ROLE OF ATTACHMENT OF *XYLELLA FASTIDIOSA* TO GRAPE AND INSECTS IN ITS VIRULENCE AND TRANSMISSIBILITY

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ABSTRACT
Attachment of *Xylella fastidiosa* to xylem vessels and insect vectors may be required for virulence and transmission; therefore we have individually disrupted *fimA*, *fimF*, *xadA*, and *hecA* 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 combination of two of the mutants and observation of the phenotypes of these mutants under a scanning electron microscope is underway. Patterns of cell adhesion and aggregation of mutants on surfaces lead us to hypothesize that *fimA* and *fimF* are important in cell-to-cell aggregation while *xadA* and *hecA* are involved in the first steps of adhesion of bacteria to the plant host. Rooted grapevine cuttings were inoculated with FimA-, FimF-, XadA-, HecA-, and wild-type *X. fastidiosa* 'Temecula' or 'STL'. A higher incidence and severity of disease was observed in vines inoculated with the wild-type *X. fastidiosa* strain compared with FimA-, FimF-, XadA- or HecA- mutant strains. Similarly, wild-type strain STL strain of *X. fastidiosa* 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 *X. fastidiosa* requires both cell-to-cell and cell-to-surface attachment. To distinguish the various mutants from each other in mixed inoculations and to determine what factors affect attachment of the mutants we have constructed disrupted *fimA* vectors for use in a *gfp* marked *Xylella fastidiosa*. This will allow us to distinguish the FimA- cells from other cells in a mixture adhesion assay using fluorescence microsopy and to follow these cells in grape following inoculation with these mutants. Because *hecA* is a large gene, we are also disrupting various locations within the HecA gene. We will test these different HecA- mutants in inoculation experiments to determine the role of HecA in virulence of *X. fastidiosa* to grape.

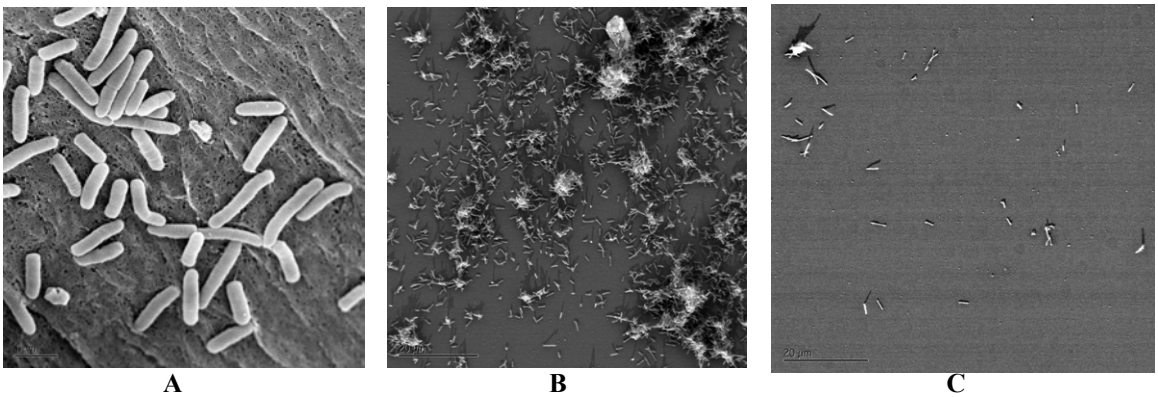
INTRODUCTION
Adhesion is a well-known strategy used by phytopathogenic bacteria to initiate colonization of their plant hosts and a precursor step to invasion (Romantschuk et al. 1994). *Xylella fastidiosa* possesses many genes involved in attachment or adhesion. Simpson et al. (2000) identified 26 genes encoding proteins involved in the biogenesis and function of Type 4 fimbriae filaments (*pilA*, *B*, *C*…). We have focused on the fimbrial operon, which is composed of 6 genes (*fimA*, *ecdD*, *fimC*, *D*, *E*, and *F*). Even though the fimbrial mutant cells had less fimbriae than the wild type cells as seen in scanning electron micrographs, the cells seemed to still be able to attach to surfaces by another mechanism (Feil et al. 2003) (Figure 1A). This suggested that fimbriae are more important in cell-to-cell adhesion than in cell-to-surface adhesion. While FimA and FimF were found to be important in cell-to-cell aggregation (Feil et al. 2003) the initial attachment of *X. fastidiosa* to plants must involve other factors. The goal of this research was thus to assess the relative role of different fimbrial and non-fimbrial adhesins in the attachment process and to determine their role in the disease process. Among the afimbrial adhesins of *X. fastidiosa* we chose XadA and HecA to study because genes homologous to these in other bacteria were found to be virulence determinants.

