EFFECTS OF FIMBRIAL (FIMA, FIMF) AND AFIMBRIAL (XADA, HXFB) ADHESINS ON THE ADHESION OF *XYLELLA FASTIDIOSA* TO SURFACES

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ABSTRACT
We investigated the role of fimbrial and afimbrial adhesions in the attachment of *Xylella fastidiosa* (*Xf*) to grape. We have individually disrupted FimA, FimF, XadA, and HxfB to assess their role in adhesion to plants and in the disease process. We performed adhesion assays using each mutant and wild-type separately as well as a combination of two mutants at one time to observe the phenotypes of these mutants using fluorescence or confocal microscopy. The fimbrial mutants FimA- or FimF- did not aggregate nor did they attach to the glass surface whereas the adhesion mutants XadA- or HxfB- did not attach to glass but did form aggregates and attached to cells that had adhered to a surface. All mutants had fewer single cells or aggregates that remained attached to glass than wild-type cells did after washing steps. We observed that afimbrial mutant cells (i.e. XadA- or HxfB-) were clumped on top of fimbrial mutant cells (i.e. FimA- or FimF-). Both afimbrial and fimbrial proteins thus apparently play a role in attachment of cells to glass in the early phases of adhesion while fimbrial proteins appear more important in cell-to-cell aggregations than afimbrial proteins. To determine if these adhesions are important in virulence, rooted grapevine cuttings were inoculated with FimA-, FimF-, XadA-, HxfB-, and wild-type *Xf* 'Temecula' or 'STL'. A higher incidence and severity of disease was observed in vines inoculated with the wild-type *Xf* strain compared with FimA-, FimF-, XadA- or HxfB- mutant strains. Similarly, wild-type strain *Xf* 'STL' resulted in more vines with symptoms than FimA-, FimF- or XadA- mutants of this strain indicating that the process of attachment appears to involve similar genes in both the 'Temecula' and 'STL' strains. It thus appears that successful colonization of plants by *Xf* requires both cell-to-cell and cell-to-surface attachment.

INTRODUCTION
Attachment is the first step in the colonization process of bacterial pathogens. Attachment of *Xf* to xylem vessels and insect vectors may be required for both virulence and transmission. We therefore investigated the role of various fimbrial and afimbrial adhesions produced by *Xf*. Amongst the afimbrial adhesions, *Xf* has a homolog to XadA, an adhesion shown to be important in virulence of *Xanthomonas oryzae* pv. *oryzae* to rice. Since *Xf* is also a xylem inhabiting plant pathogen we hypothesized that XadA would also be a virulent determinant for *Xf* in grape. Similarly HecA was shown to be a virulent factor for *Erwinia chrysanthemi* to tobacco seedlings. *Xf* has four homologs to the HecA adhesion, among them are HxfA (PD2118) and HxfB (PD1792). These hemagglutins are the largest genes of the *Xf* genome and we hypothesized that these adhesions are important in the colonization process. Previous studies showed that HxfA and HxfB caused early grapevine death (hypervirulence) and mediated contact between *Xf* cells, which resulted in colony formation and biofilm maturation within the xylem vessels (Guilhabert and Kirkpatrick 2005). In the present study, inoculations were performed several times, and to ensure virulence was not diminished due to a high number of passage in the laboratory, we recreated the mutants multiple times using a low passage wild-type strain of *Xf* 'Temecula'. Inoculations were repeated several times with either low passage wild-type cells or each of the recreated mutants (FimA-, FimF, XadA-, and HxfB-).

OBJECTIVES
1. Determine the role of adhesions, in particular of the adhesion XadA and hemagglutinin HxfB in the attachment and virulence of *Xf* in grape.
2. Develop adhesion assays to characterize the behavior of the fimbrial and adhesion mutants of *Xf.*

RESULTS
Objective 1
XadA and HxfB (PD1792) mutants of *Xf* grape strains 'Temecula' and 'STL' were produced using the method described previously (Feil et al. 2003). Because the hemagglutinin HxfB is a large gene (10 kb), we constructed several vectors to disrupt this gene to maximize our chance to disrupt an important domain in the HxfB protein. Characterization of HxfB mutants was done by PCR and sequencing.

