

RpfF-Dependent Regulon of *Xylella fastidiosa*

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ABSTRACT

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Xylella fastidiosa regulates traits important to both virulence of grape as well as colonization of sharpshooter vectors via its production of a fatty acid signal molecule known as DSF whose production is dependent on *rpfF*. Although *X. fastidiosa* *rpfF* mutants exhibit increased virulence to plants, they are unable to be spread from plant to plant by insect vectors. To gain more insight into the traits that contribute to these processes, a whole-genome Agilent DNA microarray for this species was developed and used to determine the RpfF-dependent regulon by transcriptional profiling. In total, 446 protein coding genes whose expression was significantly different between the wild type and an *rpfF* mutant (false

discovery rate < 0.05) were identified when cells were grown in PW liquid medium. Among them, 165 genes were downregulated in the *rpfF* mutant compared with the wild-type strain whereas 281 genes were over-expressed. RpfF function was required for regulation of 11 regulatory and σ factors, including *rpfE*, *yybA*, PD1177, *glnB*, *rpfG*, PD0954, PD0199, PD2050, *colR*, *rpoH*, and *rpoD*. In general, RpfF is required for regulation of genes involved in attachment and biofilm formation, enhancing expression of hemagglutinin genes *hxfA* and *hxfB*, and suppressing most type IV pili and *gum* genes. A large number of other RpfF-dependent genes that might contribute to virulence or insect colonization were also identified such as those encoding hemolysin and colicin V, as well as genes with unknown functions.

Pierce's disease of grape, a chronic problem in the grape industry in California now promises to be a far more devastating disease due to the introduction of the glassy-winged sharpshooter (*Homalodisca vitripennis*), a more effective vector of the pathogen *Xylella fastidiosa* (10,43). This disease has long limited production of grape in many other areas of North America where warm climatic conditions and the presence of sharpshooter vectors coincide (43). *X. fastidiosa* apparently causes disease by multiplying within and, thus, blocking xylem vessels (33,35). The process of grape vessel and insect colonization by *X. fastidiosa* has many similarities to the process of microbial biofilm formation in other aquatic systems, where thick layers of cells can form on solid surfaces that are exposed to a flow of liquids. Cells in these structures are often embedded in an EPS matrix after initial attachment to the surface (52). Cells in biofilms are often more resistant to many stresses such as antimicrobial compounds, viruses, and predators. The EPS matrix can also facilitate growth of the cells by accumulating various types of nutrients in a way analogous to an ion-exchange column (56) as well as other extracellular products such as enzymes and signal molecules. Small molecules such as *N*-acyl homoserine lactones (AHLs), peptides, and fatty acids play key roles as signals that coordinate biofilm formation in numerous species of bacteria (6,55). The signals, which increase in concentration with population density, also often coordinate the expression of genes involved in exploitation of a host organism. The virulence of many pathogens is usually greatly reduced when the ability to produce signaling molecules is disrupted.

Considerable evidence indicates that *X. fastidiosa* regulates both virulence to plants and its ability to be acquired by insect vectors via its production of one or more fatty acid signal molecules known as DSF, whose production is dependent on regulation of pathogenicity factors (*rpf*) gene, *rpfF*. The identity of the DSF molecule or molecules made by *X. fastidiosa*, while currently unknown, is likely similar but probably different from those made by *Xanthomonas* spp. because a *Xanthomonas campestris* pv. *campestris* DSF biosensor strain recognizes DSF produced by *X. fastidiosa*, although weakly (10,41). DSF in *Xanthomonas campestris* pv. *campestris* has recently been shown to consist of three closely related unsaturated fatty acids, each of which has at least some ability to act as a signal molecule capable of inducing the expression of an *eng:gfp* reporter gene in *Xanthomonas campestris* pv. *campestris* (29). In both pathogens, the several genes associated with DSF production and sensing (*rpf*) have high homology and are organized in clusters, although in *X. fastidiosa*, two genes, *rpfA* and *rpfB*, are located elsewhere in chromosome. Other components of the signaling pathway in *Xanthomonas campestris* pv. *campestris* have been characterized (16,18,31) but only *rpfB*, *rpfF*, and *rpfC* have been studied in detail in *X. fastidiosa*. The role of these proteins in *X. fastidiosa* is apparently similar to that in *Xanthomonas campestris* pv. *campestris*, with RpfB presumably altering the substrate pools used by RpfF for synthesis of DSF (1,41) and RpfC being part of a putative two-component regulator that senses DSF (10). The DSF-mediated regulation of virulence factors is essential for host plant colonization in *Xanthomonas campestris* pv. *campestris*, with *rpfF* and *rpfB* associated with DSF production (5) and *rpfC* and *rpfG* with signal perception and transduction (16,18,30,31), leading to downstream control of gene transcription via modulation of cyclic di-GMP levels. In contrast to *Xanthomonas campestris* pv. *campestris*, *rpfF* mutants in *X. fastidiosa* exhibit dramatically increased virulence to plants (41). In addition to suppressing *X. fastidiosa* colonization of plants, DSF is required for its

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplementary figures and one supplementary table.

colonization of insect vectors, an essential component of the disease cycle (41). Cells must attach to and colonize the cuticular surface of specific regions of the vector foregut, forming microcolonies that eventually develop into mature biofilms of polarly attached cells (2). A DSF-deficient mutant was not able to colonize the foregut of vectors and was not transmissible to plants (41).