OBJECTIVES
1. Determine the role of adhesins other than those found in the fimbrial operon, in particular of the adhesin XadA and hemagglutinin HecA in the attachment and virulence of *X. fastidiosa* in grape.
2. Characterize the behavior of the fimbrial and adhesion mutants of *Xylella fastidiosa* in grape and to compare this behavior over time via expression analysis.
3. Determine what factors affect attachment of wild-type or mutant cells to grape
4. Determine if these mutants can attach to the insect vector and be transmitted to grape.

RESULTS

XadA and HecA mutants of the 'Temecula' strain of *X. fastidiosa* were produced using the method described previously (Feil et al. 2003). Characterization of HecA mutants was done by PCR and sequencing. To confirm that HecA was disrupted at the HecA site, 3 kb fragments of DNA from HecA- mutant cells containing the kan insert were sequenced. Using Blast search, we found that the sequences of the mutant were identical to those of HecA on one side and to N-manoacetyltransferase on the other, indicating that the kan gene was inserted in the HecA region we wanted to disrupt. There are four large HecA homologs in the *X. fastidiosa* genome. The HecA we mutated is the third from the origin of replication of the genome. Dr. Tom Burr group at Cornell University has mutated the 3' HecA homolog using transposon mutagenesis and is characterizing this mutant. We compared wild-type to FimA-, FimF-, XadA- , and HecA- cells using the adhesion assay on silicon surfaces and SEM. We have performed adhesion assays using each mutant and wild-type separately as well as combination of two of the mutants.

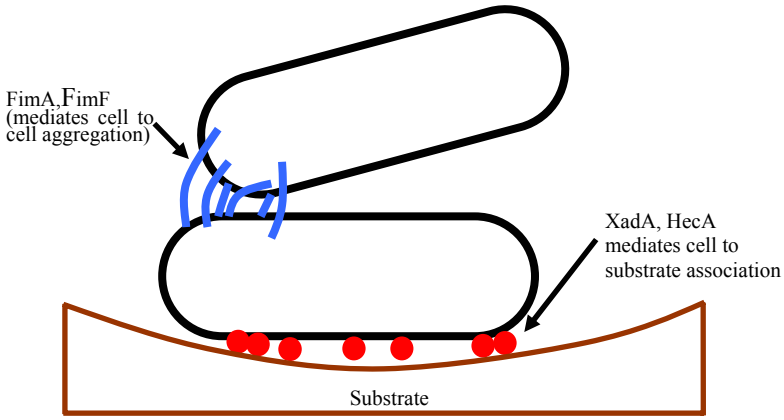
We have found that XadA appears to play a major role in the early steps of bacterial adhesion to host surfaces. We observed phenotypic difference between XadA- mutant and wild-type cells of *X. fastidiosa* in culture. In particular, no rings on the sides of the flask were formed when XadA- mutant cells were grown in fructose-based medium whereas a thick ring appeared around the flask when wild-type cells were grown in the same medium. In the adhesion assay using xylem sap, more than 100-fold fewer XadA- cells adhered to a glass surface than of the wild-type cells when observed under SEM, indicating that the XadA- cells are surface adhesion-deficient (Figure 1, B and C).



A **B** **C**

Figure 1. SEM micrographs of FimA- *X. fastidiosa* (A), wild-type (B), and XadA- .

We thus have hypothesized that the afimbrial adhesins are responsible for initial attachment of *X. fastidiosa* to grape xylem vessels. Below is a cartoon depicting a summary of the hypothetical role for each mutant.



