MANAGEMENT OF PIERCE'S DISEASE OF GRAPE BY INTERFERING WITH CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA*

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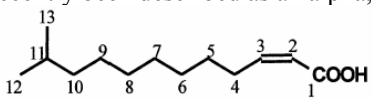
ABSTRACT
Xylella fastidiosa (Xf) is an endophyte that is restricted to the xylem, a network of vessels for water transport, in which it forms an aggregated biofilm. It is transmitted from plant to plant by xylem sap-feeding insects, and forms a polar biofilm in these insects' foreguts. In other systems, biofilms are characterized by community behavior under the control of cell density-dependent gene expression, which requires cell-cell signaling. *Xf* has homologs of the cell-cell signaling genes found in the important plant pathogen *Xanthomonas campestris* pathovar *campestris (Xcc)* and produces a similar alpha,beta unsaturated fatty acid signal molecule called DSF that coordinates gene expression in a community (2, 7). We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease. We have determined that the *rpfF* gene is necessary and sufficient for DSF signal synthesis and that *rpfF* mutants of *Xf* are hypervirulent and non-transmissible. Lack of transmissibility was linked to an inability of the *rpfF* mutant to form a biofilm in the insect foregut; while taken up by insects, the mutant strain is not retained. *Xf* strains that overproduce DSF produce disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We screened several collections of bacterial strains isolated from plants and identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF, by degrading DSF, or by in some way interfering with recognition of DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DFS degrading strains greatly reduced the indicidence of disease in grape; DSF-producing strains consistently were the most effective in reducing disease. Given that DSF appears to mediate an attenuation of virulence in *Xf* we are in the process of transforming grape with the *rpfF* gene to enable DSF production in planta. Preliminary results indicate that transient expression of *rpfF* in *Nicotiana benthamiana* following infiltration with appropriate *Agrobacterium tumefaciens* strains resulted in high levels of DSF production, suggesting that it is likely that grape cells will produce DSF when transformed with the bacterial *rpfF* gene. Non-endophytic bacterial species were also established in high numbers inside grape leaves and petioles following spray application to plants with a high concentration of a silicon-based surfactant with a low surface tension, suggesting that it may be possible to produce protective compounds such as DSF in plants by a variety of bacteria.

INTRODUCTION
Endophytic bacteria such as *Xylella fastidiosa (Xf)* colonize the internal tissues of the host, forming a biofilm inside the plant. A key determinant of success for an endophyte is the ability to move within the plant. We expect activities required for movement to be most successful when carried out by a community of cells since individual cells may be incapable of completing the feat on their own. Cells assess the size of their local population via cell-cell communication and coordinately regulate the expression of genes required for such processes. Our study aims to determine the role of cell-cell communication in *Xf* in colonization and pathogenicity in grapevines and transmission by the insect vector.

Xf shares sequence similarity with the plant pathogen *Xanthomonas campestris* pathovar *campestris (Xcc)* (7). In *Xcc,* expression of pathogenicity genes is controlled by the Rpf system of cell-cell communication, enabling a population of cells to launch a pathogenic attack in a coordinated manner (1). Two of the Rpf proteins, RpfB and RpfF, work to produce a diffusible signal factor (DSF) (1) which has recently been described as an alpha,beta unsaturated fatty acid (9):



As the population grows, the local concentration of DSF increases. Other Rpf proteins are thought to sense the increase in DSF concentration and transduce a signal, resulting in expression of pathogenicity factors (8). The *Xf* genome not only contains homologs of the *rpf* genes most essential for cell-cell signaling in *Xcc*, but also exhibits striking colinearity in the arrangement of these genes on the chromosome (2). We now have shown that *Xf* makes a molecule that is recognized by *Xcc*

but probably slightly different than the DSF of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of Rpf regulation would be genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel. Similarly, we would expect the density-dependent genes to be expressed during the time when a population of *Xf* is ready to move into uncolonized areas.

