervirulence and more rapid colonization of xylem vessels (Guilhabert and Kirkpatrick 2005). The premise of this research is to determine whether over-expressing *Xf* HA adhesion domains in the xylem, either by transformation of grapevines or inoculation of grapevines with HA expressing endophytes, the HA will act as a "molecular glue" which clumps the *Xf* cells and retards their ability to systemically colonize grapevine and cause Pierce's disease (PD).

Because of the large size of the HA genes (10kb), we cannot 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 we believe will contain the cell-cell AD. Recombinant proteins derived from these fragments are being expressed in *E. coli*, purified and injected into rabbits to produce AD specific antisera. The resulting antisera will be 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 can later be transformed into plants.

## OBJECTIVES
1a. Use antibodies we have prepared against a conserved, putative binding domain (AD2) that is present in both *Xf* hemagglutinins (HA), which we have named HxfA and HxfB, 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.

2a. 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.

3a. Transform bacterial grapevine endophytes with portion(s) of the Xf HA domains that mediate cell-cell clumping. Determine if transformed endophyte cells can bind *Xf* cells in vitro.
b. Transform tobacco, an experimental host of *Xf* and an easily transformable plant, with *Xf* HA binding domains. Use antibodies prepared in Objective 1 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.

4a. Mechanically inoculate grapevines with *Xf* HA-expressing grapevine endophytes.
b. Mechanically inoculate endophyte colonized grapevines and HA-transgenic tobacco with wild type (wt) *Xf* cells. Compare disease progression and severity in endophyte colonized grapevines and transgenic tobacco with non-protected controls.

**RESULTS**

**Objective 1a. Cloning of adhesion domain 2 (AD2) of HxfA and antibody generation**

Because of the sequence similarity between AD2 of HxfB and HxfA, a 1133bp fragment from HxfA (Figure 1) was PCR-amplified, cloned in the expression vector pet29b and expressed as His-fusion protein in *E. coli.* The expressed protein was purified by Ni-column chromatography and the identity and integrity of the fusion protein was verified by sequencing. A total of 1mg purified protein was injected into a NZW rabbit after pre-immune serum was taken. Five bleeds were taken and the resulting anti-AD2 antibodies were analyzed by ELISA. Only a 2-fold signal increase occurred in indirect ELISA analyses of post-injection antisera compared to the pre-immune serum; this result indicated the AD2 antigen was not especially antigenic or that insufficient antigen was used to elicit high quality Abs. Although the antiserum was of comparative low titer, we used it in *Xf* cell clumping and Western blot analyses.

**Determination of native size and location of *Xf* HA in *Xf* cultured cells and grapevines by Western blot analysis**

Several Western blot experiments with a variety of protein samples of wild type Temecula, $HxfA^-$ and $HxfB^-$ mutants were conducted. The cells were grown in liquid $PD_3$ or $XDM_2$ (Leite et al. 2003) media for 10 days and 100ml of conditioned medium was concentrated using Centricon plus 70 filters (Millipore). The conditioned medium was purified by dialysis and ReadyPrep 2-D Cleanup Kit Biorad) and protein concentration was determined by using a Bradford assay. Western blots of whole cells and medium proteins were performed using the AD2 antibody. A faint band of ~200kDa could be detected in wild type whole cell protein, but not in proteins in purified medium samples suggesting that HAs are possibly not secreted but associated with the outer membrane of *Xf* cells. A corresponding band was not detected in whole protein extracts of the HxfA and B mutants (Figure 2). However, the signal was weak and there were non-specific reactions between other *Xf* cellular proteins and the AD2 Abs and additional experiments, preferably using higher quality antibodies produced against the other *XF* HA fusion proteins, will need to be done in order to confirm these preliminary results.

**Objective 1b. Determine if AD2-antibodies or conditioned media can facilitate clumping of *Xf*.**

A mixture of the $4^{th}$ and $5^{th}$ bleed AD2-antibodies was used to assess their ability to clump *Xf* cells in liquid culture. If the antibodies bound to HA domains that are responsible for cell-cell clumping, we might expect to see a decrease in clumping if the HAs were not located on the surface of the *Xf* cell which is what would be expected because other bacterial HAs are secreted. If the HA was physically linked to the *Xf* cell we would have expected an increase in clumping due to cross-linking *Xf* cells by the antibody. We observed no significant differences in clumping between antibody-treated and pre-immune serum which suggests that the AD2 domain is not present on the outside of *Xf* cells if the HA is associated with the outer membrane or that the AD2 does not mediate cell-cell interactions. These results indicate that additional putative ADs must be evaluated.