Since we have infected grape with each of these mutants (FimA, FimF, XadA, and HecA) and wild-type cells of the 'Temecula' grape strain we will soon be able to assess the pattern of colonization of the plant with the various mutants. Microscopic observation of these tissue sections will be done to visualize *X. fastidiosa* in plants and to compare the extent of colonization between mutant and wild *X. fastidiosa* strains. With a similar approach, we are determining the role of the *fimA*, *fimF*, and *xadA* genes in attachment to insects (BGSS and GWSS). We have fed BGSS in plants infected with these mutant strains and are preparing to visualize the bacterial cells in the insects to determine if different patterns of colonization of the insect have resulted form the adhesion mutation. We will also determine if the insects remain competent to transmit the various mutant strains as well. An initial experiment on acquisition/transmission using FimA, FimF and XadA mutants and

wild-type cells was not conclusive (only two plants out of 100 tested positive following transmission assays using the blue-green sharpshooter as insect vectors). We will repeat these experiments. Insects will be placed on grapes infected with the various mutants (FimA, FimF, XadA, HecA, and wild-type), and acquisition-transmission experiments will be performed. We will keep the insects for further microscopy to determine variation in attachment of the various cells to the insect.
To further test our model of the multifunctional adhesion process we will make FimA-, FimF-, XadA-, and HecA- mutants in a gfp marked *X. fastidiosa* strain (Newman et al. 2003). This will allow us to distinguish each gfp mutant from other cells in mixture experiments during adhesion assays using fluorescence microscopy. This will also enable us to use confocal microscopy to determine the three-dimensional structure of cell aggregates formed by various mixtures of *X. fastidiosa* mutants. This mixture study should enable us to verify, for example, that FimA- mutants will be found attached to the glass or plant surface, while XadA- mutants (but not FimA- mutants) will be attached to each other (and to the FimA- mutants). We will use the FimA mutants in gfp marked *X. fastidiosa* to compare attachment of these cells and wild-type cells in fructose broth. We will observe putative differences in attachment to glass and grape tissue. Difference in ring formation will also be evaluated to determine phenotypic difference.

To assess the virulence of adhesion mutants we have infected grape with each of these mutants (FimA, FimF, XadA, and HecA) and wild-type cells of the 'Temecula' grape strain and recorded the number of diseased plants over time. At a given sample time wild-type *X. fastidiosa* incited a higher incidence of disease in grapevines than either FimA- , FimF-, XadA-, or HecA- mutants (Figure 1). HecA- inoculations generally resulted in the least number of diseased vines.

