Cell–cell signaling controls Xylella fastidiosa interactions with both insects and plants

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Contributed by Steven E. Lindow, December 16, 2003

Xylella fastidiosa, which causes Pierce’s disease of grapevine and other important plant diseases, is a xylem-limited bacterium that depends on insect vectors for transmission. Although many studies have addressed disease symptom development and transmission of the pathogen by vectors, little is known about the bacterial mechanisms driving these processes. Recently available X. fastidiosa genomic sequences and molecular tools have provided new routes for investigation. Here, we show that a diffusible signal molecule is required for biofilm formation in the vector and for vector transmission to plants. We constructed strains of X. fastidiosa mutated in the rpfF gene and determined that they are unable to produce the signal activity. In addition, rpfF mutants are more virulent than the wild type when mechanically inoculated into plants. This signal therefore directs interaction of X. fastidiosa with both its insect vector and plant host. Interestingly, rpfF mutants can still form in planta biofilms, which differ architecturally from biofilms in insects, suggesting that biofilm architecture, rather than a passive response to the environment, is actively determined by X. fastidiosa gene expression. This article reports a cell–cell signaling requirement for vector transmission. Identification of the genes regulated by rpfF should elucidate bacterial factors involved in transmission and biofilm formation in the insect.

The Gram-negative bacterium Xylella fastidiosa colonizes the xylem, a water transport network of interconnected vessels composed of dead cells, forming an aggregated biofilm inside the plant. Vessels can become occluded by dense colonization, and high frequencies of blocked vessels are associated with disease symptom development (1–3). X. fastidiosa is responsible for diseases that cause economic loss in many agricultural plants; however, it can also live in symptomless hosts that serve as a source of inoculum (4). In susceptible host plants, X. fastidiosa spreads throughout the xylem network from the site of inoculation. Although X. fastidiosa cells likely move along individual xylem vessels with the sap flow, it is currently thought that X. fastidiosa moves from one vessel to another through the bordered pits (intervessel channels) only after degrading the membranes that guard them (1, 5). Xylem sap-feeding insects can acquire and transmit X. fastidiosa, which forms a biofilm of polarly attached cells inside their foreguts (6, 7). Once infected with X. fastidiosa, insects remain infective with the pathogen, which multiplies in the foregut (8, 9). Xylella fastidiosa, insects remain infective with the pathogen, which multiplies in the foregut (8, 9).

Abbreviations: DSF, diffusible signaling factor; Xcc, Xanthomonas campestris pv. campestris; KanR, kanamycin resistance.

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DSF Assays. *X. fastidiosa* strains were grown to OD_{600} 0.045 (stationary phase) in 50 ml of periwinkle wilt medium (PW) (21) at 28°C, shaking at 160 rpm, then 50 ml of water-saturated ethyl acetate was added to the culture supernatant, vortexed, and centrifuged. The ethyl acetate phase was concentrated by evaporation to 20 μl and spotted onto a paper disk, which was placed on a King’s B agar (KB) plate. Ten microliters of an OD_{600} 0.25 suspension of 8523 (pKLN55) was pipetted near the disk, and plates were incubated for 48 h. *Xcc* strains were streaked on KB plates, incubated 1 day at 28°C. Resulting colonies were oversprayed with an OD_{600} 0.25 suspension of 8523 (pKLN55) by using an airbrush and incubated 2 additional days. In these assays, signal diffuse from the sample to the reporter, resulting in green fluorescence. Fluorescence was viewed on a Zeiss SV11 stereoscope with Kramer epifluorescence/Optronix Color DEI450.