Although numerous genes with various functions were identified to be controlled by RpfF in *Xanthomonas campestris* pv. *campestris* (30), *Xanthomonas citri* subsp. *citri* (27), and *Xanthomonas oryzae* pv. *oryzae* (9), relatively little is known about the genes that are dependent on RpfF in *X. fastidiosa*. Afimbrial adhesins such as hemagglutinin-like proteins (Hxfs) are under DSF regulation and are important for cell attachment to vectors (36,37). Afimbrial adhesins are generally upregulated by DSF, suggesting that, when *X. fastidiosa* reaches high local densities in host plants, there is a restriction of further migration of cells within the plant; however, such cells are facilitated in their adhesion to insect vectors (25,37). In contrast, some genes that would facilitate movement of the pathogen and growth in plants such as those conferring production of pili and extracellular enzymes are upregulated in the absence of DSF (10) and do not appear to be essential for vector transmission (37), suggesting that cell-to-cell signaling coordinates the complex life style of this bacterium (10). Because the traits that would contribute to insect colonization by *X. fastidiosa* seem to conflict with plant colonization, spatially structured cells of *X. fastidiosa* may occur within xylem vessels, and the dichotomous characteristics of these phenotypic states may be regulated by differential accumulation of DSF (8).

Although RpfF clearly has a major impact on the behavior of *X. fastidiosa*, we still lack insight into what constitutes virulence factors in such a pathogen besides those few extracellular enzymes and cell surface features that have been examined (8). Thus, in this study, we performed global transcriptional profiling by designing a whole-genome Agilent DNA microarray to more fully identify those traits in *X. fastidiosa* that influence the interactions of the pathogen with plants and insects that are dependent on *rpfF* and, thus, cell-to-cell signaling.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type (WT) strain *X. fastidiosa* Temecula (American Type Culture Collection number 700964) and an isogenic *rpfF* mutant (41) were grown at 28°C in PW liquid media (33,41) with shaking at 160 rpm. The *rpfF* mutant was confirmed to grow in media containing kanamycin (50 µg/m) (Sigma-Aldrich, St. Louis) but was grown without antibiotics for transcriptome analysis.

Microarray design. A DNA microarray containing sequences for all open reading frames (ORFs) in both the genome and plasmid of *X. fastidiosa* was designed using eArray (Agilent Technologies, Santa Clara CA). Briefly, ≤10 probes with a length of 60 nucleotides were designed for each of 2,058 ORFs. Some target sequences had <10 probes because of short sequence length or inappropriate base composition. In all, 100 negative probes sharing no similarity with *X. fastidiosa* sequences but, having good temperature and BC scores, were designed based on either *Escherichia coli* or *Arabidopsis thaliana* gene sequences. The probes were printed in 105-k × 2 multiplex format to reduce the variation within arrays at Agilent Technologies following their standard procedure. In total, 76,788 tiling probes were also included in this array to cover the whole genome and were not used in this current expression analysis of the RpfF regulon. In addition, this array also contained probes for genes of *X. fastidiosa* strain 9a5c.

RNA isolation. RNA was isolated using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Cells were pelleted directly into

RNAprotect Bacteria Reagent (Qiagen Inc.) 6 days after inoculation. For inoculation in PW broth media, initial concentration (optical density at 600 nm [OD₆₀₀] = 0.05) of WT and *rpfF* strains were used. Contaminating DNA was digested with a TURBO DNA-free Kit (Ambion, Austin, TX). RNA concentration was determined with a NanoDrop Spectrophotometer (NanoDrop Technologies, Inc.) and sample quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray analysis. Labeled cDNA was generated using the Agilent Two-Color Microarray-Based Prokaryote Analysis kit (Fairplay III labeling; Agilent Technologies). cDNA synthesized from 5 µg of total RNA using AffinityScript HC and random primers was then labeled with either cy3 or cy5 and purified following the manufacturer's instructions. A total of 825 ng of labeled cDNA per sample was used in hybridizations on each microarray. A dye swap was performed to reduce any bias from the labeling dyes. Two biological replicates were used for this study. Hybridization was performed in an oven for 17.5 h at 65°C using a Gene Expression Hybridization Kit (Agilent Technologies) according to the user's manual. The arrays were washed using the manufacturer's recommended protocols and scanned using a dual-laser DNA microarray scanner (Model G2505C; Agilent Technologies). Fluorescence intensities of each element were extracted from the scanned image using Feature Extraction 10.1.1.1 software (Agilent Technologies).

Data preprocessing and differential expression analyses of mean signals provided by the Agilent Feature Extraction were done using the Bioconductor Limma package (51). Data were first normalized within the array by Lowess algorithm (58), then normalized between arrays using a quantile approach. Probe-level expression values were summarized into gene-level signal data. A model-based cluster analysis approach was applied to normalized data to assign detection calls based on negative control probe information. Probe sets lacking detection calls were removed in all the samples before further analysis. For differential expression analysis, the expression value for each gene was fit to a linear model to assess the significance of the difference between mutant and WT strains. Limma applies empirical Bayes methods to construct moderated statistics and incorporates statistical tools to adjust for the multiplicity of the tests (51). The Benjamini and Hochberg approach was used to compute the false discovery rate (FDR). Only protein coding genes were subjected for further analysis. The J. Craig Venter Institute (JCVI) database was used to group the RpfF-dependent regulon into different role categories.

All data from transcriptome experiments as well as experimental protocols used are available from Gene Expression Omnibus datasets, the National Center of Biotechnology Information (accession number GSE38469).

Quantitative reverse-transcription polymerase chain reaction. Quantitative reverse-transcription polymerase chain reaction (QRT-PCR) assays were conducted as described previously (26) using either an ABI 7500 or 7300 system (Applied Biosystems, Inc., Foster City, CA). Primers used in this study were designed using Lasergene v8.1 (DNASTar, Madison, WI). The *dnaQ* encoding the ε subunit of DNA polymerase III was used as an endogenous control. Values of fold change of QRT-PCR were means of four replicates. Experiments were repeated twice with similar results and only one representative result was presented. The ΔΔcycle threshold method was used to calculate the fold change and standard error as described previously (39).

