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 probably shares a similar means of coordinating gene expression in a community (2, 7). Using the *Xcc* paradigm as a guide, we have investigated 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 *Xf* produces a cell-cell signal and that the *rpfF* gene is necessary and sufficient for signal synthesis. We compared *rpfF* mutants to the wild type and found they 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. We are in the process of investigating the mechanism of hypervirulence. To further elucidate the behavior of *Xf in planta,* we created a green fluorescent strain of *Xf* and used confocal laser scanning microscopy to observe this strain of *Xf* within the xylem of plants. We found that vessel plugging is the colonization feature most tightly correlated with disease symptom expression, providing strong evidence that vessel plugging causes disease. We screened several collections of bacterial strains isolated from plants and identified bacterial strains that can interfere with *Xf* signaling. We are in the process of testing how these strains interact with *Xf* in the xylem, identifying to which species they belong and isolating the genes responsible for signal interference activity.

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, sending out "scouts" to colonize new areas within the host. 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 investigate cell-cell communication in *Xf* to determine its role 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). 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). Thus *Xf* likely employs a cell-cell signaling apparatus similar to that of *Xcc.* Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of Rpf regulation would be genes necessary for colonizing the xylem and spreading from vessel to vessel. For example, expression of extracellular polysaccharides, cellulases, proteases and pectinases might be induced by the signal. 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.

It is conceivable that cell-cell signal interference may be used by other organisms to interfere with density-dependent behaviors, such as pathogenicity or spreading through the habitat. Several recent studies indicate that other organisms can disrupt or manipulate the cell-cell signaling system of bacteria (4, 5). Examination of *Xf* population size in plants where *Xf* lives as an endophyte versus those in which *Xf* causes the xylem blockage symptoms of Pierce's disease demonstrates a positive relationship between population size and symptom development (3). We hypothesize that an interaction between *Xf*

and other organisms, such as another endophyte or the host plant itself, may modulate density-dependent behaviors in *Xf* by interfering with cell-cell signaling.

OBJECTIVES

1. Characterize cell-cell signaling factors in *Xf*.
2. Determine role of signaling factors on virulence and transmissibility of *Xf*.
3. Identify degraders of signaling factors of *Xf*.
4. Identify inhibitory analogs of signaling factors of *Xf*.

RESULTS

Objective 1. We have constructed "signal-sensing" strains of *Xcc* to determine whether *Xf* uses the same butyrolactone signal as *Xcc*. These strains carry a green fluorescent protein (gfp) gene under the control of a promoter that is up-regulated in response to the signal. We have successfully detected a signal from *Xf* using this system (Figure 1a,b), however the response is much weaker than that of *Xcc*. We conclude that *Xf* may make high concentrations of the signal only under specific conditions, such as *in planta*. A second possibility is that the *Xf* signal differs slightly from the *Xcc* signal and cannot fully activate the *Xcc* signal sensor except at high concentrations. To further investigate the ability of *Xf* to make DSF, we cloned the *Xf rpfF* gene, which is predicted to function as the signal synthase, into an *Xcc rpfF* mutant and determined that it could restore DSF production to the *Xcc* strain (Figure 1h). Interestingly, the *Xf* gene could not achieve the same level of rescue as the *Xcc* gene (Figure 1g), further suggesting that the *Xf* signal may have a slightly different structure that the *Xcc* signal.

