IDENTIFICATION OF TRAITS OF *XYLELLA FASTIDIOSA* CONFERRING VIRULENCE TO GRAPE AND INSECT TRANSMISSION BY ANALYSIS OF GLOBAL GENE EXPRESSION USING DNA MICROARRAYS

Project Leader:
Steven Lindow
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Cooperators:
Nian Wang
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Bill Feil
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Reporting Period: Results reported here are from work conducted September 2005 to September 2006.

ABSTRACT
Xylella fastidiosa (*Xf*) regulates virulence factors important in both virulence to grape as well as colonization of sharpshooter vectors via its production of a fatty acid molecule (known as DSF) whose production is encoded by *rpfF*. The RpfF homologue of *Xf* strains that cause Pierce's disease (PD), synthesizes a fatty acid cell-cell signal (DSF) that is apparently similar to that produced by *Xanthomonas campestris* pv. *campestris (Xcc).* *Xf rpfF* mutants exhibit increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors. While we have identified a key regulator of virulence and insect transmission in *Xf* we lack an understanding of the traits that are regulated by this pathogen in response to the DSF signal molecule. We thus are initiating studies to determine the rpf-regulation in *Xf*. We are exploiting a DNA microarray developed in another project that addresses host specificity genes in *Xf* to assess gene expression differences in isogenic $RpfF^+$ and $RpfF^-$ strains of *Xf* strain Temecula. The microarray contains 2555 gene-specific 70 bp oligodeoxynucleotides including negative and positive controls. Microarray analysis was performed to identify genes that are controlled by DSF and/or RpfC. DSF bioassay with reporter strain *Xcc* 8523 (pKLN55) indicated that DSF production is most abundant 10 days after inoculation when *rpfF* expression is most active. Preliminary results reveal that at least 124 genes are controlled in response to *rpfF* in *Xf*, including those encoding gum production, type IV pili and hemagglutinin. Clearly this regulator has a large effect on the physiological function of *Xf*. Microarray analysis revealed that more than 300 genes are also controlled by RpfC, including some of the same genes regulated by rpfF as well as genes such as *tonB*. Microarray-based gene expression results are being verified using quantitative Reverse Transcriptase-PCR. Comparison of the RpfF and RpfC regulons reveals that a complex pattern of expression of potential virulence genes contribute to the virulence of *Xf* and explains the hypervirulence of *rpfF* mutants and the reduced virulence of *rpfC* mutants. Work is also underway to determine the subset of *Xf* genes that is plant-inducible and the identity of those whose plant-inducible genes whose expression is also dependent on DSF production.

INTRODUCTION
PD 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, which is a far more effective vector of the pathogen *Xf* (Purcell 1997). *Xf* apparently causes disease by multiplying within, and thus blocking, xylem vessels (Hill and Purcell 1995, Hopkins 1989). The colonization by *Xf* in grape and sharpshooters shows great similarities to microbial biofilms that form in other aquatic systems. Biofilms of bacteria develop on solid surfaces that are exposed to a continuous flow of nutrients to form thick layers. These structures consist primarily of an EPS matrix in which the bacteria are embedded. Cells in biofilms are inherently more resistant to many stresses such as antimicrobial compounds, viruses, and predators. The EPS matrix aids in the nutrition on the cells by accumulating various types of nutrients in a way analogous to an ion exchange column (Wolfaardt et al. 1994). Thus, cells in such aggregates are much more able to grow and survive than planktonic cells, which might be thought of as "scouts" for other colonization sites. Small molecules such as *N*-acyl homoserine lactones (AHLs), small peptides, butyrolactone derivatives or a fatty acid (known as DSF), play key roles as signals (Bassler 2002,Whitehead et al. 2001) in biofilm formation in numerous species of bacteria. The signals, which increase in concentration with population density, typically 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 compounds is disrupted by mutation.