*Xcc* rpfF Rescue. rpfF was amplified from the Xcc and *X. fastidiosa* genomes by using primers “XccrpflFFXhoI” (5′-ACTCGAGATTAGGAGGAAAACATATGTCCGCTGTACA-3′) and “XccrpfrFRSpeI” (5′-GACTAGTCAAGCCCGCTGCAGGCTTAGAGGGCAGCCTAGGAGAGCCGACCT-3′), or “XfrrpflFFXhoI” (5′-ACTCGAGATTAGGAGGAAAACATATGTCCGCTGTACA-3′) and “XfrrpfrFRSpeI” (5′-GACTAGTCAAGCCCGCTGCAGGCTTAGAGGGCAGCCTAGGAGAGCCGACCT-3′), and ligated into the same sites of pBBR1MCS-3 (25, 50), respectively, and ligated into pGEM-T (Promega) to make pXccrpflFF-T and pXfrrrpflFF-T, respectively. rpfF was cut out from pXccrpflFF-T and pXfrrrpflFF-T with XhoI and SpeI and ligated into the same sites of pBRR1MCS-3 (22) to make pKLN69 and pKLN70, respectively. pKLN69, pKLN70, and pBRR1MCS-3 were mated into *Xcc* strains 8004 (18) and 8523 (20).

Transmission Tests. Blue-green sharpshooter leafhoppers, *Gra- phocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae) were maintained and used in transmission tests as described (1). Adults were caged on symptomatic grapevines infected with Temecula, KLN61, or mock inoculated for a 4-day acquisition access period. Each insect was then transferred to a healthy grapevine for a 7-day inoculation access period. Grapevines were observed for symptoms in the greenhouse for 6 months. *X. fastidiosa* infection was verified by culturing bacteria from plant macerates (23), then genotyping bacteria by PCR with primers “rpfFKOFNotI” and “rpfFRBglII” (5′-GGAAGATTCCTACACAGCTTTAAATGTCCTCATGTT-3′).

Detection of *X. fastidiosa* from Insects. Forty-one insects for each treatment (Temecula, KLN61, and buffer) were exposed to infected plants for 4 days. Then, 19 were killed for bacteriological culture, and 22 were moved to uninfected grapevines for 7 days before culturing. For culturing, insect heads were severed, surface sterilized, macerated in 100 μl of buffer, and dilution plated onto periwinkle wilt medium with gellan gum (PWG) (23). We calculated our limit of detection to be 25 colony-forming units per head. For statistical analysis, we analyzed six different 2 × 2 contingency tables representing all treatment combinations to determine significant differences among treatments. We used a Bonferroni correction method, α∗ = α/k (24) to determine the correct α (0.0083), where α = 0.05 and k = 6. Excrement was collected from leafhoppers during the final day of a 4-day access period on an infected grapevine, and bacterial cells were pelleted, resuspended in buffer, heated to 95°C, and used as templates in PCR with the *X. fastidiosa*-specific primers RST31 and RST33 (25).

Pathogenicity Assays. Greenhouse-grown *Vitis vinifera* “Cabernet sauvignon” grapevines were mechanically inoculated by using standard procedures (26). The number of ascisced leaf blades from the first 20 nodes distal to the inoculation site was noted.

**Fig. 1.** (A and B) pKLN61 and pKLN62 plasmids used to create rpf mutant strains KLN61 and KLN62, respectively. The vector backbones are pGEM-5ZF(+) (Promega). The gray-shaded region represents the rpf coding sequence and flanking DNA (start and stop codons are indicated, if present). Regions of the coding sequence were removed before insertion of the KanR gene. Double recombination with the *X. fastidiosa* genome occurs in the gray-shaded regions flanking the KanR gene, resulting in exchange of the wild-type allele for the deletion-harboring KanR allele. No genes are cotranscribed with rpf in *X. fastidiosa; therefore, disruption of expression of genes other than rpf in strains KLN61 and KLN62 is highly unlikely. We note that homologous DNA in allelic exchange construct pKLN62 was only 97 bp long on one end, indicating that very short stretches of sequence are sufficient for homologous recombination in this organism. (C) Alignment of the predicted RpfF proteins from *X. fastidiosa* and Xcc. The sequences are 290 aa in length and are 66% identical and 80% similar overall. The coding sequence for the solid underlined region is deleted in strain KLN61 (codons 4–89). The coding sequence for the dashed underlined region is deleted in strain KLN62 (codons 89–290). The sequence encoding amino acid 99, a glutamine, is deleted in both strains. Sequences were aligned by using CLUSTALW 1.8 (and presented in BOXSHADE 3.21). Strains infecting plants were verified by culturing and PCR-based genotyping as above.