RESULTS

Identification of genes controlled by cell-to-cell signaling in PW broth medium. Genes of *X. fastidiosa* that are regulated in an *rpfF*-dependent manner were determined by global transcriptional profiling of cells recovered from relatively dense

cultures in broth media. We reasoned that liquid medium (PW) better simulates the aqueous environment that *X. fastidiosa* encounters in the xylem than a solidified medium. As in previous studies, it was noted that the WT strain of *X. fastidiosa* formed a thick aggregative ring at the air/liquid interface of culture flasks that was much more pronounced after 14 days of growth than for the *rpffF* mutant (Supplementary Figure 1). The total population size of the two strains (sum of both planktonic and attached cells), however, was similar at a given sampling time. Aggregates of cells were also noted within the cultures, particularly for the *rpffF* mutant, but these dispersed after ≈ 11 days of incubation. Thus, transcript abundance was assessed on the total bacterial community recovered from culture flasks using an Agilent DNA microarray constructed for strain Temecula, which consisted of ≤ 10 60-bp probes for each of the 2,058 ORFs of this strain.

In total, 446 protein coding genes whose expression was significantly different between the WT and the *rpffF* mutant ($FDR < 0.05$) were identified when cells were grown in PW liquid medium for 6 days with initial concentration of $OD_{600} = 0.05$. Among them, 165 genes were downregulated in the *rpffF* mutant compared with the WT strain whereas 281 genes were over-expressed at least twofold in the *rpffF* mutant compared with the WT strain (Supplementary Table 1). The expression of 247 of these genes differed by > 2.5 -fold between the WT and the *rpffF* mutant. Of these, 150 were more highly expressed in the *rpffF* mutant than in the WT while 97 were expressed at a lower level. These *rpffF*-dependent genes clearly were involved in many cellular processes because they could be assigned to 20 functional categories according to the annotation from the JCVI role categories, including biosynthesis of cofactors, prosthetic groups, and carriers; cell envelope; cellular processes; signal transduction; metabolism of purines, pyrimidines, nucleosides, and nucleotides; DNA metabolism; energy metabolism; fatty acid and phospholipid metabolism; mobile and extrachromosomal element functions; protein fate; protein synthesis; regulatory functions; transcription; transport and binding proteins; and hypothetical proteins (Supplementary Figure 2). Interestingly, RpfF function was required for regulation of 11 regulatory and σ factors, including *rpfE* (PD0401), *yybA* (PD0595), PD1177, *glnB* (PD1025), *rpfG* (PD0405), PD0954, PD0199, PD2050, *colR* (PD1919), *rpoH* (PD2048), and *rpoD* (PD0593).

Although many of the genes of *X. fastidiosa* that are most strongly regulated in an *rpffF*-dependent manner had no obvious direct connection to virulence or insect transmission, a number of genes whose expression was less strongly dependent on RpfF ($P \leq 0.05$) that might have such a connection were found (Table 1). In total, 61 confirmed or putative virulence genes that differed in expression between a WT and *rpffF* mutant ($P \leq 0.05$) were identified (Table 1). Of these, 18 genes were downregulated and 43 genes were upregulated in the *rpffF* mutant compared with the WT strain. Among those genes upregulated in the *rpffF* mutant were the regulatory genes *csrA* (PD0095), *xrvA* (PD1905), *fur* (PD1374), PD0708, and *pilH* (PD1632), in addition to the 11 regulatory genes mentioned above. In addition, 4 colicin V-related genes, 4 gum genes, 11 type IV pili genes, and 1 type I pilus gene *mrkD* were more highly expressed in the *rpffF* mutant compared with the WT strain. The downregulated genes in the *rpffF* mutant compared with the WT consist of four hemagglutinin genes (including *hxfA* and *hxfB*), two hemolysin genes, and *yegN*, which encodes an acriflavin resistance protein (Table 1).

Confirmation of microarray data using QRT-PCR. In general, estimates of relative RNA abundance for a given gene made using DNA microarrays were confirmed using QRT-PCR. The *rpffF*-dependent expression of 24 genes was determined using both methods. Similar ratios of expression of 22 of the 24 genes in the WT strain and the *rpffF* mutant were obtained by both methods (Table 2).

DISCUSSION

Given the large number of genes that exhibit *rpffF*-dependent patterns of expression in this study, it is clear that DSF-mediated signaling plays a central role in the context-dependent behavior of *X. fastidiosa*. RpfF-mediated signaling influences a much larger number of genes (a total of 446 protein coding genes with an $FDR < 0.05$, and even as many as 247 genes with both an $FDR < 0.05$ and a fold change > 2.5) than other regulators, including GacA (27 genes), AlgU (43 genes), RpoN (38 genes), and RpoE (21 genes) (14,15,48,49). Given that it controls the expression of several central regulatory genes and σ factors, including *rpfE* (PD0401), *yybA* (PD0595), PD1177, *glnB* (PD1025), *rpfG* (PD0405), PD0954, PD0199, PD2050, *colR* (PD1919), *rpoH* (PD2048), and *rpoD* (PD0593) ($FDR < 0.05$), at least some of the regulon of the *rpff* system may be due to such secondary regulators. Under the less-stringent requirements for differential gene expression ($P \leq 0.05$), the additional regulatory genes *csrA* (PD0095), *xrvA* (PD1905), *fur* (PD1374), PD0708, and *pilH* (PD1632) were also found to be regulated by DSF (Table 1). Thus, DSF-mediated signaling seems to be tightly linked with other regulators to enable complex patterns of context-dependent gene expression to be employed by *X. fastidiosa* in the different habitats that it colonizes.

