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

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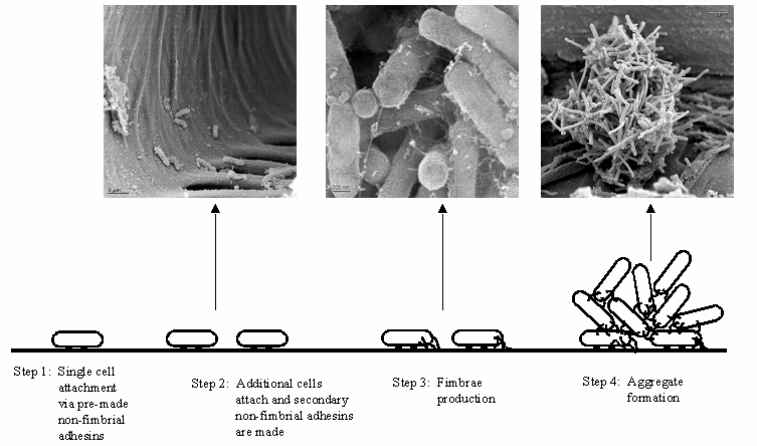
ABSTRACT
Xylella fastidiosa causes Pierce's disease, a serious disease of grape, citrus variegated chlorosis, almond and oleander leaf scorches, and many other similar diseases. Although the complete genome sequences of several strains of this organism are now available, the function of most genes in this organism, especially those conferring virulence, is lacking. We are elucidating the role of fimbriae and adhesins in the pathogenicity of *X. fastidiosa* to grape, in the attachment to grape or to insect mouthparts, and in the transmissibility of the bacteria to grape via insects. We are also investigating the role of the non-fimbrial adhesins (HecA, B, C, and XadA) in the attachment process because we believe that these genes are important in the early steps of adhesion in bacterial cell-host cell's surface attachment. We were successful in producing XadA- mutants of *X. fastidiosa* strains 'Temecula' and 'STL' and conducted various adhesion assays comparing wild-type to XadA- mutant cells' phenotypes. Polymerase chain reaction and southern blot analyses of the mutants indicated that a double crossover event had occurred exclusively within the *xadA* gene, replacing the chromosomal gene with the disrupted gene and abolishing production of the corresponding protein, XadA. Scanning electron microscopy revealed that attachment to glass was inhibited for the XadA- mutants of *X. fastidiosa* when compared with the parental strain. XadA- mutants of *X. fastidiosa* remained pathogenic to grapevines, but further characterization of virulence and insect transmissibility are underway.

INTRODUCTION
Adhesion is a well-known strategy for phytobacteria to begin colonizing their plant hosts and a precursor step to invasion (Romantschuk et al. 1994). Electron micrograph studies described *X. fastidiosa* attached to grape xylem vessels or to the cuticle lining the foregut in insect vectors (Purcell et al. 1979). Within the grape xylem vessels, *X. fastidiosa* also appeared to be embedded in a filamentous matrix (H. Feil *unpublished*). The fibril-like structures were said to be analogous to fimbriae or pili in other bacteria (Hopkins 1977). *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*). It was shown that *X. fastidiosa* contains only one chaperone/usher-dependent fimbrial operon compared with enteric bacteria that contain 10 to 15 such operons (Bhattacharyya et al. 2002). Even though the fimbrial mutants remained virulent to grape, we observed phenotypic differences in vitro between mutants and wild-type cells. Scanning electron microscopy (SEM) revealed that fimbriae size and number and cell aggregation were reduced for the $FimA^-$ or $FimF^-$ mutants of *X. fastidiosa* when compared to the parental strain suggesting that fimbriae probably play an essential role in self-aggregation of *X. fastidiosa* cells. Several questions remained to be answered:

1. How do the fimbrial mutants of *X. fastidiosa* behave in grape?
2. Are the fimbrial mutant cells still transmissible to grape via the insect vector?
3. If fimbriae are key components of the self-aggregation of *X. fastidiosa* cells, what genes contribute to the initial attachment of *X. fastidiosa* to grape vessels?