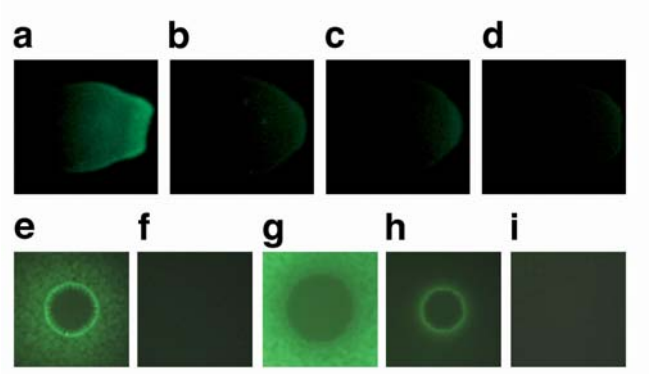

Figure 1. Detection of DSF from *X. fastidiosa* and *Xanthomonas* strains. (a-d) DSF-reporter strain grown to the left of concentrated *X. fastidiosa* culture extracts of the wild-type strain Temecula (a), sterile medium (b), or rpfF mutant strains KLN61 (c) and KLN62 (d). (e-i) DSF-reporter strain sprayed over colonies of *Xanthomonas* wild type (e), *Xanthomonas* rpfF mutant (f), *Xanthomonas* rpfF rescue strain (g), *X. fastidiosa* rpfF rescue strain (h), empty vector control strain (i). Green fluorescence indicates detection of DSF that has diffused from the culture extract or colony.

Objective 2. We have constructed strains of *Xf* Temecula in which the *rpfB* and *rpfF* genes, which are each required for production of the signal in *Xcc,* are knocked out. These mutants were constructed using exchange of the wild-type allele for a deleted copy carrying an antibiotic resistance gene on a suicide plasmid. We tested *Xf rpfB* and *rpfF* mutants for DSF production. Although *rpfB* mutants are still able to make DSF (data not shown), *rpfF* mutants can no longer make the signal (Figure 1b,c). *rpfB* and *rpfF* mutant strains were tested for their ability to infect and move within host plants and to cause Pierce's disease symptoms. Neither of these genes is strictly required for virulence as mutant strains cause symptoms similarly to the wild type. However, 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 differences if they existed. We observed colonies *in planta* via scanning electron microscopy and again found no obvious differences. We hypothesize that *rpfF* mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression. We are in the process of creating a green fluorescent *rpfF* mutant to investigate the pattern of colonization by the mutant and compare it to that of the wild type.

We have tested transmissibility of the mutant strains by an insect vector. While the *rpfB* mutant was transmitted with equal frequency as the wild type by blue-green sharpshooter leafhoppers, the *rpfF* mutant was virtually untransmissible. This defect in transmissibility by the signaling-deficient mutant was unexpected and 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 of the mutant whereas the wild type is never cleared from leafhoppers without molting (6). Scanning electron micrographs of leafhopper foreguts revealed that the *rpfF* mutants are unable to form the characteristic polar biofilm in the precibarium (Figure 2).

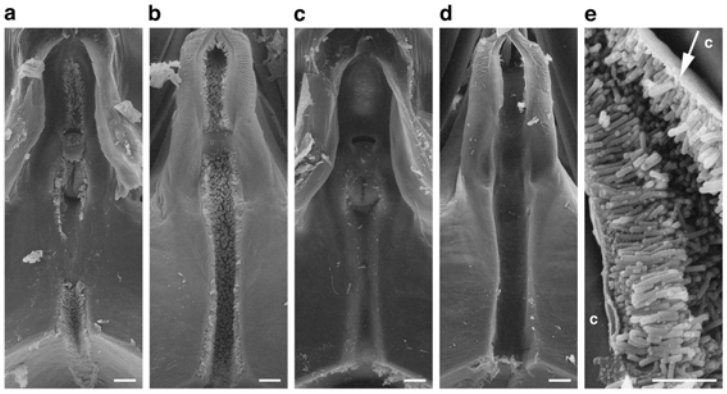

Figure 2. Formation of polar biofilm in insect foreguts. Scanning electron micrographs of the precibarium epipharynx (a,c) and hypopharynx (b,d) of blue-green sharpshooter leafhoppers fed on grapevines infected with the wild-type strain Temecula (a,b,e) or the rpfF mutant KLN61 (c,d). Xylem sap enters the precibarium from the top and runs through the canal, which is coated with a biofilm by wild-type cells (a,b) but not rpfF mutant cells (c,d). (e) High magnification of polar biofilm that has slightly detached from cuticle (c) during fixation revealing a mat-like structure at the attachment site (arrow). Bar = 10 μ (a-d) and 5 μ (e).