Experiments were also conducted with "conditioned" media. Conditioned media is PD3 medium in which wt *Xf* cells were grown to stationary phase and the cells were removed by centrifugation. Such medium would be expected to contain HA proteins if they were secreted. By adding HA-containing conditioned media in various concentrations to $HxfA^-$ and $HxfB^-$ mutant cells growing in log phase we would expect to complement with soluble HA and restore the clumping phenotype. No clumping was observed which is indirect evidence that *Xf* HA is not secreted into the medium, at least in large amounts.

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

It has been shown for FHA, the filamentous hemagglutinin of *Bordetella pertussis* (Renauld-Mongenie et al. 1996) 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 B (Guilhabert and Kirkpatrick 2005) and additional analysis revealed a FhaB domain located between position 2000-2300 of

HxfA. This conserved domain is found in other bacterial proteins that mediate adhesion and therefore they could be act as a possible adhesion domain. To evaluate this hypothesis, a knockout in that region will be done, and the FhaB domain will be replaced by the kan-cassette. We also conducted hydrophobicity plots of the proteins to determine potential antigenic sites. An RGD (Arg-Gly-Asp) site at position 2780 in HxfA and at position 3062 in HxfB was found. The RGD site in the filamentous HA proteins of *Bordetella pertussis* mediates binding of B. pertussis to lung epithelial cells (Ishibashi et al. 2001).

The *in silico* analyses led us to divide HxfA and HxfB into three regions named AD1-3, AD4 and AD5, each for HxfA and HxfB (Figure 1). All six ADs were cloned into expression plasmid pet30b, the identity and integrity of the amplified fragments was verified by sequencing and the constructs were transformed into *E.-coli*. Vectors containing AD5 of HxfA and AD4 of HxfB and were transformed into the new expression *E. coli* host (ArcticExpress DE3, Stratagene). AD4 of HxfB was expressed to high levels and the integrity of the protein verified by sequencing. AD 4 antigen has been prepared and will be injected into rabbits on October 27. AD1-3 from HxfA and B, as well as AD4 from HxfA were also expressed at high levels and we are now verifying the sequence of these proteins with the anticipation that they will also be ready to inject into rabbits. Recombinant fusion proteins of AD5 of both HxfA and B seemed to be slightly smaller in size than expected so additional characterization of these proteins will be needed before preparing antisera.

**Objectives 3 and 4:** It will be necessary to identify HA cell-cell binding domains before these fragments can be transformed into plant hosts (tobacco and grapevines) or bacterial endophytes of grapevines. Good progress has been made towards identifying fragments of the *Xf* HAs that mediates cell-cell binding in the three months this project has received funding.

In addition, a collaborative effort between the Bruening and Kirkpatrick labs has identified M13 phage that can specifically bind to HxfA (see Bruening 2006 PD/GWSS report for details). This phage can be used much like the antibodies that are being raised against the various *Xf* HAs ADs described in this report. Our labs are now pursuing collaborative research to use the phage to determine the size and location of native *Xf* HAs and to use the specificity of the HxfA-specific phage to better understand the interactions that mediate *Xf* cell-cell attachment.