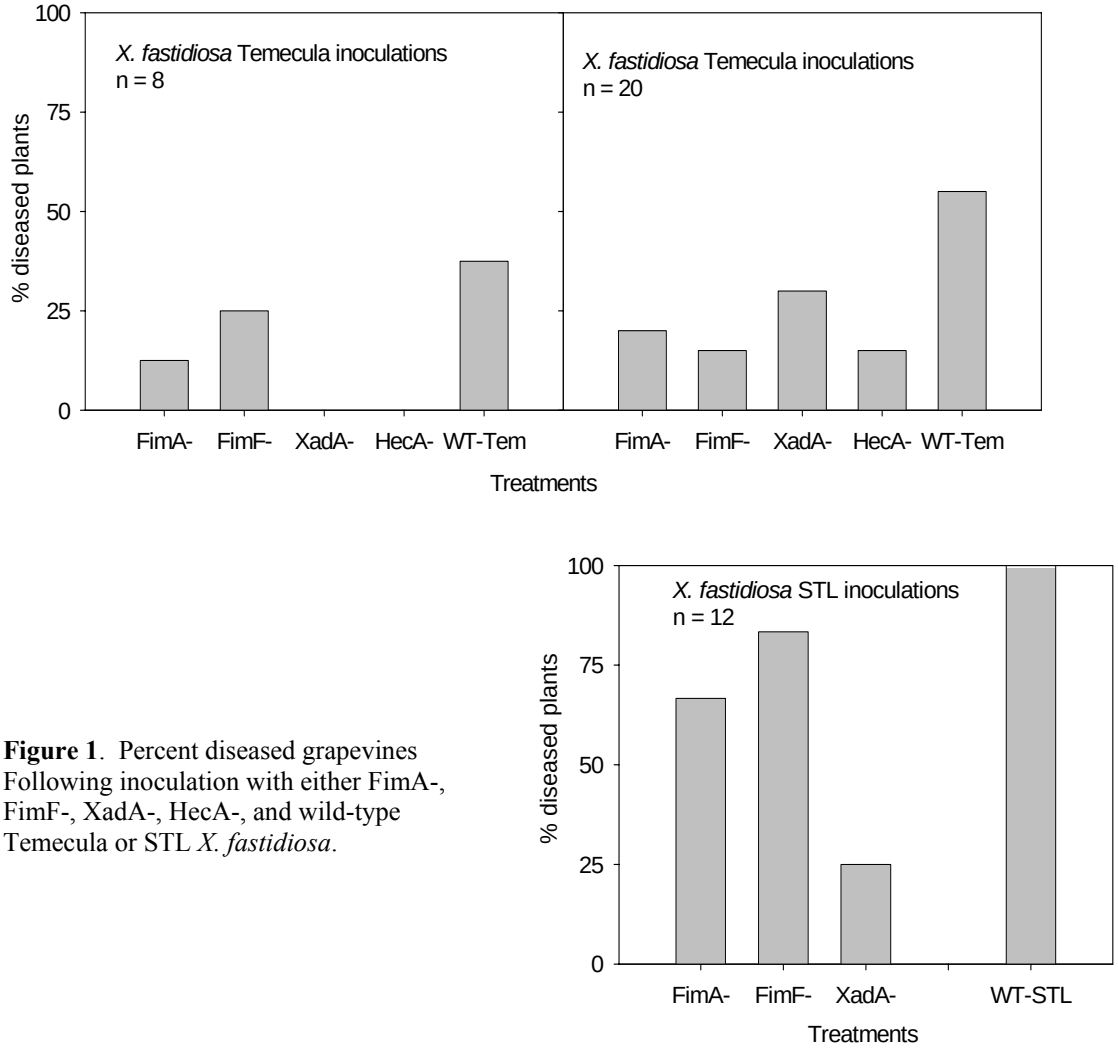


Figure 1. Percent diseased grapevines Following inoculation with either FimA-, FimF-, XadA-, HecA-, and wild-type Temecula or STL *X. fastidiosa*.

CONCLUSIONS

Since disease development was reduced in grapevines inoculated with FimA-, FimF-, XadA- or HecA- mutants compared to wild type *X. fastidiosa* strains we have shown that attachment is important for disease development. Targeting the FimA, FimF, XadA, or HecA genes could be one way to reduce disease incidence in grapevine-growing regions affected by Pierce's disease. We have now observed substantially differential attachment phenotypes for the various attachment mutants under various experimental conditions. The results clearly show that attachment is a complex process, probably involving the sequential contribution of non-fimbrial and fimbrial 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. Attachment is also affected by chemical components and now that we know the relative role of different attachment factors we will assess the role of different media components and other compounds that might be feasible for introduction into plants to determine their effects on attachment.

REFERENCES

Feil, H., Feil,W. S., Detter, J. C., Purcell, A. H., and S. E. Lindow. 2003. Site-directed disruption of the *fimA* and *fimF* fimbrial genes of *Xylella fastidiosa* Phytopathogy 93:675-682.

Newman, K. L., Almeida, R. P. P., Purcell, A. H., and S. E. Lindow. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. Applied and Environmental Microbiology 69:7319-7327.

Romantschuk, M., Roine, E., Ojanen, T., van Doorn, J., Louhelainen, J., Nurmiaha-Lassila, E-J., and K. Haahtela. 1994. Fimbriae (pilus) mediated attachment of *Pseudomonas syringae*, *Erwinia rhapontici*, and *Xanthomonas campestris* to plant surfaces. In: Molecular Mechanisms of Bacterial Virulence. 67-77. C. I. Kado and J. H. Crosa (eds). Kluwer Academic Publishers. Netherlands.

Simpson, A. J. G., et al 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature. 406:151-159.

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