Many of the *rpffF*-dependent genes found in *X. fastidiosa* have been studied only in other related pathogens such as *Xanthomonas campestris* pv. *campestris* and *Xanthomonas citri*; hence, their roles in *X. fastidiosa* remain unclear. ColR, MarR, PilH, Fur, XrvA, and RpfG have all been reported to be involved in the regulation of virulence genes in other pathogens (20,46,54,60). ColR is a global regulator involved in virulence, hypersensitive response, and stress tolerance of *Xanthomonas campestris* pv. *campestris* and *X. axonopodis* pv. *citri* (57,60). Members of the MarR family such as HpaR were reported to be involved in pathogenesis, hypersensitive response, and extracellular protease production in *Xanthomonas campestris* pv. *campestris* (54). *xrvA*, encoding an H-NS-like protein in *X. oryzae* pv. *oryzae*, contributes to its virulence, because a knockout mutant exhibited a significant reduction in virulence to rice (20). Disruption of RpfG, involved in the DSF signal transduction system along with RpfC and RpfF, led to reduced expression of virulence functions such as extracellular enzymes, biofilm structure, and motility in *Xanthomonas campestris* pv. *campestris* (46) and reduced virulence in *X. citri* (27). PilH is a regulator of type IV pili (13) and expression of type IV pili contributes to virulence of *Acetovorax avenae* subsp. *citrulli* (4). Type IV pili are essential for twitching motility and, thus, active movement of *X. fastidiosa* in xylem vessels and various *pil* mutants are less virulent to grape (38,40). Fur is central to the regulation of iron homeostasis and is required for full virulence of many bacteria, including *Bacillus cereus* (28), *Erwinia chrysanthemi* (22), and *X. oryzae* pv. *oryzae* (53), and DSF signaling in *X. oryzae* pv. *oryzae* has been linked to iron metabolism (9). Thus, the context-dependent expression of the homologs of these virulence regulators in *X. fastidiosa* might be expected to be involved in controlling expression of those traits that are appropriate for its solely vascular existence and, perhaps, also of its colonization of the surface of insect mouthparts.

Attachment and biofilm formation are the most coherent virulence phenotypes that are RpfF-dependent. A large collection of genes that lead to the adhesiveness of *X. fastidiosa*, including those encoding hemagglutinins (*hxfA* and *hxfB*), were expressed at a lower level in the *rpffF* mutant. In contrast, 11 type IV pili genes and 4 gum genes were expressed at higher levels than in the WT strain (Table 1). Thus, this study extends the initial reports of the regulation of these traits seen in earlier studies (10,37). In general, the patterns of expression of the genes involved in adhesion and motility are consistent with a model proposed by Chatterjee et al. (8) that describes a radical shift in the traits

expressed by *X. fastidiosa* as it transitions from a plant exploratory phase to a more adhesive form that is capable of being vectored by insects. This model accounts for the hypervirulence of *rpfF* mutants that, in turn, are incapable of colonizing insects and, thus, of being transmitted, highlighting that the traits that allow movement through the plant are incompatible with insect transmission. In aggregate, nearly all of the genes that would encode structures or molecules that would tend to cause cells to adhere to surfaces were upregulated in a WT strain compared with the *rpfF* mutant. The lower expression of this suite of ad-

hesins in an *rpfF* mutant also accounts for the fact that it is reduced in biofilm formation and attachment to flasks. That is, downregulation of the hemagglutinin genes would lead to less attachment and thus biofilm formation. Higher expression of type IV pili genes, which are involved in twitching motility (40), in an *rpfF* mutant accounts for its increased movement and colonization of plants (10). Previous studies linked Pierce's disease symptom development with the rate and extent of *X. fastidiosa* colonization of the xylem (23,34,44). The finding of downregulation of the suite of hemagglutinin genes in an *rpfF* mutant is consistent with

TABLE 1. Relative expression of putative virulence genes of a wild-type strain and an *rpfF* mutant of *Xylella fastidiosa*^a