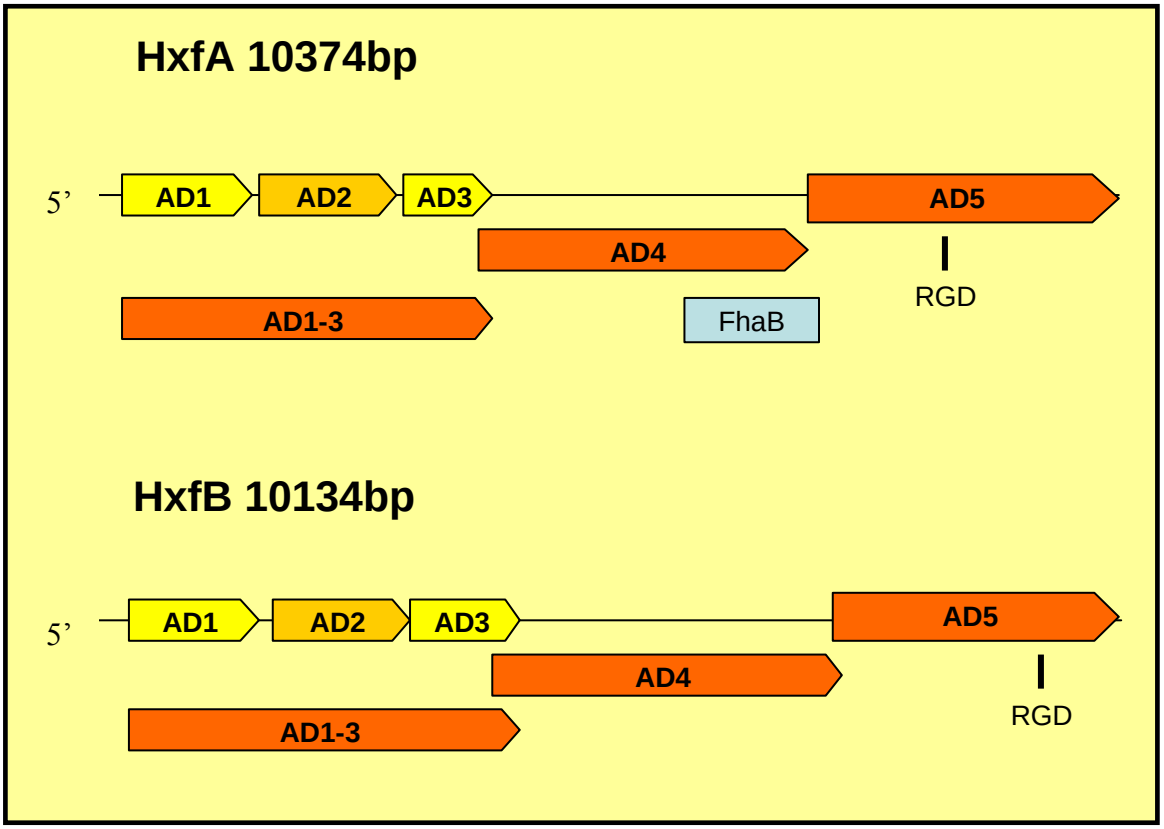
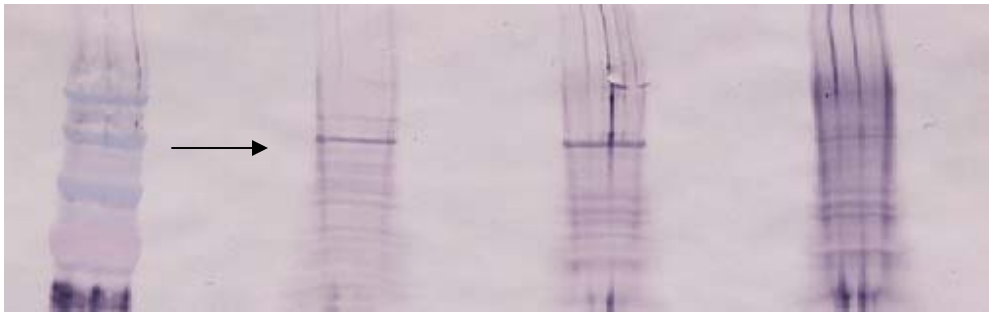



**Figure1.** Identification putative *Xf* HA adhesion domains (AD) based on data base analysis with other bacterial HAs and hydrophobicity plots. Antibodies made against AD2 of HxfA provided preliminary information regarding the size and locations of native HA. AD1-3, AD4 and AD5 of both HxfA and HxfB were cloned and expressed in *E. coli*. AD4 and AD1-3 from both HxfA and B are ready to be injected into rabbits while further characterization of AD5 from both HAs will be needed.



**wt Temecula** **HxfA$^-$** **HxfB$^-$**

**Figure 2.** Western Blot analysis of whole cell proteins from wild type Temecula, HxfA and HxfB mutants. Arrow denotes a protein band of ~200kDa that is detectable in the wild type, but not in the mutant cells.