To assess the virulence of adhesion mutants we have infected grape with each of these mutants FimA, FimF, XadA, and HxfB (mutants were derived from both grape strains of *Xf* 'Temecula' or 'STL') and wild-type cells of the 'Temecula' or 'STL' grape strain and recorded the number of diseased plants over time. We created the mutants two separate times in a low-passage 'Temecula' background and repeated the inoculations twice for a total of three separate experiments. All these experiments gave the same results. Specifically, HxfB- mutants were always less virulent than wild-type cells. This result contrasts with previous studies on HxfB- mutant by Guilhabert and Kirkpatrick (2005). One reason could be that the site of

disruption in this large gene was different for the two mutants in these two studies and could therefore lead to different phenotypes. Samples were tested for the presence of *Xf* by culturing. The percent diseased grapevines following inoculation with either FimA-, FimF-, XadA-,or HxfB- was reduced compared to the percent diseased vines inoculated with the wild-type *Xf* 'Temecula' or 'STL.' At a given sample time wild-type *Xf* incited a higher incidence of disease in grapevines than either FimA- , FimF-, XadA-, or HxfB- mutants.

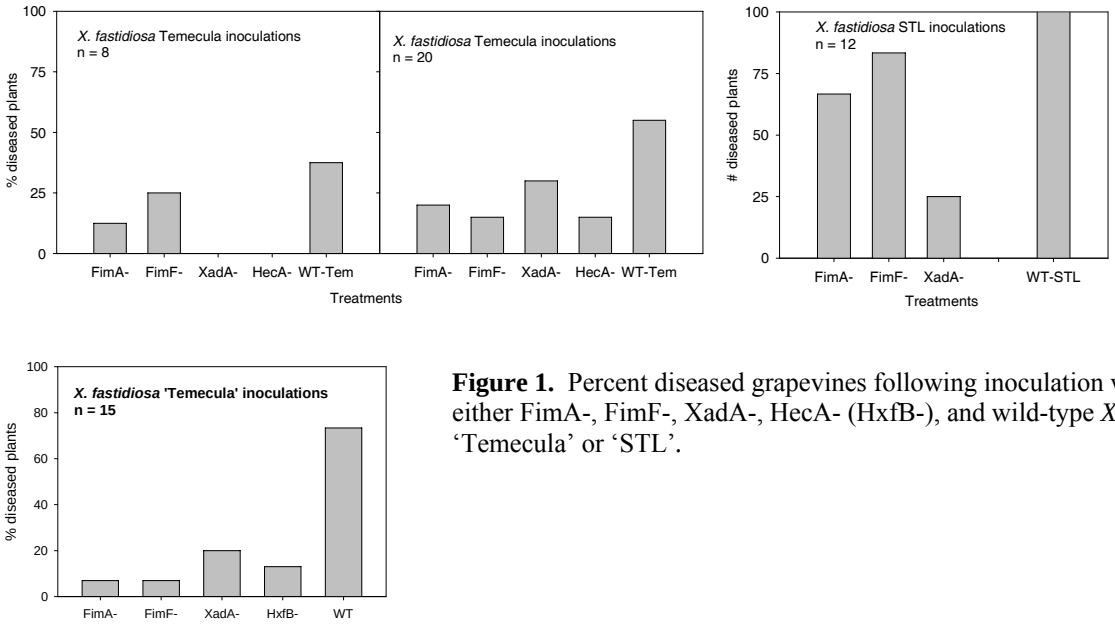


Figure 1. Percent diseased grapevines following inoculation with either FimA-, FimF-, XadA-, HecA- (HxfB-), and wild-type *Xf* 'Temecula' or 'STL'.