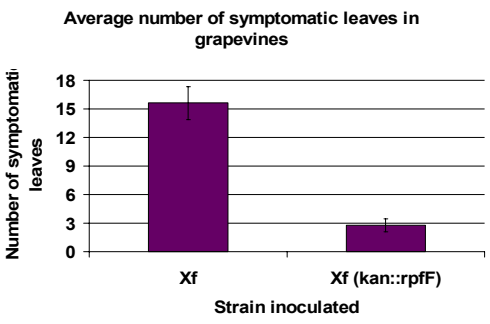
Other organisms can apparently interfere with the density-dependent behaviors of *Xf*. Several recent studies indicate that other organisms can disrupt or manipulate the cell-cell signaling system of bacteria (4, 5). We have found that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*, but until this study we did not know of the manner in which the interaction occurred nor whether such strains had the potential to affect the virulence of *Xf* in grape. In this period we have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants and insects as well as the manner in which other bacterial strains affect such cell signaling and determined the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling.

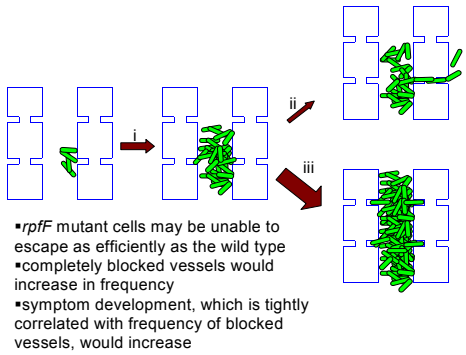
OBJECTIVES

1. Determine role of signaling factors on virulence and transmissibility of *Xf*.
2. Identify degraders and producers of diffusible signaling factors used by *Xf*.
3. Perform Pierce's disease (PD) biocontrol tests on grapevines using DSF-interfering bacteria
4. Isolation of mutant strains of DSF-degrading and DSF activating bacteria that no longer interfere with cell-cell signaling in *Xf*. to verify that disease control is linked to cell-cell signal interference
5. Creation of grapevines expressing gen4s conferring DSF-degradation and DSF-synthesis activites to test for PD resistance
6. Engineer grapevine endophytes such as *Alcaligenes xylosoxidans denitrificans* to express genes conferring DFS-degradation or DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control

RESULTS

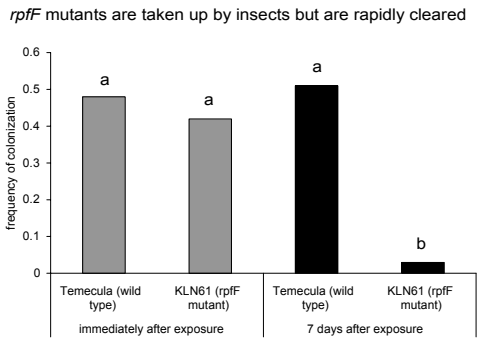
We have constructed a strain of *Xf* Temecula in which the *rpfF* gene, which is required for production of the signal in *Xcc,* is knocked out. This mutant was constructed using exchange of the wild-type allele for a deleted copy carrying an antibiotic resistance gene on a suicide plasmid. The *rpfF* mutant of *Xf* does not make DSF as determined using previously constructed "signal-sensing" strains of *Xcc* to determine DSF production by *Xf* and other bacterial strains. *rpfF* mutants strains were tested for their ability to infect and move within host plants and to cause Pierce's disease symptoms. The *rpfF* gene appears to play a role in modulating disease progress because the timing and severity of symptom development are greatly exacerbated in grapevines infected with *rpfF* mutants when compared to the wild type. We have investigated the mechanism behind these differences. We have found no detectable difference in populations or movement between the wild type and *rpfF* mutants, although our sampling methods would not be able to detect small increases in colonization if they existed. We hypothesize that *rpfF* mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression. We have recently made a green fluorescent *rpfF* mutant to investigate the pattern of colonization by the mutant and compare it to that of the wild type. Importantly, when rpfF was over-expressed in *Xf* under the control of a high and constitutive promoter, the severity of disease in plants was greatly reduced (below). The *Xf* strain that overproduced DSF caused disease symptoms in grape, but only at the site of inoculation. The mutant cells did not move within the plant as did wild-type strains. These results all support our model that DFS regulates genes required for movement of *Xf* from colonized vessels.





