A cell–cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*

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Cell–cell signaling in *Xylella fastidiosa*, a xylem-colonizing plant pathogenic bacterium, mediated by a fatty acid Diffusible Signaling Factor (DSF), is required to colonize insect vectors and to suppress virulence to grape. Here, we show that a hybrid two-component regulatory protein RpfC is involved in negative regulation of DSF synthesis by RpfF in *X. fastidiosa*. *X. fastidiosa* *rpfC* mutants hyperexpress *rpfF* and overproduce DSF and are deficient in virulence and movement in the xylem vessels of grape. The expression of the genes encoding the adhesins FimA, HxfA, and HxfB is much higher in *rpfC* mutants, which also exhibit a hyper-attachment phenotype in culture that is associated with their inability to migrate in xylem vessels and cause disease. *rpfF* mutants deficient in DSF production have the opposite phenotypes for all of these traits. RpfC is also involved in the regulation of other signaling components including *rpfG*, *rpfB*, a GGDEF domain protein that may be involved in intracellular signaling by modulating the levels of cyclic-di-GMP, and the virulence factors *toxC* and *pglA* required for disease. *rpfC* mutants are able to colonize the mouthparts of insect vectors and wild-type strains but are not transmitted as efficiently to new host plants, apparently because of their high levels of adhesiveness. Because of the conflicting contributions of adhesiveness and other traits to movement within plants and vectoring to new host plants, *X. fastidiosa* apparently coordinates these traits in a population-size-dependent fashion involving accumulation of DSF.

biofilm | endophytes | Pierce's disease | quorum sensing | adhesins

Xylella fastidiosa is an important plant pathogen that causes disease in many economically important plants including grape, citrus, coffee, and many species important in horticulture (1). *X. fastidiosa* is restricted to the xylem vessels of plants and is transmitted by xylem sap feeding insects such as sharpshooter species (2, 3). The mechanisms of virulence of *X. fastidiosa* are not entirely clear, but the most characteristic symptoms of diseases that it causes, such as Pierce's disease of grape, are leaf scorching and are associated with the colonization of large numbers of xylem vessels, likely leading to diminished water flow to distal parts of the plant (4–6). Thus, the virulence of *X. fastidiosa* is associated with traits including those that enable it to move within and between xylem vessels and to grow to large population sizes in such vessels.

Cell–cell signaling plays an important role in virulence of many plant pathogenic bacteria including *X. fastidiosa*. In this pathogen, and in many species of *Xanthomonas*, a cell–cell signaling system involving the production of a Diffusible Signaling Factor (DSF) modulates their virulence (7–9). The *rpfF* gene has been shown to be required for DSF production in both *X. fastidiosa* and *Xanthomonas* species (7–9). In *Xanthomonas campestris* pv. *campestris* (*Xcc*), a pathogen of crucifers, and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a pathogen of rice, DSF-deficient mutants have reduced virulence, although DSF seems to regulate different virulence traits in each of these species (8, 9). However, in *X. fastidiosa*, *rpfF* mutants that are deficient in DSF production are hypervirulent on grape (7).

Along with signal synthesis, proper signal sensing is also required for coordination of virulence factors (10, 11). In *Xcc* it

has been shown that DSF sensing requires RpfC, one part of a putative two-component regulator. *rpfC* mutants of *Xcc* are de-repressed for DSF production and are also deficient in production of virulence factors such as extracellular polysaccharide (EPS) production and secretion of extracellular enzymes (12). Unlike in *Xcc*, *rpfC* mutants of *Xoo* are deficient in symptom development but grow and migrate like the wild-type strain inside rice leaves (13). In *Xcc*, *rpfC* has been implicated in sensing DSF (12) and initiating downstream signal transduction to regulate gene expression (14). *rpfC* mutants of *Xcc* thus have the same phenotypes (virulence deficiency and lack of virulence-associated functions like EPS and extracellular enzymes) as an *rpfF* mutant that is deficient in DSF biosynthesis (12). In contrast, *rpfF* mutants of *X. fastidiosa* are hypervirulent (7) and the signal transduction pathway for DSF regulation of virulence is unknown. In this study we show that *X. fastidiosa* has an *rpfC* homolog that is involved in autoregulation of DSF synthesis and traits contributing to virulence to grape. We will show that *rpfC* mutants of *X. fastidiosa* do not have the same phenotypes as *rpfF* mutants, being deficient in virulence to grape but able to colonize insect vectors while being deficient in transmission to new hosts, and that these phenotypes involve opposite patterns of expression of a variety of adhesins. We propose a model by which DSF-mediated regulation of virulence in which adhesion to surfaces is a prominent feature is controlled in this species.