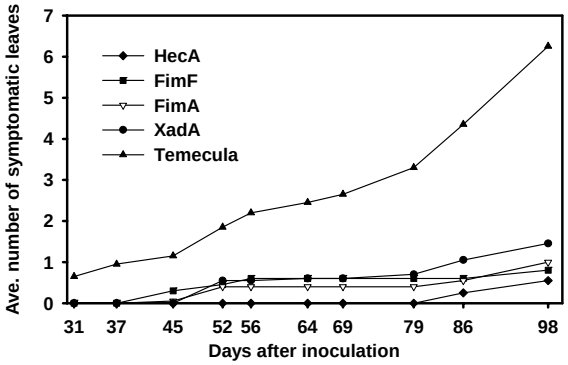


Figure 2. Average number of symptomatic leaves following inoculation with either FimA-, FimF-, XadA-, HecA- (HxfB-), and wild-type *Xf* 'Temecula' or 'STL.'

Disease severity was much reduced for each mutant compared to wild-type cells. For all mutant- inoculated grapevines, onset of symptom development was delayed by at least two weeks from the one for wild-type-inoculated grapevines. These results were confirmed by taking samples from all vines and culturing the bacteria from the samples.

Objective 2

Wild-type, FimA-, FimF-, XadA-, and HxfB- cells were scrapped from plates and placed in PWG broth to an OD of 1 (~ 10^8 cells per ml). 300 µl of each suspension was placed on a glass slide and incubated at room temperature in a moist chamber for four hours. The slide was then rinsed with sterile deionized water by submerging it in water twice. 20 µl of DAPI stain was placed on the slide to stain any attached cells. The stained cells were viewed under a fluorescent microscope and the number of cells attached counted.

FimA- FimF- XadA- HxfB- Wild-type

Figure 3. FimA-, FimF-, XadA-, HxfB- or Wild-type cells remaining attached to glass after four hours.

Wild-type cells remained attached to glass after four hours either as single cells, or small or large aggregates. Neither small nor large aggregates of FimA- or FimF- cells could be observed attached to glass whereas XadA- or HxfB- cells remained attached in aggregates to the glass surface if rinsing was gentle (but not if more vigorous rinsing was employed.). XadA- or HxfB- had fewer single cells remaining attached to glass after rinsing than FimA- or FimF- cells. Overall, the fimbrial and non-fimbrial mutants were attachment deficient when compared to the wild-type cells. This indicates that attachment of *Xf* requires both fimbrial and afimbrial adhesions.

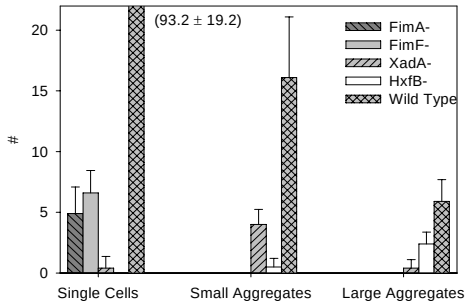
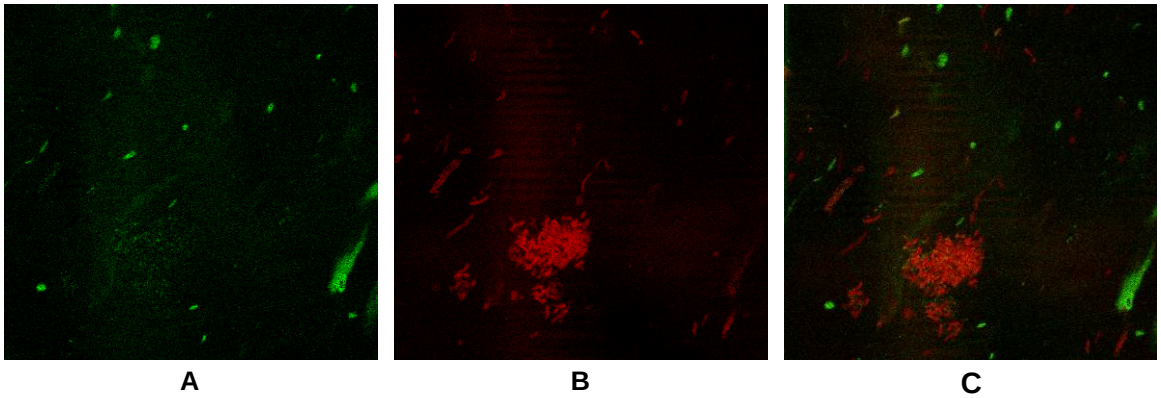


Figure 4. Number of cells remaining attached to glass after four hours for FimA-, FimF-, XadA-, HxfB- or wild-type cells.