Scanning Electron Microscopy. Grapevine petiole segments and leafhopper heads were fixed and dried according to the University of California Electron Microscope Lab protocol at biology.berkeley.edu/EML/psem.html. Samples were dissected, coated with 13 nm of gold/palladium, and viewed on an ISI DS-130 (Fig. 4) or 3 nm of platinum and 13 nm of carbon and viewed on a Hitachi S-5000 (Fig. 6) scanning electron microscope at the University of California Electron Microscope Lab.

**Results**

*X. fastidiosa* Requires rpfF to Make a Diffusible Signal Factor. We constructed *X. fastidiosa* strains impaired in signaling by deleting rpfF via allelic exchange mutagenesis (Fig. 1). Two independent rpfF deletion mutants were constructed, KLN61 and KLN62, linking mutant phenotypes definitively to this locus. To detect signal produced by X. fastidiosa strains, we developed a DSF reporter strain of Xcc, 8523 (pKLN55), which expresses GFP in the presence of DSF. This reporter expressed GFP in response to ethyl acetate extracts of an *X. fastidiosa* culture (Fig. 2A) but not sterile media (Fig. 2B), demonstrating that a DSF-like signal was extracted from the *X. fastidiosa* culture. No signal was
detected in extracts of the rpfF mutant strains KLN61 or KLN62 (Fig. 2 C and D), indicating that rpfF is required for signal production in X. fastidiosa.

To confirm that the X. fastidiosa RpfF protein functions as a DSF synthase, we rescued Xcc rpfF mutant strain 8523 (20) with X. fastidiosa rpfF (Fig. 2 E–I). X. fastidiosa rpfF conferred DSF production to strain 8523 (Fig. 2 F and H), although less efficiently than did Xcc rpfF (Fig. 2 F and G). The reason that rescue was less efficient with the X. fastidiosa gene than with the Xcc gene is unclear. We speculate that X. fastidiosa RpfF may have lower activity than Xcc RpfF in Xcc cells. Alternatively, the X. fastidiosa RpfF may synthesize a somewhat different signal molecule that is less active in Xcc. The Xcc rpfF gene in the rescued Xcc rpfF mutant strain produced higher levels of DSF than did the wild-type strain (Fig. 2 E and G). This finding might be expected due to an increase in copy number and transcriptional activation of the Xcc rpfF gene in the rescued strain compared with the wild type.

rpfF Mutants Are Defective in Insect Transmissibility. We investigated whether the signaling mutants were transmissible by an efficient insect vector, the blue-green sharpshooter leafhopper. Because strains KLN61 and KLN62 were equally impaired in signal production, we chose strain KLN61 for this analysis. We used a protocol that maximized insect acquisition and transmission opportunities (see Materials and Methods). After inoculation by leafhoppers, Temecula-infected grapevines had multiple symptomatic leaves by an average of 57 ± 6 SE days, whereas KLN61-infected plants were symptomless for 6 months after inoculation. Strain KLN61 was never recovered from these insect-inoculated plants whereas Temecula was always recovered (n = 8 replicates). This experiment was repeated twice with similar results (n = 11 and n = 17). The average transmission efficiency for these experiments was 0.72 ± 0.15 SE for Temecula compared with only 0.03 ± 0.03 SE for KLN61 (one case of transmission). Therefore, the rpfF mutant is highly impaired in transmissibility compared with the wild type.