Such results suggest that elevating DSF levels in plants should reduce movement of *Xf* in the plant.

We have tested transmissibility of the *rpfF* mutant strain by an insect vector. The *rpfF* mutant was virtually non-transmissible. This defect in transmissibility by the signaling-deficient mutant reveals the importance of cell-cell signaling in insect transmission. Leafhoppers fed on *rpfF*-infected plants ingested *rpfF* cells but were able to rapidly clear themselves whereas the wild type is never cleared.

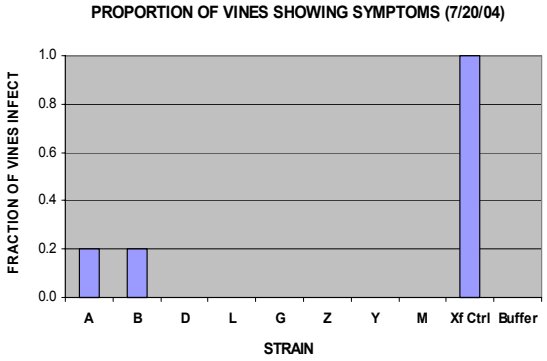


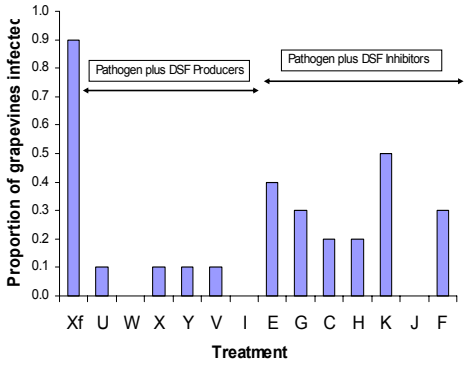
We have isolated a variety of bacteria from grapevines from vineyards affected by Pierce's disease as well as tomato and cruciferous crop plants infected with the signal-producing pathogens *Xanthomonas campestris* pv. *vessicatoria* and *Xcc,* respectively and tested them for their ability to interfere with cell-cell signaling in *Xf* in an assay using the signal-sensing strain described above. We found several strains that negatively affected signaling in *Xcc* while several strains were found to produce DSF. By adding purified DSF to either cell-free extracts of the strains with a negative influence on signaling or to whole cells we found that at least two mechanism of interference with signaling could be observed. Some strains such as strains C,E,G, H, and J are able to degrade DSF while other inhibitor strains did not do so, and apparently have another means of interfering with DSF perception by *Xcc*. The several strains that produced DSF were all identified as *Xanthomonas* species. We sequenced the 16S rRNA gene from these strains to determine their species identity.

Strain	Genus	Origin	Mechanism of DSF Interference
A	*Paenibacillus*	Grape	Unknown inhibition
B	*Paenibacillus*	Grape	Unknown inhibition
C	*Pseudomonas*	Cabbage	Enzymatic digestion
D	*Staphylococcus*	Grape	Unknown inhibition
E	*Bacillus*	broccoli	Enzymatic digestion
G	*Pseudomonas*	Cabbage	Enzymatic digestion
H	*Pseudomonas*	Cabbage	Enzymatic digestion
J	*Pseudomonas*	Tomato	Enzymatic digestion
L	*Staphylococcus*	Grape	Unknown inhibition
I	*Xanthomonas*	Tomato	DSF production
U	*Xanthomonas*	Broccoli	DSF production
V	*Xanthomonas*	Broccoli	DSF production
W	*Xanthomonas*	Broccoli	DSF production
X	*Xanthomonas*	Broccoli	DSF production
Y	*Xanthomonas*	Tomato	DSF production
Z	*Xanthomonas*	Grape	DSF production