Much evidence now indicates that *Xf* regulates virulence factors via its production of a fatty acid molecule (known as DSF) whose production is encoded by *rpfF*. *Xf rpfF* mutants exhibit dramatically increased virulence to plants, however, the *rpfC* mutant showed decreased virulence to plants (Newman et al. 2004). Numerous genes with various functions were identified to be controlled by *rpfF* in the plant pathogens *Xanthomonas campestris* pv *campestris (Xcc)*, and *Xanthomonas oryzae* pv *oryzae (Xoo)* (Chatterjee and Sonti 2002). The RpfF homologue of *Xf* strains that cause PD, synthesizes a fatty acid cell-cell signal (DSF) similar to that of *Xcc* (Newman et al. 2004, Scarpari et al. 2003). Once DSF concentration reaches a threshold level, *Xf* senses DSF by RpfC, a two-component regulator containing both sensor kinase and response regulator domains, which then controls downstream genes. While DSF levels clearly are involved in regulation of virulence and behavior of *Xf*, we still do not understand what virulence factors are, nor how they are regulated in this pathogen. We thus are investigating the regulon dependent on DSF (rpfF regulon) as well as those genes dependent on rpfC both in culture and in plants to determine those factors that influence the interactions of the pathogen with plants and insects.

OBJECTIVES

1. Determine those genes in *Xf* whose transcription is controlled by *rpfF*, the regulator of virulence and insect transmission, by assessing global gene expression using DNA microarrays.
2. Determine the number and identity of genes in *Xf* that are expressed in grape plants but not in culture by assessing global gene expression using DNA microarrays.
3. Assess the contribution of individual genes of *Xf* whose transcription is dependent on *rpfF* in its virulence and insect transmissibility.

RESULTS

Dynamics of DSF production by *Xf* Temecula

To investigate the dynamic production of DSF by *Xf* on PWG medium, wild type strain Temecula was inoculated onto PWG medium and cell number and rpfF expression was measured with time. *Xf* reaches its stationary phase (maximum growth) around 10 days after inoculation (DAI) (Figure 1). Expression of *rpfF* reaches its peak in the early stationary phase and began to decline in late stationary phase (Figure 1). Likewise, the accumulation of DSF also was maximum at about eight days. This indicated that DSF production was most abundant in the early stationary phase.

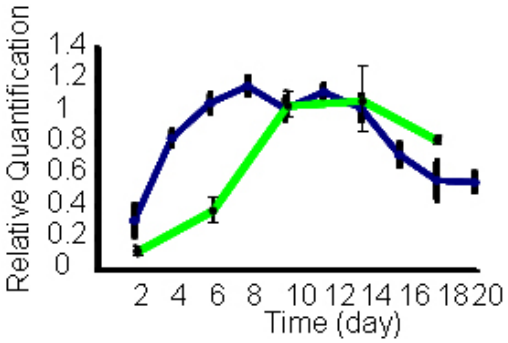


Figure 1. Dynamic of rpfF expression in *Xf*. *Xf* were cultured on PWG medium at 28°C. Green line indicates the growth curve of *Xf*; blue line indicates the expression level of rpfF measured by quantitative real time PCR.

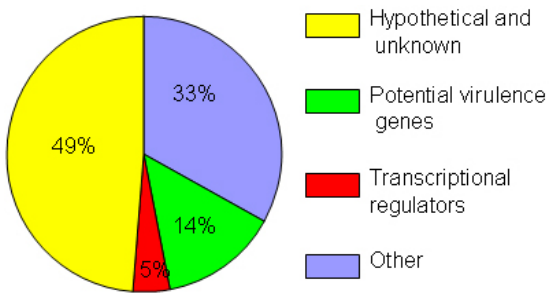


Figure 2. Categories of genes controlled by DSF in *Xf*.

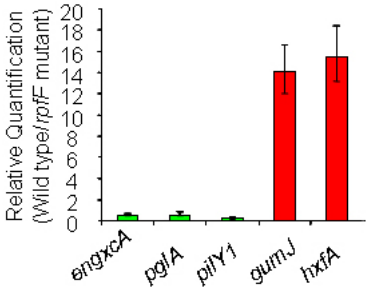


Figure 3. Virulence genes controlled by *rpfF* in planta identified with QRT-PCR. *engxcA*: endo-1,4-beta-glucanase, *pglA*: polygalacturonase, *pilY1*: type IV pili assembly protein, *gumJ*: EPS biosynthesis protein, *hxfA*: Hemagglutinin adhesions.