Colonization of Insects Is Impaired in rpfF mutants. Transmission of X. fastidiosa by the insect depends on uptake of bacteria by the insect during feeding. To investigate the ability of the insect to ingest KLN61 from infected source plants, we collected excrement produced during feeding on grapevines mechanically infected with either Temecula or KLN61, or mock inoculated. Both Temecula and KLN61 were detected in excrement, confirming that insects ingested both wild-type and rpfF bacteria during feeding although the frequency of detection of bacteria in excrement was extremely low [only 1 (KLN61) or 2 (Temecula) positive samples were obtained from 10 insects per treatment]. Immediately after removal from infected plants, we recovered Temecula from 8/19 (42.1%) and KLN61 from 10/19 (52.6%) leafhoppers (Fig. 3), indicating that both strains are inside leafhoppers at similar frequencies during exposure to an infected plant. When leafhoppers were then transferred to uninfected grapevines for 7 days, there was no change in the total proportion of leafhoppers retaining Temecula [11/22 (50%); χ² = 0.26, df = 1, P = 0.6]. However, KLN61 was retained by only 1/22 leafhoppers (4.5%), revealing a significant defect in KLN61 retention by leafhoppers (Fig. 3; χ² = 9.34, df = 1, P = 0.0005). No bacteria were recovered from insects or excrement when fed on mock-inoculated grapevines. These results suggest that the rpfF mutant is poorly transmitted because it is not retained by leafhoppers as efficiently as the wild type, as must be required for efficient vector transmission.

rpfF Mutants Cannot Form Biofilms in Insects. We examined leafhopper foreguts 7 days after transfer from infected to uninfected grapevines by using scanning electron microscopy. All insects fed on Temecula-infected plants (n = 8) displayed characteristic bacterial colonization throughout the precibarium, a section of the foregut, with cells attached in a polar biofilm in both the epitharynx (Fig. 4 A and E; dorsal) and hypopharynx (Fig. 4B; ventral). Although only a small number of X. fastidiosa cells are required for efficient transmission (27), we did not find any bacteria within the precibarium of insects fed on KLN61-infected plants (n = 12; Fig. 4 C and D). We conclude that polar biofilm formation in the precibarium requires rpfF, which may regulate genes involved in initial attachment or proper formation of the biofilm.

rpfF Mutants Are Hypervirulent in Plants. We compared development of Pierce’s disease symptoms in grapevines mechanically inoculated with rpfF and wild-type bacteria. Pierce’s disease symptoms typically progress from loss of chlorophyll in leaf margins, to leaf scorch, and finally to the highly characteristic abscission of the leaf blade from the petiole, or leaf stem (Fig. 5 A and C). Grapevines were inoculated with the X. fastidiosa strains Temecula, KLN61, or KLN62, or buffer, and symptom development was monitored. After 4 months, grapevines inoculated with KLN61 or KLN62 lost about three times as many leaves (9.83 ± 1.60 SE and 14.0 ± 1.73 SE, respectively) as grapevines inoculated with Temecula (4.14 ± 0.51 SE). Mock-
Populations at 3 weeks postinoculation were highly variable with no apparent trend, but, grapevines per treatment per time point. Populations at 3 weeks inoculated grapevines before symptom expression. Plant samples 15, 30, and 60 cm distal to the inoculation site at 3, 5, and 7 weeks postinoculation were cultured from grapevines infected with the wild-type strain Temecula (A, B, and C) or the rpfF mutant KLN61 (C and D). The stylets, which are inserted into the plant, are located above, and the cibarium (pumping chamber) is located below, the frame of the images. Xylem sap enters the grapevine xylem by scanning electron microscopy of petioles of symptomatic leaves and found that both strains formed matrix-both were associated with long strands of matrix when found in symptomatic leaves and found that both strains formed matrix-which are inserted into the plant, are located above, and the cibarium (pumping chamber) is located below, the frame of the images. Xylem sap enters the grapevine xylem by scanning electron microscopy of petioles of symptomatic leaves and found that both strains formed matrix-both were associated with long strands of matrix structure. This hypervirulence was surprising, given that Xcc rpfF mutants are hypovirulent and suggests that, in X. fastidiosa, cell–cell signaling tempers virulence in the plant.

To understand the mechanism of hypervirulence by the rpfF mutant, we monitored bacterial populations in mechanically inoculated grapevines before symptom expression. Plant samples 15, 30, and 60 cm distal to the inoculation site at 3, 5, and 7 weeks postinoculation were cultured from grapevines infected with the wild-type strain Temecula (A, B, and C) or the rpfF mutant KLN61 (C and D). Populations at 3 weeks postinoculation were highly variable with no apparent trend, but, at 3, 5, and 7 weeks, populations for all strains and distances were similar, $10^8$ colony-forming units/g (data not shown). These data suggested that large increases in population size or in swiftness of spread from the inoculation site are not the cause of rpfF hypervirulence although only substantial differences in populations would have been detected in our study.