One reason that could explain why the fimbrial mutants remained virulent is that since the mutant cells don't aggregate so well they are freer to move within the xylem vessels of grape and colonize other vessels more rapidly than the wild-type cells; such cells might prove to be hypervirulent. We hypothesized that the lack of fimbriae in the mutants may reduce the adhesion capacity of the cells to plant tissue and/or to insect mouthpart thereby diminishing the transmissibility of the bacteria to plants via the insect. We propose to further investigate the attachment of the mutants to grape and to insect by conducting inoculation and acquisition/transmission experiments with the BGSS, the mutant and wild-type *X. fastidiosa.*

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 surface by another mechanism (Feil et al. 2003). This suggests that fimbriae are more important in cell-to-cell adhesion than in cell to surface adhesion. We hypothesized that the afimbrial adhesins are responsible for early attachment of *X. fastidiosa* to grape xylem vessels. The cartoon below depicts our proposed diagram of the steps in the adhesion of *X. fastidiosa* to xylem vessels:



In step 1, upon first contact to the host cell's surface, the *X. fastidiosa* cells stick to the surface via non-fimbrial adhesins. These adhesins allow the bacteria to stick to the surface by binding the xylem vessel's surface. Step 2 represents the secondary contact via more adhesins. These adhesins form a matrix around the cells allowing for more binding between the bacterial surface and the host cell's surface. In step 3, fimbriae are formed between bacterial cells to enhance cell-to-cell attachment. Finally, step 4 depicts bacterial cells aggregated to one another via fimbriae and fibrils to form a mass of cells within the xylem vessel. Fimbriae appear to be more important for cell-to-cell aggregation and therefore enter late in the adhesion process.

Because we think the early steps in adhesion are crucial to successful colonization of grape xylem vessels by *Xylella fastidiosa*, we are directing our research towards the investigation of the role of other adhesin genes, which have been shown to reduce virulence in other bacterial system. Different strains of *X. fastidiosa* were shown to have different afimbrial adhesins suggesting a role for these genes in host specificity (Bhattacharyya et al. 2002). Other host specific adhesin may include the *hia* gene, homolog of the major adhesin of *Haemophilus influenzae*. Other adhesins present in the *X. fastidiosa* genome are HecA, B, and C. *hecA*, a hemagglutinin homolog in *Erwinia chrysanthemi*, the causal agent of soft-rot disease of chrysanthemum, played a role in the attachment, aggregation, cell killing, and virulence of this organism to tobacco seedlings (Rojas et al. 2002). Epifluorescence and confocal laser-scanning microscopy revealed that this mutant was reduced in its ability to attach, to form aggregates and to kill epidermal plant cells. The genome of *X. fastidiosa* has three hemaglutinins (*hecA*, *B*, and *C*) genes with similarity to *hecA* and to a *Neisseria meningitidis* secreted protein (Tettelin et al. 2000). These genes are large (over 10 Kb each) and distributed within the genome at least 400 Kb apart. They share high homology especially in the upstream sequence of the gene. The downstream third of their sequence has less identity to the other two sequences. This suggests that their respective specificity resides in the expression of the 3' end of their sequences. We are targeting the 3' end of each of these genes in producing mutants to study their role in attachment and pathogenicity.

Another adhesin, XadA, an outer membrane protein found in *Xanthomonas oryzae* pv *oryzae* and in *Xanthomonas campestris* pv. *vesicatoria,* has been implicated in virulence for these two organisms. XdaA-deficient mutants of the rice pathogen, *Xanthomonas oryzae* pv *oryzae* are less virulent (i.e. cause smaller lesion on rice than that of the wild-type cells) and altered colony morphology (Suvendra et al. 2002). Recently *xadA* has been found in *Xanthomonas campestris* pv. *campestris* and in *Xanthomonas axonopodisis* pv. *citri* (Da Silva et al. 2002). The genome of *X. fastidiosa* also has a *xadA*-homolog suggesting that this conserved gene may have a general importance in pathogenicity for the plant pathogens. We disrupted this gene using the same site-directed method used for the fimbrial mutant and are determining the role of this gene in attachment and pathogenicity.

We have investigated the role of several fimbrial genes in pathogenicity (Feil et al. 2003) and propose to further elucidate the role of fimbriae and adhesins in the pathogenicity of *X. fastidiosa* to grape, in the attachment to grape or to insect mouthparts, and in the transmissibility of the bacteria to grape via insects. However since fimbriae are important in cell-to-cell attachment, which is probably relatively late in the adhesion process, we also investigated the role of the non-fimbrial adhesins (HecA, B, C, and XadA) in the attachment process. We believe that these genes are important in the early steps of adhesion in bacterial cell-host cell's surface attachment. We also chose these particular non-fimbrial adhesins because they were found to play a significant role in virulence for other plant bacterial pathogens. A better understanding of this important aspect of the biology of Pierce's disease should allow potential new ways to control this serious plant pathogen to be developed and will elucidate the processes that occur during colonization of both grape as well as sharpshooter vectors.