DSF controls the expression of many genes of various functions

Microarray analysis was used to identify genes controlled by DSF by comparison of expression profiles of wild type strain Temecula and *rpfF* mutant, which is DSF deficient. Considering the timing of DSF production in the wild type strain, samples of RNA were obtained at 10 DAI to maximize DSF-dependent gene expression. In total, 124 genes were identified to be dependent on *rpfF* for expression. Around 49% of the *rpf*F-dependent genes are hypothetical or unknown genes, 14% are potential virulence genes, 5% are regulatory genes and 33% belong to other categories including house keeping genes (Figure 2). Some of the most interesting genes, including most of the genes normally considered to be putative virulence genes in *Xf* are listed in Table 1. Such virulence genes include gum genes, hemagglutinin genes involved in attachment, and genes involved in secretion of extracellular products. The genes for hemagglutinins, and gum as well as several others were up-regulated in the presence of DSF (Table 1). In contrast, type IV pili genes, and cell wall degrading genes are downregulated in the presence of DSF (Table 1). These data are consistent with the apparently lowered production of GUM and high motility of the *rpfF* mutant as well as the fact that the RpfF mutant is much less adherent to surfaces in culture studies. The fact that DSF affects expression of some virulence genes indicates that DSF play key roles in regulation of virulence of *Xf*.

QRT-PCR was performed to confirm the expression profile of several key virulence genes. Both *gumJ* and *hexA* are upregulated by *rpfF* (Figure 3). Type IV pili gene *pilY1* and cell wall degrading enzyme genes *engxcA* and *pglA* are all downregulated by *rpfF* (Figure 3). These are consistent with the microarray data.

Expression of virulence genes are induced in planta

In order to understand the expression profile of *Xf* in planta, QRT-PCR was used to compare the expression level of several key virulence genes in culture and in planta. All the virulence genes tested are induced 2-23-fold in planta including *gumJ*, *hexA*, *pilY1*, *engxcA* and *pglA* (Figure 4). Thus *Xf* is clearly responding to chemical or physical signals in the plant to regulate gene expression. The rather artificial conditions in culture media commonly used to grow *Xf* also may not be conducive to expression of traits that normally might be expected to be expressed in plants. Thus better defining those genes that are expressed in plants will be a major goal in our continuing work since it should provide a better insight into the behavior of *Xf* in plants. We thus will strive to define those genes that are expressed in plants in a DSF-dependent fashion. Since they should be most directly related to those involved in virulence to grape.

Identification of genes controlled by RpfC with microarray analysis

Microarray analysis was also performed to identify genes that belong to RpfC regulon. Initial results indicate that at least 300 genes are controlled by RpfC. Potential virulence genes including IV pili genes, GUM genes, and TonB and TonB-dependent transporter genes are mostly up-regulated by RpfC. In contrast, hemagglutinin genes are downregulated by RpfC. QRT-PCR also confirmed the regulation of GUM and hemagglutinin genes by RpfC (Figure 5).

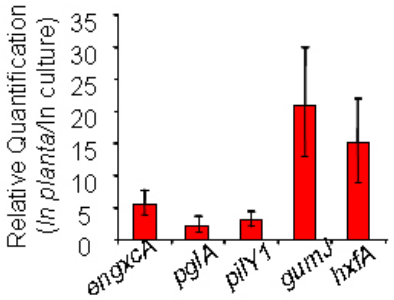


Figure 4. Comparison of gene expression of *Xf* in culture vs. in planta. *engxcA*: endo-1,4-beta-glucanase, *pglA*: polygalacturonase, *pilY1*: type IV pili assembly protein, *gumJ*: EPS biosynthesis protein, *hxfA*: Hemagglutinin adhesions.

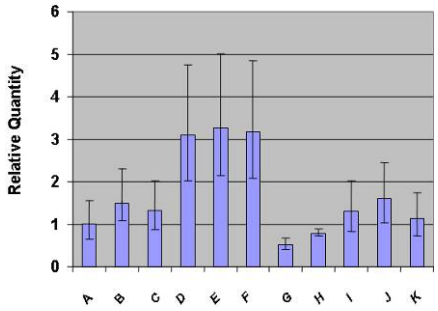


Figure 5. Effect of *rpfC* mutations on gene expression in *Xf* as measured by QRT-PCR. A: PD1851 (*engxcA*), B: PD1856 (*engxcA*), C: *fimA*, D: *gumB*, E: *gumJ*, F: *gumD*, G: *hxfA*, H: *hxfB*, I: *pglA*, J: *pilB*, K: *pilY1*.