## CONCLUSIONS

We have made good progress in our goal of identifying what HA domain(s) mediate cell-cell binding. Changes in the manner in which we express HA fragments in *E. coli*, i.e. using a different expression system (ArcticExpress DE3, Stratagene) which contains a second plasmid expressing chaperones that help fold over-expressed fusion proteins in the cytoplasm and therefore reduce their degradation and the amount of recombinanat protein that ends up in insoluble inclusion bodies, should yield both better antigens and more soluble proteins to perform *Xf* cell agglutination assays. We will also inject HA ADs into 2 rabbits instead of 1 to increase the likelihood of producing higher quality antibodies. The discovery made by Feldstein and Bruening of M13 phage that specifically binding to HxfA should also facilitate the localization and characterization of native HAs associated with *Xf* cells in culture and *in planta*. Once identified, cell-cell HA binding domains will be expressed in bacterial endophytes that will be inoculated into 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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# DETECTION OF SIDEROPHORES IN THE ENDOPHYTIC BACTERIA *METHYLOBACTERIUM* SPP. ASSOCIATED WITH *XYLELLA FASTIDIOSA*

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## ABSTRACT
We analyzed the production of siderophores by endophytic bacteria *Methylobacterium* spp., which occupy the same niche as *Xylella fastidiosa* (*Xf)* in citrus plants. All strains of *Methylobacterium* spp. tested were CAS-positive for siderophore production. *Methylobacterium* spp. produced hydroxamate-type, but not catechol-type siderophores. Specific primers for pyoverdin, a hydroxamate type ferrisiderophore receptor gene were used to amplify this gene from *Methylobacterium* strains. The growth of *Xf* was stimulated by the presence of a supernatant-siderophore of endophytic *Methylobacterium mesophilicum*. If *Xf* is able to use heterologous siderophores during its establishment inside the host plant, it may benefit from production of siderophores by endophytic symbionts.

## INTRODUCTION
Endophytes colonize the living, internal tissues of plants without causing any immediate, over negative effects (Hallmann et al., 1997; Azevedo et al., 2000). Research has shown that endophytic microorganisms isolated from surface disinfected plant tissues exhibit a potential as biocontrol agents against phytopathogens (Sturz et al., 1998) and insects (Azevedo et al., 2000) as well as increasing plant growth and hastening plant development (Lodewyckx et al., 2002). However, synergistic interactions between endophytes and phytopathogens have not been studied yet.

Bacterial siderophores are low-molecular-weight compounds with high iron (III) chelating affinity (Sharma and Johri, 2003) that are responsible for the solubilization and transport of iron (III) into bacterial cells. Iron is an essential mineral and its sequestration by specific bacterial siderophores may induce the development of plant disease (Nachin et al., 2003; Etchegaray et al., 2004). Acquisition of iron from siderophores produced by other microbial species has already been described for *Escherichia coli*, *Salmonella typhimurium* (Martinez et al., 1990), *Actinobacillus pleuropneumoniae* (Diarra et al., 1996), *Streptomyces* sp. (Imbert et al., 1995), and *Arthrobacter flavescens* (Winkelmann, 1991).

*Xylella fastidiosa* (*Xf*) is the causal agent of Citrus Variegated Chlorosis (CVC), which is an important disease of citrus species (Hartung et al., 1994). In Brazil, over 70 million sweet orange trees (38%) are affected, and CVC is responsible for losses of US$ 100 million per year to the Brazilian citrus industry, affecting all commercial sweet orange varieties (de Souza et al., 2005). *Xf* was the first plant pathogen to have its genome completely sequenced and putative genes for membrane receptors, including siderophores, were found (Simpson et al., 2000).

The genus *Methylobacterium*, which occupies the same ecological niche as *X. fastidiosa*, was the most frequently isolated endophytic bacterium from CVC-symptomatic citrus plants (*Citrus sinensis*). Recently, an interaction between *Methylobacterium* species and *Xf* was strongly indicated (Araújo et al., 2002; Lacava et al., 2004).

## OBJECTIVES
The aim of this study was to evaluate the ability of *Methylobacterium* spp., isolated as citrus endophytic bacteria (Araújo et al., 2002), to produce siderophores and to investigate the capacity of *Xf* to use siderophores produced by *Methylobacterium mesophilicum* (*M. mesophilicum*) for growth and development.

## RESULTS
All strains of *Methylobacterium* spp. tested were CAS-positive for siderophores production (Table 1), and the siderophores production tested by the CAS-agar assay revealed that 66% of CVC-symptomatic, 55% of uninfected, 20% of asymptomatic and 10% of tangerine strains of *Methylobacterium* spp., showed very high production. Also, all strains of *Methylobacterium*