Results

***rpfC* Is Required for Regulation of DSF Production in *X. fastidiosa*.** Sequence analysis of RpfC of *X. fastidiosa* (strain Temecula) indicated that it is truncated at the N-terminal compared with the RpfC of *Xcc* and *Xoo*, and other strains of *X. fastidiosa* [supporting information (SI) Fig. 6]. Except for absence of the first 52 aa of the N-terminal region, the *X. fastidiosa* Temecula RpfC is highly similar to RpfC in other pathogenic bacteria (SI Fig. 7).

We constructed two independent *rpfC* mutants of *X. fastidiosa* by allelic exchange mutagenesis, which we designated SC6 (*rpfC*-Kan19) and SC7 (*rpfC*-Kan20), and a *rpfF*-*rpfC* double mutant to determine the phenotypes controlled by *rpfC* and to determine how *rpfC* and *rpfF* coordinate gene expression and virulence. DSF production of these mutants was assessed by using an *Xcc* GFP-based reporter capable of detecting DSF from *X. fastidiosa* and from *Xcc* (7). The *X. fastidiosa* *rpfC* mutants greatly overproduced DSF (7.9-fold higher) compared with the wild-type strain (SI Fig. 8). Expression analysis also indicated that *rpfF*, encoding the DSF synthase, is expressed at a much higher level in these *rpfC* mutants than in the parental strain (6.6-fold; Table 1). The *X. fastidiosa* *rpfC* can complement the

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Fig. 2. Severity of Pierce's disease incited by wild-type (solid bars), an *rpfC* mutant (cross-hatched bars), and a *rpfF* mutant (striped bars). The vertical bars represent the standard error of the mean of disease severity determined from 15 replicate plants for each treatment.

41 plants) showed typical symptoms of Pierce's disease 12 weeks after sharpshooters that had fed on plants infected with wild-type *X. fastidiosa* were allowed to feed on them. In contrast, only 5 plants of 66 that had been fed on by sharpshooters that had previously fed on plants infected with an *rpfC* mutant of *X. fastidiosa* exhibited symptoms of Pierce's disease even 5 months after transmission (SI Fig. 10). A 2×2 Chi-Square test ($df = 1$), $P \leq 0.05$, showed that the difference in transmission frequency was significant. The absence of symptom development on plants inoculated with the *rpfC* mutant by insects was not associated with asymptomatic colonization of the plants because cells of the *rpfC* mutant were recovered by culturing from each of the five plants that had exhibited symptoms after sharpshooter inoculation, but not from any of the asymptomatic plants.

To determine whether the insect transmission deficiency exhibited by the *rpfC* mutant is caused by its inability to colonize the insect vector, we quantified bacterial cells in insect heads 4 days after acquisition from source plants by culturing as described in ref. 7. The frequency of retention of the wild-type *X. fastidiosa* strain by the leafhoppers was 0.58, whereas the average retention frequency of the *rpfC* mutant under similar conditions was 0.41 (SI Fig. 10), a difference that was not significant (Chi-Square $P = 0.32$). Although sharpshooter heads had a similar frequency of colonization, slightly fewer cells of the *rpfC* mutant were detected by culturing of those heads that were colonized; a total of $\log 3.8 \pm 1.3$ and $\log 2.7 \pm 1.6$ cells were detected per head of sharpshooters colonized by the wild-type and *rpfC* mutant, respectively. Furthermore, the average numbers of bacteria isolated from insect heads that were successful

Fig. 3. *rpfC* mutants of *X. fastidiosa* are deficient in movement and multiplication within grapevines. Bacterial population size from petioles of grape (Cabernet sauvignon) inoculated with wild-type *X. fastidiosa* strain Temecula (triangles), *rpfC* mutant of this strain (PSC6) (squares), and an *rpfF* mutant (circles) were determined in petioles harvested at different heights above the point of inoculation. The vertical bars represent the standard error of the mean of log-transformed cell numbers from eight replicates of each treatment. Similar results were observed in two independent experiments (data not shown).

Fig. 4. Estimates of the total number of cells per culture flask present as planktonic cells in the culture medium or attached as a biofilm on the glass surface. The average number of cells from four independent replicate flasks per treatment are shown. The vertical bars represent the standard errors of log-transformed population size. Similar results were obtained in three independent experiments (data not shown).

in transmitting the wild-type and *rpfC* mutants were $\log 4.26 \pm 2.1$ and $\log 2.75 \pm 1.3$, respectively. The average numbers of bacteria isolated from insect heads that were not successful in transmitting the wild-type and *rpfC* mutants were $\log 3.75 \pm 1.4$ and $\log 2.71 \pm 1.6$, respectively, indicating that inoculation efficiency was not tightly linked to cell numbers in the vector. These results indicated that, although the *rpfC* mutant is acquired by and colonizes insect vectors, it is deficient in subsequent transmission to new host plants.