We observed wild-type and rpfF mutant colonization of the grapevine xylem by scanning electron microscopy of petioles of symptomatic leaves and found that both strains formed matrix-encased biofilms when found packed in crowded vessels, and both were associated with long strands of matrix when found in less crowded communities (Fig. 6). This matrix likely represents exopolysaccharide that we believe is secreted by the bacteria, suggesting that the X. fastidiosa rpfF mutant still produces exopolysaccharide, unlike the Xcc rpfF mutant, which cannot (20). Interestingly, rpfF mutations in a third related plant pathogen, Xanthomonas oryzae pv. oryzae, cause reduced virulence without affecting exopolysaccharide production (28).

**Discussion**

After the first identification of cell–cell signaling by way of DSF in Xcc and demonstration of its role in virulence gene expression, DSF was shown to be produced by several other bacterial species, in the Xanthomonas, Pseudomonas, Mycobacterium, and Erwinia genera (14, 15). However, with the exception of X. oryzae pv. oryzae (28, 29), the role of DSF signaling has not been investigated for any of these other species. Here, we demonstrate that a DSF activity is detected in X. fastidiosa media extracts by an Xcc-based GFP reporter (Fig. 2 A and B), suggesting that cell–cell signaling occurs in X. fastidiosa. We found that DSF reporter activation by the X. fastidiosa Temecula strain was much weaker than that by Xcc perhaps because high concentrations of the signal are produced only under specific conditions, such as in planta, or because the X. fastidiosa signal is structurally different and cannot fully activate the Xcc-based DSF reporter. These results are in agreement with those obtained for a citrus strain of X. fastidiosa (17).

We constructed two strains of X. fastidiosa Temecula in which the rpfF gene was disrupted and found that they do not have signal activity (Fig. 2 B and C), indicating that rpfF is required for DSF biosynthesis in X. fastidiosa. We are unable to rule out that RpfF may play a role in the synthesis of other molecules in addition to DSF. Expression of the X. fastidiosa rpfF gene from a constitutive promoter in an Xcc rpfF mutant restored DSF activity production (Fig. 2H). Interestingly, the X. fastidiosa gene could not confer the same level of DSF reporter activation as the Xcc gene did when expressed in either Xcc (Fig. 2G) or in Escherichia coli strain DH5α, which was used to prepare the plasmids (data not shown). That the difference in activities occurs even when both genes are placed in a heterologous host (E. coli) further suggests that the X. fastidiosa signal may have a slightly different structure than the Xcc signal. In addition, the rpfF gene alone confers production of DSF to E. coli (which does not express the gene in planta).

**Fig. 4.** Formation of polar biofilm in insect foreguts. Scanning electron micrographs of the precibarium epipharynx (A and C) and hypopharynx (B and D) of blue-green sharpshooter leafhoppers fed on grapevines infected with the wild-type strain Temecula (A, B, and E) or the rpfF mutant KLN61 (C and D). The stylets, which are inserted into the plant, are located above, and the cibarium (pumping chamber) is located below, the frame of the images. Xylem sap enters the grapevine xylem by scanning electron microscopy of petioles of symptomatic leaves and found that both strains formed matrix-both were associated with long strands of matrix structure (arrow). Rod-shaped structures are bacterial cells. (Bar = 10 μm (A–D) and 5 μm (E).)

**Fig. 5.** Pierce’s disease symptoms in grapevines. Cabernet sauvignon grapevines infected with the wild-type strain Temecula (A), the rpfF mutant KLN61 (B), or mock inoculated (C). Yellow arrows (A and B) indicate leaves with leaf scorch, an early disease symptom, and white arrows (B) indicate leaves that have detached at the blade–petiole junction, an advanced disease symptom.
not produce DSF on its own), indicating that RpfF is sufficient for DSF biosynthesis.