Locus tag	Product name	Ratio (Mut/WT)	P value
PD0215	Colicin V precursor	9.12	1.30E-14
PD0216	Colicin V precursor	7.89	5.96E-13
PD0895	Outer membrane protein P6 precursor	4.23	5.02E-07
PD1632	PilH family regulatory protein	3.38	2.21E-05
PD0496	Colicin V secretion protein	3.29	3.24E-05
PD0283	DnaK suppressor	3.23	4.45E-05
PD0146	Outer membrane protein	3.06	9.62E-05
PD1923	Fimbrial assembly protein	2.92	1.85E-04
PD0852	Colicin V production protein	2.90	2.09E-04
PD0708	Virulence regulator	2.70	5.45E-04
PD0855	VirK protein	2.69	5.73E-04
PD0845	Pilus protein	2.64	7.23E-04
PD0405	Response regulator	2.53	0.001
PD0012	Biopolymer transport ExbD2 protein	2.46	0.002
PD1691	Fimbrial assembly protein	2.44	0.002
PD1393	GumE protein	2.43	0.002
PD0058	Fimbrial adhesin precursor	2.40	0.002
PD0757	Peptidoglycan-associated outer membrane lipoprotein precursor	2.36	0.003
PD0846	Pilus biogenesis protein	2.31	0.003
PD1279	DnaJ protein	2.21	0.01
PD1682	Outer membrane hemin receptor	2.21	0.01
PD1395	GumC protein	2.13	0.01
PD0234	Aconitate hydratase	2.11	0.01
PD1807	Outer membrane protein	2.10	0.01
PD0755	cAMP-regulatory protein	2.10	0.01
PD0011	Biopolymer transport ExbD1 protein	2.08	0.01
PD0024	PilE protein	2.07	0.01
PD1147	Twitching motility protein	2.01	0.01
PD0233	Putative long-chain fatty acyl CoA ligase	2.00	0.02
PD1924	Fimbrial protein	1.96	0.02
PD1374	Ferric uptake regulator	1.95	0.02
PD1709	Outer membrane protein	1.94	0.02
PD1905	Virulence regulator	1.93	0.02
PD1396	GumB protein	1.91	0.02
PD1148	Twitching motility protein	1.90	0.02
PD1611	Fimbrial assembly protein	1.88	0.03
PD0369	Proteic killer active protein	1.88	0.03
PD1392	GumF protein	1.88	0.03
PD1735	Type 4 fimbrial biogenesis protein	1.88	0.03
PD1981	TonB protein	1.84	0.03
PD0095	Carbon storage regulator	1.84	0.03
PD0125	Cysteine protease	1.79	0.04
PD0143	Hemolysin III protein	1.77	0.05
PD2110	Hemagglutinin-like secreted protein	0.58	0.05
PD0843	TonB protein	0.57	0.05
PD0020	Pre-pilin leader sequence	0.55	0.04
PD1403	Acriflavin resistance protein D	0.54	0.03
PD0536	Hemolysin	0.54	0.03
PD1427	Bacteriocin	0.53	0.03
PD2112	Hypothetical protein PD2112	0.52	0.02
PD1506	Hemolysin-type calcium binding protein	0.51	0.02
PD2114	Hypothetical protein PD2114	0.49	0.01
PD2118	Hemagglutinin-like secreted protein	0.48	0.01
PD1589	TonB-dependent receptor	0.46	0.01
PD1554	Hypothetical protein PD1554	0.42	0.003
PD1517	Hypothetical protein PD1517	0.41	0.002
PD1787	Hypothetical protein PD1787	0.38	6.63E-04
PD1792	Hemagglutinin-like protein	0.38	6.36E-04
PD0986	Hemagglutinin-like secreted protein	0.35	2.17E-04
PD2108	Hypothetical protein PD2108	0.33	8.97E-05
PD1790	Hypothetical protein PD1790	0.28	9.87E-06

^a FC: fold change of gene expression; Mut: *rpfF* mutant of *X. fastidiosa*; WT: wild-type strain of *X. fastidiosa*.

the demonstration of these genes as encoding antivirulence traits (25).

The RpfF regulon of *X. fastidiosa* is apparently quite different from that of *Xanthomonas campestris* pv. *campestris*, even though they do share some similarity. Many of the homologous genes that are regulated by RpfF in all of these species exhibit different patterns of regulation. For example, among the 165 RpfF-dependent genes identified in *Xanthomonas campestris* pv. *campestris* (30), 91 have homologues in *X. fastidiosa*, 7 have a similar trend in expression, and 26 have an opposite trend, while 58 are not regulated by RpfF in *X. fastidiosa*. RpfF positively regulates expression of genes encoding chemotaxis (*cheY*), iron uptake (*tonB*), drug resistance (*ftsL* and *acrD*), transcription regulation (*slyA* and PD0747) and fatty acid metabolism (*phaF*) in both species. However, RpfF negatively regulates Gum genes and *pil* genes in *X. fastidiosa* while the opposite is true in *Xanthomonas campestris* pv. *campestris* or *X. citri* (27,30). Other examples of genes that show opposite patterns of regulation include *exbD1* and *exbD2*, which are involved in iron uptake; *ahpF*, involved in oxidative stress resistance; and genes encoding ribosomal proteins (*rpsC*, *rpsD*, *rpsL*, *rpsO*, *rpsS*, *rplE*, *rplP*, and PD0125); genes involved in the TCA cycle (*sucC*, *sucD*, *sdhC*, and *sdhD*); genes involved in respiration (*cyoA*, *cyoC*, and *cyoD*); genes encoding membrane components and transporters (*atpB*, *atpE*, and *atpF*); and the hypothetical gene PD0710.

The different patterns of regulation might be facilitated by structural differences in the *rpf* clusters of *X. fastidiosa* and *Xanthomonas campestris* pv. *campestris*. The *rpf* cluster of *Xanthomonas campestris* pv. *campestris* contains 16 genes whereas the *X. fastidiosa* *rpf* cluster consists of only 12 genes. Both *rpfH* and *rpfI* are not present in *X. fastidiosa*. RpfH is structurally related to the sensory input domain of RpfC while RpfI positively regulates the synthesis of proteases, endoglucanases, and EPS in *Xanthomonas campestris* pv. *campestris* (19,50). Likewise, *rpfB*, which is involved in DSF synthesis by apparently modulating the mixture of chemical species of DSF that are produced by these species (1,29), is located elsewhere in the chromosome of *X. fastidiosa*, and is not linked transcriptionally with *rpfF* as in *Xanthomonas campestris* pv. *campestris*.

There is some evidence that the different chemical forms of DSF produced by *X. fastidiosa* each lead to somewhat different patterns of gene expression (1). Thus, the unlinking of *rpfF* and *rpfB* expression in *X. fastidiosa* may enable a more nuanced pattern of DSF signaling to occur compared with *Xanthomonas campestris* pv. *campestris*, which could explain the substantial differences in the *rpfF* regulon in these two species. The opposite patterns of RpfF-dependent expression of a variety of virulence genes clearly explain why the *rpfF* mutants of *X. fastidiosa* are hypervirulent whereas those of other *Xanthomonads* are less virulent (5,27,41). As noted above, the lifestyle of *X. fastidiosa*, which colonizes both xylem vessels and the mouthparts of insects, is both more complex and different from that of other *Xanthomonads* that are restricted to the vasculature of plants; thus, it would be expected that it would need to employ different behaviors to colonize these two habitats compared with pathogens such as *Xanthomonas campestris* pv. *campestris* and *Xanthomonas citri*. In addition, differences in the test conditions (e.g., time points and media) might also contribute to the different patterns of regulation of *X. fastidiosa* and *Xanthomonas campestris* pv. *campestris*.