***rpfC* Mutants Have a Hyperattachment Phenotype.** Because inoculation of plants by colonized insects and movement within plants presumably would be influenced by the degree to which *X. fastidiosa* remained attached to surfaces, we examined the ability of *rpfC* mutants to adhere to a glass surface. *X. fastidiosa* forms a ring of attached cells on glass surfaces at the culture medium/air interface when grown in liquid PD medium (15; SI Fig. 11). Interestingly, the *rpfC* mutant, which also forms "sticky" colonies on agar plates, formed a much more pronounced attached ring of cells on the glass surface (SI Fig. 11). In contrast, we observed that an *rpfF* mutant, which forms less "sticky" colonies than the wild-type strain on agar, is severely reduced in its formation of an attached ring on the glass surface and the majority of the bacterial cells were planktonic in the culture medium (Fig. 4). An *rpfF-rpfC* double mutant also exhibited a reduced-attachment phenotype similar to the *rpfF* mutant (data not shown). The average ratio of the number of cells attached to the glass surface compared with those in the planktonic phase for the wild type, *rpfC*, and the *rpfF* mutant was ≈ 0.1 , 1,985, and 0.00001, respectively (Fig. 4). The *rpfC* mutants clearly have an enhanced ability to attach to the glass surface that is impaired in the DSF-deficient *rpfF* mutant.

***rpfF* and *rpfC* Mutants of *X. fastidiosa* Have Different Patterns of Expression of Adhesins and *rpf* Signaling Components.** To investigate the role of *rpfC* and *rpfF* in regulation of other *rpf* signaling components and putative virulence genes, we used quantitative real-time reverse transcriptase PCR (Q RT-PCR) to estimate the abundance of their transcripts in different mutant backgrounds. *rpfF* was expressed at a level at least 6-fold higher in *rpfC* mutants than in the wild-type strain (Table 1). Interestingly, the expression of *rpfC* in a *rpfF* mutant was 4-fold higher than in the wild-type strain (Table 1). The expression of *rpfG*, *rpfE*, and *rpfB* also differed in the *rpfF* and *rpfC* mutant backgrounds, being higher in the *rpfC* mutant than in the wild-type strain (2.1-fold, 1.8-fold, and 2.2-fold, respectively; Table 1). In contrast, these genes were all expressed at a lower level in an *rpfF* mutant than in the wild-type strain (Table 1). Because GGDEF-EAL domain proteins have been shown to be involved in

Fig. 5. A proposed model for DSF-mediated cell-cell signaling in *X. fastidiosa*. *rpfF* encodes the DSF synthase. *RpfC* encodes a hybrid two-component sensor of DSF. DSF levels negatively regulate the expression of *rpfF* by negative feedback. DSF binding to a two-component intracellular DSF sensor leads to autophosphorylation and phosphorelay to a two-component response regulator, likely *RpfG*, which positively regulates genes required for attachment and biofilm formation (*hxfA*, *hxfB*, *fimA*, and *gumJ*). *RpfC* likely interacts with *RpfF*, whereas both *RpfF* and *RpfC* may interact with a putative intracellular repressor "R." The amount of free R would depend on the levels of *RpfF*, *RpfC*, and DSF. Free repressor would repress virulence genes like *tolC*, *pglA* and PD0279 (encoding GGDEF domain protein), which are required for extensive colonization and movement of *X. fastidiosa* in the plant.

in an *rpfF-rpfC* double mutant, the expression of these genes is apparently coordinated by the presence of both *RpfF* and *RpfC*. In an *rpfF* mutant background there is overexpression of *rpfC*. A putative repressor (which can interact with both *RpfF* and *RpfC*) may be sequestered with *RpfC* when it is in excess and, therefore, cannot repress *tolC*, *pglA* and PD0279 (Fig. 5). Because *rpfC* mutants both overexpress *rpfF* and overproduce DSF, DSF may still be sensed by the low-affinity intracellular DSF sensor leading to overexpression of genes like *fimA*, *hxfA*, and *hxfB* required for biofilm formation. However, an excess of *RpfF* would bind with the repressor blocking its ability to repress *tolC*, *pglA* and PD0279. When there is neither *RpfF* or *RpfC*, there would be a high proportion of free repressor that would repress *tolC*, *pglA* and PD0279. Because *RpfC* from *Xcc* and *Xac* was found to bind to several proteins in addition to *RpfF* (14, 25), such an analysis of *RpfC*-binding proteins in our system should be fruitful in identifying the putative repressor. It is likely that intermediate regulators such as *Clp* are under the control of *RpfG* as in *Xcc* (26) and more directly control expression of genes such as *fimA*, *hxfA*, *hxfB*, and some *rpf* genes.