OBJECTIVES

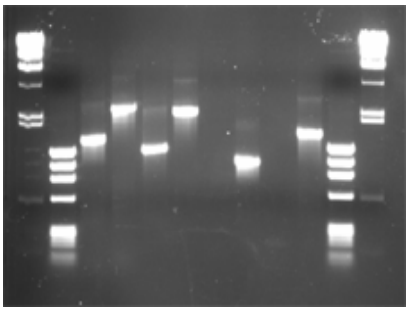
1. To further characterize the behavior of the fimbrial mutants of *Xylella fastidiosa* in grape.
2. To determine if these mutants can attach to the insect vector and be transmitted to grape.
3. To determine the role of adhesins other than those found in the fimbrial operon, in particular of the hemagglutinins and the adhesin XadA in the attachment and virulence of *X. fastidiosa* in grape.

RESULTS AND CONCLUSIONS

Objective 1: We have infected grape tissue with mutants of FimA, FimF, and XadA or wild-type cells of the 'Temecula' grape strain. Sampling for presence of bacteria in sections at several point in time following inoculation are underway. Disease symptoms are being followed over time to ascertain the hyper or decreased virulence of these mutants. Microscopic observation of these tissue sections are being done to visualize *X. fastidiosa* in plants and to compare the extent of colonization between mutant and wild *X. fastidiosa* strains.

Objective 2: With a similar approach than for objective 1, we are determining the role of the *fimA*, *fimF*, and xadA genes in attachment to insects (blue-green sharpshooter and glassy-winged sharpshooter). Plants infected with the FimA, FimF, and XadA mutants are being used as source plants for insect transmission. The efficiency of transmission of these mutants will be compared with that of the wild-type strain in greenhouse feeding studies.

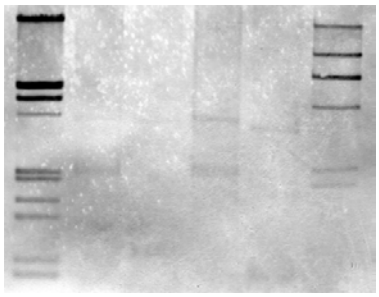
Objective 3: We constructed vectors to disrupt the hemagglutinin genes (*hecA*, *B*, and *C*) and the adhesin gene (*xadA*) of *X. fastidiosa*. We were successful in producing XadA- mutants of the 'Temecula' and 'STL' strains of *X. fastidiosa*. We are in the process of making hemagglutinin mutants. We further characterized the Xada- mutants by PCR (Figure1), southern blot (Figure 2), and sequencing.



ladders 1 2 3 4 5 6 7 8 ladders

Figure 1. PCR of wild-type vs. XadA- DNA

1. Wild-type (WT) DNA/primers outside *xadA*
2. XadA- mutant DNA/primers outside *xadA*
3. Wild-type (WT) DNA/ *xadA* primers
4. XadA- mutant DNA/ *xadA* primers
5. Wild-type (WT) DNA/ *kan* primers
6. XadA- mutant DNA/ *kan* primers
7. Wild-type (WT) DNA/*xadA* forward-*kan* reverse primers
8. XadA- mutant DNA/ *xadA* forward-*kan* reverse primers



ladder XadA WT XadA WT ladder

Figure 2. Southern blot of wild-type vs. XadA DNA. The probe was the xadA gene. The larger size of the XadA digest indicates insertion of the *kan* gene within *xadA*

We tested how different the adhesion of the Xada- mutants was as compared to the adhesion of wild-type cells on various substrates (balsa wood, glass, silicon chip). We also tested if adhesion of the mutant or wild-type cells was affected by various media (PW broth, Fructose broth, xylem sap). We observed that a thick ring of cells formed around the glass flasks for wild-type cells whereas no ring was detected using the Xada- cells (Figure 3).

Figure 3. Wild-type (WT) vs. XadA- mutant cells growing in flasks in fructose broth for 10 days. A thick ring can be seen around the flask with the wild-type cells whereas no ring is observed for the XadA- cells.

This is the first phenotypic difference observed between wild-type and XadA- cells and it suggests that adhesion to host surfaces may be impaired in the XadA- cells.

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