A variety of genes whose linkage to virulence of *X. fastidiosa* is less clear are also controlled by RpfF. At least three hemolysin-related genes were dependent on RpfF for proper expression (Table 1). Hemolysin is a member of a protein family that includes pore-forming toxins and has been implicated in bacterial virulence (32). However, its role in virulence of *X. fastidiosa* is unclear and needs further characterization. Curiously, the hemolysins encoded by PD0536 and PD1506 are upregulated by RpfF whereas that of PD0143 is downregulated (Table 1), suggesting that any role that they play may occur at different stages of the infection process, or at least in cells differing in local cell density. The expression of PD1517, encoding a homolog of invasion-associated protein P60, is upregulated in the presence of RpfF. P60 homologs are major extracellular proteins involved in the invasion of pathogens into their host cell, functioning as a presumptive murein hydrolase and host-membrane binding protein in *Listeria* spp. (42). Four genes encoding colicin V-related proteins were suppressed by RpfF (Tables 1 and 2). In fact, these genes were among those most highly suppressed in the presence of

TABLE 2. Relative expression of various *Xylella fastidiosa* genes in a wild-type and an *rpfF* mutant strain as measured by both quantitative reverse-transcription polymerase chain reaction (QRT-PCR) and microarray analysis

Locus tag	Name	Average FC (Mut/WT) ^a		Function
		QRT-PCR (mean ± SD)	Microarray	
16S	16S	1.38 ± 0.13	ND	16S rRNA
PD0001	<i>dnaA</i>	1.55 ± 0.04	4.72	Chromosomal replication initiator
PD0019	<i>fimT</i>	1.17 ± 0.04	ND	Pre-pilin like leader sequence
PD0024	<i>pilE</i>	1.88 ± 0.21	2.07	PilE protein
PD0062	<i>pilA</i>	0.88 ± 0.06	ND	Fimbrial subunit precursor
PD0216	...	1.89 ± 0.06	7.89	Colicin V precursor
PD0313	<i>pspB</i>	1.07 ± 0.11	ND	Serine protease
PD0406	<i>rpfC</i>	2.67 ± 0.16	ND	Regulator of pathogenicity factors
PD0496	<i>cvaA</i>	1.93 ± 0.37	3.29	Colicin V secretion protein
PD0593	<i>rpoD</i>	1.91 ± 0.12	3.72	RNA polymerase σ -70 factor
PD0852	...	1.62 ± 0.16	2.9	Colicin V production protein
PD1485	<i>pglA</i>	0.86 ± 0.12	ND	Polygalacturonase precursor
PD1611	<i>pilY1</i>	2.96 ± 0.47	1.88	Fimbrial assembly protein
PD1632	<i>pilH</i>	2.06 ± 0.05	3.38	Regulatory protein pilH family
PD1672	<i>bfr</i>	2.18 ± 0.12	4.15	Bacterioferritin
PD1691	<i>pilQ</i>	1.41 ± 0.09	2.44	Fimbrial assembly protein
PD1792	<i>pspA</i>	0.63 ± 0.03	0.38	Hemagglutinin-like protein
PD1851	<i>engXCA</i>	0.53 ± 0.08	ND	Endo-1,4-beta-glucanase
PD1856	<i>engXCA</i>	0.85 ± 0.13	ND	Extracellular endoglucanase
PD1919	<i>colR</i>	1.77 ± 0.14	3.34	Two-component system, regulatory protein
PD1922	<i>pilD</i>	1.38 ± 0.23	ND	Pre-pilin leader peptidase
PD1923	<i>pilC</i>	2.44 ± 0.12	2.92	Fimbrial assembly protein
PD1960	<i>rpsB</i>	0.64 ± 0.06	ND	30S ribosomal protein S2
PD2048	<i>rpoH</i>	3.10 ± 0.55	5.45	RNA polymerase σ -32 factor

^a FC: fold change of gene expression; Mut: *rpfF* mutant of *X. fastidiosa*; WT: wild-type strain of *X. fastidiosa*. SD: standard deviation; ND: no difference.

TABLE 3. Expression of colicin V-related genes in *Xylella fastidiosa* under different conditions^a

	Gene name	ORFs	<i>rpjF</i> /WT	HIC	LIC	3G10R/ PW	Gomesin/no gomesin	<i>gacA</i> / WT	<i>rpoN</i> / WT	Heat shock/ control	Pathogenic/ nonpathogenic condition
Immunity protein	<i>cvi</i>	PD0214	NC	Up	Up
Precursor	<i>cvaC</i>	PD0215, PD0216	Up	Up	Up	Up, NC	Up	...	Up, down	Down	Up
Secretion protein	<i>cvaA</i>	PD0496	Up	Up	Up	Down	...
ABC transporter	<i>cvaB</i>	PD0499	NC	Up
Production protein	<i>cvpA</i>	PD0852	Up	Up
Reference	This study	59	59	11	21	49	15	14	17

^a ORFs: open reading frames; HIC: high iron concentration (100 μ M ferric pyrophosphate); LIC: low iron concentration (treated with the iron chelator, 200 μ M 2,2'-dipyridyl); WT: wild type; NC: no change.