Recently it has been reported that cyclic di-GMP may participate as an intracellular signaling molecule that couples extracellular DSF signaling to modulation of expression of virulence-associated functions (17, 27). Three protein domains, GGDEF, EAL, and HD-GYP, are implicated in the synthesis and degradation of cyclic di-GMP (16, 17, 27). Analysis of the genome sequence of *X. fastidiosa* indicated the presence of GGDEF-EAL domain proteins (PD1994 and PD1617). Interestingly, PD0279 has a GGDEF domain but not an EAL domain, indicating that it may have a dedicated function for cyclic di-GMP synthesis. Expression analysis indicated that the production of this protein is strongly regulated by DSF (Table 1). It seems reasonable to conclude that DSF-dependent signaling involves the modulation of cyclic di-GMP levels in *X. fastidiosa*.

Our preliminary results of studies of mutants blocked in production of this GGDEF domain protein indicate that it is involved in attachment and biofilm formation (unpublished results). It is possible that *RpfG* directly influences cyclic di-GMP levels in *X. fastidiosa* by the direct action of the HD-GYP domain as in *Xcc* (17, 27).

Biofilm formation is regulated by cell-cell signaling in many pathogenic bacteria that live in aquatic habitats. To establish a successful infection, such bacteria coordinate the development of the planktonic and attached (biofilm) components of their population in a cell density-dependent manner. Attachment thus plays an important role in enabling the accumulation of a large biofilm community, but such attachment must be overcome or down-regulated or attached cells to disperse and develop into a new biofilm (28). *X. fastidiosa* apparently must express traits involved in attachment differently in different phases of its lifestyle. For example, cells must be able to move within plants to spread from one xylem vessel to another, and hence attachment might be repressed under such conditions. Conversely, cells acquired by insect vectors while feeding on infected plants must adhere to the mouthparts of the vector so that they are retained in the presence of what must be a rapid flow of xylem fluids that are consumed during feeding (29, 30). Studies of DSF-deficient *rpfF* mutants and DSF-overproducing *rpfC* mutants suggest that DSF accumulation promotes biofilm formation in *X. fastidiosa*. The proportion of the *X. fastidiosa* population that formed a biofilm in cultured cells was dramatically higher in *rpfC* mutants than for wild-type cells, but dramatically lower for *rpfF* mutants than for wild-type cells (Fig. 4). Such results are consistent with the observation that *rpfF* mutants of *X. fastidiosa* did not form a biofilm in sharpshooter vectors after ingestion from infected plants (7). An *rpfF-rpfC* mutant exhibited similar virulence to grape as the wild-type strain, but was much more virulent than the *rpfC* mutant (SI Table 3). This was associated with a lack of overexpression of genes conferring production of adhesins as in the *rpfC* mutants and similar levels of expression of other virulence genes like *tolC* (18) and *pglA* (19; Table 1) which may enabled the transverse spread of *Xf* along and between xylem vessels. It seems likely that fimbrial and afimbrial adhesins that have been shown to play a role in attachment of *X. fastidiosa* to surfaces and to each other (20–22) are regulated in a DSF-dependent fashion to produce either a mixed population of cells that are capable of adherence to surfaces or free to move at a given time. Alternatively, cells with differing abilities to form a biofilm may be spatially or temporally segregated in the plant in a manner that involves DSF production.

The poor insect transmissibility of the *rpfC* mutant of *X. fastidiosa* illustrates that a balance of attachment capabilities controls the processes of acquisition and transmission of this pathogen by sharpshooter vectors. An *rpfF* mutant of *X. fastidiosa* is completely deficient in insect colonization and hence transmission to new host plants (7); although it is acquired from the plant during feeding of the insect, it is not retained as a biofilm. This mutant also is severely constrained in forming a biofilm *in vitro* (Fig. 4). Although *rpfC* mutants were able to colonize the insect vector they were severely deficient in transmission (SI Fig. 10). Because the *rpfC* mutant is much more proficient in forming a biofilm than the wild-type cells (Fig. 4), the apparent lower population size attained in the insect might reflect fewer planktonic cells of this mutant available for acquisition on feeding on xylem vessels or the lower population size of the mutant in the plant (Fig. 3). The slightly lower populations might also simply reflect an underestimation of cell numbers during culturing because of their "sticky" nature, which promotes aggregation. Because a strong correlation between the population size of *X. fastidiosa* in vectors and probability of transmission has not been found (3, 29, 31), it seems unlikely that the slightly reduced population size in the vector was the cause