To better direct our analyses, we have constructed a strain of *Xf* that constitutively expresses Gfp in order to bring the *in planta* growth habit of *Xf* during symptom formation into sharper focus. By observing differences in colonization between symptomatic and asymptomatic samples we have developed a clearer image of the mechanism of symptom development and the best strategies for preventing it. We found that symptomatic leaves had a forty-fold higher frequency of plugged vessels that asymptomatic leaves and that vessel plugging was the colonization feature most highly correlated with symptom expression (Figure 3a, b, d). By contrast, the frequency of colonized vessels was only five-fold higher in symptomatic leaves than asymptomatic leaves. (Figure 3c) This observation suggests that it is unlikely that bacterial toxins or plant-initiated vessel failure lead to disease symptoms. We also found evidence for *Xf* movement into adjacent xylem vessels via the bordered pits (Figure 3e), supporting the current hypothesis that this bacterium uses extracellular enzymes to digest through the pit membranes and gain access to new habitat.

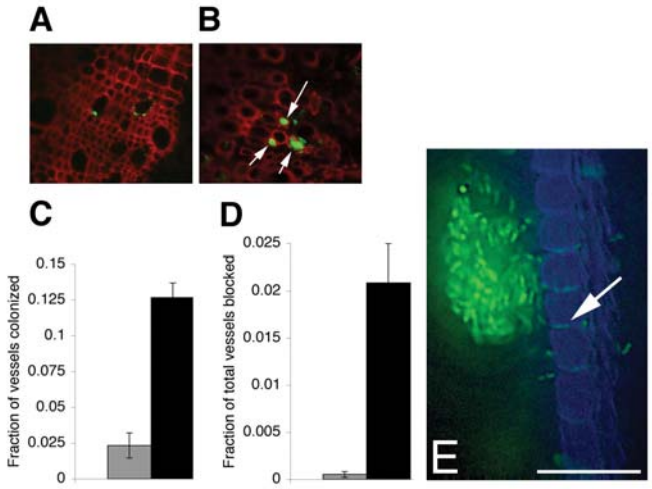

Figure 3. Analysis of xylem colonization using a gfp-marked strain. Confocal laser scanning micrograph of representative xylem bundles from asymptomatic (a) and symptomatic (b) leaf petioles. Arrows mark plugged vessels. Histograms comparing the frequency of vessels colonized (c) or plugged (d) in a ~0.9 micron section of asymptomatic leaves (grey bars) versus symptomatic leaves (black bars). Deconvolution micrograph of *Xf* cells gaining access to a new vessel (right side) from an adjacent vessel (left side) through the bordered pits (e.g., arrow).

Objectives 3 and 4. We have collected 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. We have recovered bacteria from these samples to generate a comprehensive collection of bacterial strains that grew in contact with the signal molecule. These strains have been tested for the ability to interfere with cell-cell signaling in *Xf* in an assay using the signal-sensing strains from Objective 1. We have isolated several strains that inhibit or activate cell-cell signaling. We have introduced these strains, along with *Xf,* into greenhouse-grown grapevines to monitor their effect on Pierce's disease development. We are sequencing 16S rRNA gene sequences from these strains to determine their species identity. Candidates from the interfering strains are being chosen for mutational analysis of the interfering activity. 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 timing and severity of disease is currently being monitored.

CONCLUSIONS

We have investigated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease. In this vein we have found that *Xf* indeed produces a cell-cell signal. We have shown that the *rpfF* gene is necessary and sufficient for synthesis of *Xf*'s cell-cell signal. We found that *Xf* strains that cannot signal are also not transmissible by an efficient insect vector. We show that this lack of transmissibility stems from the *rpfF* mutant being unable to form a biofilm in the insect foregut. 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. While we are still in the process of investigating the mechanism of hypervirulence, this finding suggests that cell-cell signaling is used by *Xf* to control virulence to the plant. If such a hypothesis is correct, it may 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. To that end, we characterized the behavior of *Xf in planta* using confocal laser scanning microscopy to observe a green fluorescent strain of *Xf* within the xylem of grapevines. Our analysis showed tight correlation between vessel plugging and symptom development, providing strong evidence that vessel plugging causes disease. This finding will be helpful in designing strategies to reduce disease by indicating, for example, that treatments that reduce vessel plugging may diminish disease symptoms. We have identified bacterial strains that can interfere with *Xf* signaling. These strains may be useful as protective agents for grapevines exposed to *Xf*. Alternatively, when we isolate the gene conferring the interfering activity, we may be able to directly introduce the protective trait into the plant itself.

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