TABLE 4. Expression of *Xylella fastidiosa* genes encoding gum, hemagglutinin, and hemolysin under different conditions^a

Locus tag	Name	Function	<i>rpjF</i> / WT	Gomesin treated/ nontreated	<i>gacA</i> / WT	Heat shock/ control	HIC	LIC
PD1396	<i>gumB</i>	GumB protein	Up
PD1395	<i>gumC</i>	GumC protein	Up	Up	Down
PD1394	<i>gumD</i>	GumD protein	NC	Up
PD1393	<i>gumE</i>	GumE protein	Up	Up
PD1392	<i>gumF</i>	GumF protein	Up	Up	...
PD1388	<i>gumK</i>	GumK protein	NC
PD1387	<i>gumM</i>	GumM protein	NC	Down	Down
PD1792	<i>hxfB</i>	Hemagglutinin-like protein	Down	Up
PD0986	<i>pspA</i>	Hemagglutinin-like secreted protein	Down
PD2110	<i>pspA</i>	Hemagglutinin-like secreted protein	Down
PD2116	<i>pspA</i>	Hemagglutinin-like secreted protein	NC
PD2118	<i>hxfA</i>	Hemagglutinin-like secreted protein	Down	Up	...	Up
PD0536	<i>tlyC</i>	Hemolysin	Down
PD1933	<i>hecB</i>	Hemolysin activation protein	NC
PD0143	...	Hemolysin III protein	Up
PD1787	...	Hemolysin	Down	Up
PD1790	...	Hemolysin	Down
PD2108	...	Hemolysin	Down
PD2112	...	Hemolysin	Down
PD2114	...	Hemolysin	Down
Reference	This study	21	49	14	59	59

^a WT: wild type; HIC: high iron concentration (100 μ M ferric pyrophosphate); LIC: low iron concentration (treated with the iron chelator 200 μ M 2,2'-dipyridyl); NC: no change.

RpjF. Colicin V has been suggested to play an important role for *X. fastidiosa* by acting as an antimicrobial agent, enabling the colonization of xylem vessels and the insect foregut despite the presence of other endophytic bacteria that have been reported to be present (3,7,12) and which might compete with it in the absence of such a defensive arsenal (59). The negative regulation of colicin V genes by RpjF signaling suggests that, if colicins play such a role, it is most useful when *X. fastidiosa* populations are low. Presumably once *X. fastidiosa* is well established inside the xylem, competition from other species is less important and, hence, the production of colicins is reduced.

The expression of 19 phage-related genes was at least 2.5-fold higher in the WT strain compared with the *rpjF* mutant while the expression of 2 was lower. This suggests that, at high cell populations, most prophage-like regions are activated. Interestingly, a high proportion of phage genes in *X. fastidiosa* were upregulated when bacteria were placed under stress conditions such as heat shock (14) and nitrogen starvation (15). The activation of phage-related genes was suggested to trigger induction of the lytic cycle, which ultimately results in the formation of virus-like particles. The activation of phage-related genes by RpjF indicates that they may be preferentially upregulated at high population sizes, a stress condition stimulated by the blockage of the xylem that would typically occur in later stages of infection. A similar induction of virus particles and resultant lysis of bacteria by exogenous AHL signal molecules was observed in a mixed bacterial community recovered from soil (24), suggesting that, although the virus may well have usurped the bacterial signaling pathway to enable escape from cells as they become stressed, the bacterial

host might well benefit in some way from enabling such an induction of virus production as well. However, the roles of the phage-related genes are unclear. In addition, RpjF also controls 127 hypothetical genes with unknown functions. Those genes need further characterization to understand how *X. fastidiosa* survives in the nutrient-poor xylem and causes disease.

To determine whether cell density signaling may be integrated with other environmental contexts, the published stimulons and regulons of *X. fastidiosa* in different conditions were compared with that of the RpjF regulon. It is noteworthy that many of the genes that were most strongly regulated in one environmental context, such as those encoding colicin V, type I and IV pili, gum, hemagglutinins, and hemolysins, were also strongly regulated in others (Tables 3 to 5). Interestingly, many pili- and colicin-encoding genes in the RpjF regulon are in common with those in the high iron stimulon. Furthermore, whereas most colicin genes were suppressed by RpjF, they were upregulated in high iron conditions. Likewise, although high iron conditions did not lead to upregulation of all *pil* genes, those that did exhibit this pattern of expression tended to also be suppressed by RpjF (Tables 3 to 5). Because RpjF function (DSF accumulation) is associated with high bacterial concentrations which would logically be found in blocked xylem vessels, twitching motility would probably be futile and, thus, *pil* genes are suppressed. The flow of nutrients such as iron through such vessels would also be low. Therefore, the cell might use the higher iron levels expected in vessels that are not heavily colonized as a cue for exploratory twitching motility. RpjF signaling may reinforce the lack of this cue. However, such a model needs further investigation.