We have found that the rpfF mutant was rarely transmitted by a highly efficient vector of X. fastidiosa, the blue-green sharpshooter leafhopper. This transmissibility defect 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 there is no evidence that the wild type can be cleared from leafhoppers except through molting (8). We show that this lack of retention (and thus transmissibility) is correlated to an inability of the rpfF mutant to form a biofilm in the insect foregut (Fig. 4). This result reveals an important and previously unappreciated connection between cell–cell signaling and transmission, as well as an apparent requirement for biofilm formation for efficient transmission. These findings may contribute to new strategies for controlling Pierce’s disease by targeting transmission.

It is intriguing that biofilm formation by rpfF mutants is affected in insects (Fig. 4) but not plants (Fig. 6). In both environments, bacteria are subjected to high fluid shear force, are bathed by xylem sap, and require detachment to spread to new habitats. The main difference is in the attachment substrate (insect cuticle or xylem vessel). In plants, cells attach in any orientation to the xylem wall and aggregate in matrix-enclosed communities that frequently include space where xylem sap can flow (Fig. 6). In insects, cells attach in a polar fashion to the insect cuticle and form a mat-like structure at the point of attachment (Fig. 4E). Whether such differences in architecture result from environmental forces or bacterial activity is currently under debate (30). Impairment in only one type of biofilm formation in rpfF mutants suggests that the differences in biofilm architecture are subject to control by the organism as well as by the environment. The role of cell–cell signaling in biofilm formation and architecture has been detailed for other organisms such as Pseudomonas aeruginosa, in which it is required for maturation of the biofilm architecture (10). Motility via type IV pili and flagellae has been implicated in the achievement of mature architecture (31). Although the X. fastidiosa genome lacks genes required for flagella biosynthesis (32), it includes genes similar to those encoding type IV pili and other genes encoding putative adhesins, which may be important for attachment to the insect cuticle (33).

rpfF mutations have opposite effects on virulence to plants in X. fastidiosa and Xcc, highlighting our limited understanding of X. fastidiosa virulence. Hypervirulence in rpfF mutants suggests that X. fastidiosa employs a strategy to temper virulence to hosts. Extensive exopolysaccharide production and cell division lead to vessel plugging in the plant, presumably followed by nutrient shortage and decreased spread to new hosts as sap-feeding insects avoid blocked vessels. X. fastidiosa may use cell–cell signaling-based strategies to seek the most advantageous balance between pathogenicity and growth and spread to new hosts, as has been suggested by in planta microscopic studies (1).

DSF signaling in X. fastidiosa may regulate exoenzyme and exopolysaccharide biosynthesis, as it does in Xcc (14). Current theory proposes that X. fastidiosa expresses exoenzymes to degrade pit membranes. It is assumed, but unproven, that X. fastidiosa must break down these membranes to disperse effi-
ciently throughout the plant and cause disease. However, if exoenzymes are positively regulated by DSF signaling, as they are in *Xcc*, then *rpfF* mutants of *X. fastidiosa* should be deficient in exoenzyme production in * planta*. In that case, one can theorize that hypervirulence of the *rpfF* mutants may arise from failure to escape crowded vessels into which *X. fastidiosa* may occasionally move by natural portals. We hypothesize that this failure would be expected to increase vessel plugging and exacerbate Pierce’s disease symptoms (Fig. 7). Thus, although the bacteria may not colonize as many vessels, it may more frequently occlude those vessels to which it spreads and thus incite more disease symptoms. DSF regulation of exopolysaccharide production may also contribute to virulence and transmission in *X. fastidiosa*. In some systems, exopolysaccharides inhibit cells from forming initial, specific attachments required for biofilm formation, perhaps by masking attachment proteins (34, 35). In other cases, exopolysaccharide synthesis enhances biofilm formation (36, 37), perhaps at postattachment steps. In *X. fastidiosa*, DSF-mediated cell–cell signaling may be required for control of exopolysaccharide synthesis to allow cells to form successful biofilms on the insect cuticle.

Our finding that cell–cell signaling controls both transmission and virulence opens new avenues for exploration of host–microbe interactions. Future experiments are needed to identify the specific traits regulated by DSF signaling in *X. fastidiosa* and to investigate the role these traits play in interactions with insects and plants.

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