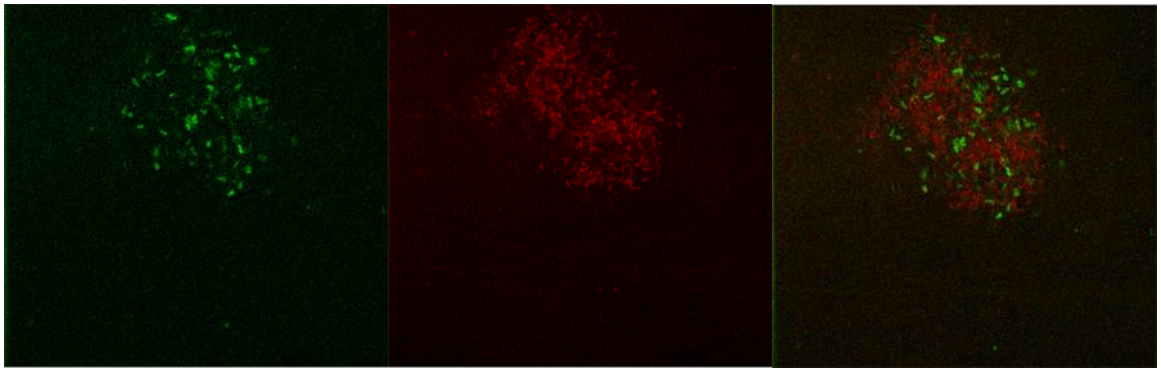
Co-inoculation experiments with epifluorescence microscopy

Mutant cells were stained using PKH67 green fluorescent dye for FimA- FimF- or wild-type 'Temecula' and PKH26 red fluorescent dye for XadA-or HxfB-. Cells were mixed two by two as follows: combination 1, FimA- and XadA-; combination 2, FimA- and HxfB-; combination 3, FimF- and XadA-, and combination 4, FimF- and HxfB-. Mixtures were placed on glass slides, placed in a moist chamber for eight hours, rinsed, and examined under the confocal laser scanning microscope. Confocal microscopy of cells stained with metabolic dyes revealed that FimA- or FimF- did not form aggregates and attached sparingly to glass whereas XadA- or HecA- did not attach to glass but were found in aggregates on top of the FimA- or FimF- cells (Figures 5 and 6). This confirms that adhesion necessitates for types of adhesions and that the fimbrial adhesions are more important in cell-to-cell aggregation than the afimbrial adhesions.



A B C

Figure 5. FimA- cells green (A) and XadA- cells red (B) separated or together (C) using the confocal with the meta detector



A **B** **C**

Figure 6. FimF-, green cells (A), HecA-, red cells (B), and FimF- mixed with HecA-, green and red cells, respectively (C).

CONCLUSIONS

The results show that attachment is a complex process, probably involving the contribution of both fimbrial and afimbrial adhesion factors. These results should help enable an understanding of the over-all process of formation of cell aggregates in xylem vessels, which presumably are major determinants of disease symptoms. Fimbrial adhesions appear more important in cell-to-cell aggregation than the afimbrial adhesions but both are responsible for attachment of *Xf* cell-to-surface. The importance in virulence for these mutants was determined by doing grapevine inoculation experiments. Inoculations were repeated three times with freshly recreated mutants each time. We counted the percent grapes vines infected following inoculation with wild-type of the two grape strains 'Temecula' or 'STL' or with either one of the four mutants tested (i.e. FimA-, FimF-, XadA-, or HxfB-). Since disease development was reduced in grapevines inoculated with FimA-, FimF-, XadA- or HxfB-, mutants compared to the wild type *Xf* strain we have shown that attachment is important for disease development. Targeting the FimA, FimF, XadA, or HxfB genes could be one way to reduce disease incidence in grapevine-growing regions affected by Pierce's disease.

REFERENCES

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