TABLE 5. Expression of type I and IV pili genes of *Xylella fastidiosa* under different conditions^a

PD	Function	Name	<i>rpfF</i> /WT	HIC	LIC	<i>rpoN</i> /WT	Gomesin/c control	<i>gacA</i> /WT
PD0019	Pili retraction protein PilT	<i>fimT</i>	NC
PD0020	Pre-pilin leader sequence	<i>pilV</i>	Down	...	Down
PD0021	Hypothetical membrane associated protein	...	NC
PD0022	Pili assembly protein PilX	<i>pilX</i>	NC
PD0023	Pili assembly protein PilY1	<i>pilY1</i>	NC
PD0024	Pili membrane scaffold protein PilE	<i>pilE</i>	Up	...	Down
PD0058	Type-1 fimbrial protein, a chain precursor	<i>mrkD</i>	Up
PD0060	Outer membrane usher protein FimC	<i>fimC</i>	NC	Down	Down
PD0061	Chaperone protein EcpD	<i>ecpD</i>	NC
PD0062	Major pilin protein FimA	<i>fimA</i>	NC	Down	Down	Up
PD0502	Pili assembly protein PilY1	<i>pilY1</i>	NC
PD0845	Pili chemotaxis chey homolog PilG	<i>pilG</i>	Up	Up
PD0846	Pili chemotaxis CheW homolog PilI	<i>pilI</i>	Up	Up
PD0847	Pili methyl chemotaxis protein PilJ	<i>pilJ</i>	NC	Up	Down
PD0848	Chemotaxis protein CheA	<i>cheA</i>	NC
PD0849	Pili chemotaxis protein methylesterase CheB (ec 3.1.1.61)	<i>cheB</i>	NC
PD1077	Pili subunit PilA1	<i>pilA1</i>	NC
PD1147	Pili retraction protein PilT	<i>pilT</i>	Up	Down
PD1148	Pili retraction protein PilU	<i>pilU</i>	Up	Down	Down
PD1497	Pili assembly protein PilZ	<i>pilZ</i>	Up
PD1610	Pili membrane scaffold protein PilE	<i>pilE</i>	NC	...	Down
PD1611	Pili assembly protein PilY1	<i>pilY1</i>	Up
PD1612	Pili assembly protein PilX	<i>pilX</i>	NC
PD1613	Pili assembly protein PilW	<i>pilW</i>	NC
PD1614	Hypothetical membrane associated protein	...	NC
PD1615	Pili retraction protein PilT	<i>pilT</i>	NC	Down
PD1623	PilF protein	<i>pilF</i>	NC
PD1691	Pili secretion protein PilQ	<i>pilQ</i>	Up
PD1692	Pili assembly protein PilP	<i>pilP</i>	NC	Up
PD1693	Pili assembly protein PilO	<i>pilO</i>	NC	Up	Up	...
PD1694	Pili assembly protein PilN	<i>pilN</i>	NC	Up
PD1695	Pili assembly protein PilM	<i>pilM</i>	NC	Up	...
PD1735	Pili retraction protein PilT	<i>fimT</i>	Up
PD1922	Type 4 prepilin peptidase	<i>pilD</i>	NC
PD1923	Pili assembly protein PilC	<i>pilC</i>	Up
PD1924	Pili subunit PilA1	<i>pilA1</i>	Up
PD1926	Pili subunit PilA2	<i>pilA2</i>	NC	Down	...	Down
PD1927	Pili assembly protein PilB	<i>pilB</i>	NC	Down	Down
PD1928	Two-component response regulator PilR	<i>pilR</i>	NC	...	Down
PD1929	Sensor protein PilS (EC 2.7.3.-)	<i>pilS</i>	NC	...	Down
Reference	59	59	15	21	48

^a WT: wild type; HIC: high iron concentration (100 μ M ferric pyrophosphate); LIC: low iron concentration (treated with the iron chelator 200 μ M 2,2'-dipyridyl); NC: no change.

The cell-to-cell signaling system of *X. fastidiosa* appears to regulate a variety of traits that would tend to prevent excessive growth in the nutrient-poor xylem as well as to enhance its tolerance of the stresses associated with high cell densities. Cells of *X. fastidiosa* within densely colonized xylem vessels are apparently mostly dead (8). Thus, although *X. fastidiosa* presumably multiplies rapidly while spreading from one relatively poorly colonized vessel to another (41), its continued growth as vessels become more heavily colonized is probably self defeating. It is noteworthy that a number of key housekeeping genes, including *rpoD* as well as many genes involved in translation, including *rpsC*, *rpsD*, *rpsL*, *rpsO*, *rpsS*, *rplE*, *rplP*, and PD0125, were suppressed by RpfF function. A similar observation was made in *Xanthomonas citri* (27). The suppression of such genes at high cell densities where DSF would accumulate would tend to reduce the growth rate of the cells, thereby avoiding cellular suicide that would result from excessive growth. It is also interesting to note that genes such as PD1403 encoding putative efflux pumps for toxic compounds are upregulated by RpfF. It might be expected that defensive compounds made in response to *X. fastidiosa* infection, or perhaps which might accumulate incidentally as plant tissues were damaged during the process of colonization, would accumulate in concert with the population size of the pathogen. It would be sensible for *X. fastidiosa* to induce traits to deal with such compounds. Such compounds are apparently present in plants because a *tolC* mutant which would be expected

to be blocked in the export of such compounds lost viability soon after inoculation into grape (45). The upregulation of a variety of fimbrial and afimbrial adhesins by RpfF probably plays several important roles. Such genes contribute to biofilm formation. The environment of cells within biofilms has been shown to lead to changes in gene expression that enhance the ability of bacteria to tolerate stress environments (47). It also results in attenuated pathogenicity compared with an *rpfF* mutant (41). Thus, by enabling biofilm formation, DSF signaling might both directly and indirectly lead to increased stress tolerance of cells in those xylem vessels in which abundant biofilm forms. As shown previously, the increased adhesiveness of cells of *X. fastidiosa* in which DSF signaling is operative, probably mostly due to elevated expression of adhesins such as HxfA and HxfB, is essential for its transmission by sharpshooters (1,37,41). Hypervirulence that is apparently avoided by RpfF signaling is probably not a desirable trait for *X. fastidiosa* because it would reduce the attractiveness of the infected plant for sharpshooter feeding. Thus, *X. fastidiosa* has evolved the RpfF-dependent cell-to-cell signaling system to live in two worlds: plants and insects.

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