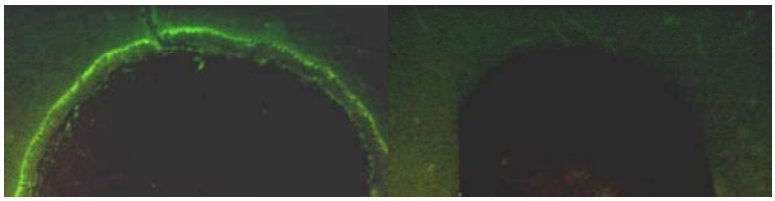
Interfering strain G, typical of strains that apparently degrade DSF, was subjected to transposon mutational analysis of the interfering activity. Several insertional mutations that block degradation of DSF have been identified and sequence analysis of the genes required for DSF degradation are being performed. We expect this analysis to reveal the identity of the gene responsible for the interfering activity. This gene can then be introduced into other organisms, such as plants.

To test the ability of bacteria that alter *Xf* signaling to alter the process of disease in plants, we co-inoculated grapevines with *Xf* and strains that either inhibit or activate cell-cell signaling in greenhouse studies. The incidence of Pierce's disease was greatly reduced by all of the signaling interfering strains that we tested. As we had expected, DSF-producing strains generally reduced disease severity more than did strains that interfered with signaling in *Xf*. These results were highly repeatable, having been observed in 2 separate experiments. We find these results to be very exciting in that they suggest that alteration of signal molecules within plants can have a profound effect on the disease process.





Given that DSF production by endophytes greatly reduces disease incidence and that DSF overproduction in *Xf* also reduces virulence, we have initiated studies to express *rpfF* in plants to achieve production of DFS in plants as a means of disease control . The *rpfF* gene from *Xf* as well as from *Xcc* was cloned into the plant transformation vector pCAMBIA to yield pKLN119. This plasmid carries a T-DNA that includes both hygromycin resistance and the *X. fastidiosa rpfF* gene driven by the CMV 35S promoter and followed by the NOS poly-A signal sequence. pKLN119 and the empty vector pCAMBIA1390 were electroporated into *Agrobacterium* strain GV3101. *Nicotiana benthamiana* plants were transiently transformed by infiltration with suspensions of *Agrobacterium* harboring T-DNA construct pKLN119 or pCAMBIA1390.
Disks of infiltrated leaves were removed after two days, placed on KB agar plates and oversprayed with the DSF bioreporter strain 8525 (pKLN55). Substantial green fluorescence was observed in leaf disks of the plants into which pKLN119 was introduced (left), suggesting that *rpfF* conferred DSF production in *N. benthamiana*.



CONCLUSIONS

Substantial data now show that cell-cell signaling plays a major role in the epidemiology and virulence of *Xf* and that disruption of cell signaling is a promising means of controlling Pierce's disease. Strikingly, *Xf* strains that cannot signal are also not transmissible by nor colonize an efficient insect vector. This result reveals an important and previously unappreciated connection between cell-cell signaling and transmission as well as the requirement for biofilm formation for transmission. These new findings will be helpful for those interested in targeting transmission as a means of disease control. We also found that mutants unable to signal are hypervirulent. Conversely, strains of *Xf* that overproduce DSF have low virulence and do not move within grape. This suggests that, it will be more efficient to elucidate and target *Xf*'s colonization strategies rather than traits predicted to contribute to virulence based on studies of other plant pathogens. We have identified bacterial strains that can interfere with *Xf* signaling. These strains proved very effective as protective agents for grapevines when co-inoculated with *Xf*. Both positive and negative interference with DSF signaling reduced disease in grape suggesting that signaling is normally finely balanced in the disease process; such a finely balanced process might be readily disrupted. Since in bacteria *rpfF* is sufficient to encode a synthase capable of DSF production, expression of DFS directly in plants is a attractive approach for disease control. Preliminary results are very encouraging that DSF can be made in plants. Alternatively, the use of various bacteria to express DSF implants may prove equally effective in altering *Xf* behavior and hence disease control.

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