Table 1. RpfF regulon of *Xf* (partial). Ratio of the abundance of RNA corresponding to a given gene in the wild-type strain to that recovered from an RpfF mutant is shown. Values less than 1.0 represent genes repressed in presence of DSF while values greater than 1.0 represent genes induced in the presence of DSF.

Gene Name	Wild type / *rpfF* mutant	Gene product
RXFZ02125	0.579	PilF protein
RXFZ02230	0.352	PilM
RXFZ02111	0.658	PilY1
RXFZ00028	0.281	PilT
RXFZ02547	0.374	PilA2
RXFZ00412	0.481	Extracellular serine protease precursor
RXFZ00730	0.288	ABC transporter ATP-binding protein
RXFZ00407	0.399	Pathogenicity-related protein
RXFZ02551	0.655	Sensor protein pilS
RXFZ00951	0.62	TonB-dependent receptor
RXFZ00320	0.687	Type I restriction-modification system
RXFZ01803	5.466	GumJ
RXFZ01637	3.485	Amino acid permease
RXFZ02199	2.01	Sensory transduction protein kinase
RXFZ02793	1.9	Hemagglutinin-like protein
RXFZ01626	1.658	Hemagglutinin-like protein
RXFZ02541	1.623	Two component system histidine kinase
RXFZ01123	1.74	Type III restriction modification system

Table 2. Comparison of the RpfF and RpfC regulons.

Gene categories	RpfF	RpfC
Type IV pili	Repressed (mostly)	Upregulated (mostly)
GUM gene (EPS)	Upregulated	Upregulated
Cell wall degrading enzymes	Repressed	Upregulated/Repressed
TonB and TonB dependent transporter	/	Upregulated
Hemagglutinin	Upregulated	Repressed

CONCLUSION

Microarray analysis and QRT-PCR have been successfully to identify genes that are differentially controlled by RpfF and RpfC. Comparison of the RpfF and RpfC regulons reveals that a complex pattern of expression of potential virulence genes contribute to the virulence of *Xf* and explains the hypervirulence of *rpfF* mutants and the reduced virulence of *rpfC* mutants (Table 2). Down-regulation of type IV pili and cell wall degrading enzymes genes and up-regulation of GUM and hemagglutinin genes may explain the lower attachment capability of the *rpfF* mutant and its high motility in planta. This leads to more blockage of the xylem, thus more virulence to grape infected by *rpfF* mutant compared to wild type strain. The up-regulation of type IV pili genes, GUM genes and down-regulation of hemagglutinin gene are probably responsible for the higher attachment capability of *rpfC* mutant compared to wild type strain. The up-regulation of some of the cell wall degrading enzyme genes might contribute to its less virulence on grapevines. The up-regulation of TonB and TonB dependent transporter by *rpfC* may also contribute to tolerance of toxic preformed plant defense chemicals in the xylem and its transportation of iron. The *rpfC* mutant should be much more susceptible to some toxic plant metabolites than the wild type strain. The damage to its iron transportation might also contribute to its weak growth of the *rpfC* mutant in the xylem, which is iron limited. Considering the induction of virulence genes in planta as identified with QRT-PCR, it is expected that more intriguing results will be done by studying its expression profiles of the wild type strain Temecula and the *rpfF* and *rpfC* mutants in planta. Together our expression profiling is providing much insight into the behaviors of the pathogen within plants and insects and should help develop methods to alter pathogen behavior for disease control.

REFERENCES

Bassler Bonnie, L. Small talk: Cell-to-cell communication in bacteria. Cell 109, 421-424 (2002).

Chatterjee, S. & Sonti, R. V. rpfF mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. Mol Plant Microbe Interact 15, 463-71 (2002).

Hill, B. L. & Purcell, A. H. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. Phytopathology 85, 1368-1372 (1995).

Hopkins, D. L. 1989. *Xylella fastidiosa*: a xylem-limited bacterial pathogen of plants. Annual Reviews in Phytopathology. 27:271-290.

Newman, K. L., Almeida, R. P. P., Purcell, A. H. & Lindow, S. E. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. PNAS 101, 1737-1742 (2004).

Purcell, A. H. 1997. *Xylella fastidiosa*, a regional problem or global threat? Journal of Plant Pathology. 79:99-105.

Scarpari, L. M., Lambais, M. R., Silva, D. S., Carraro, D. M. & Carrer, H. Expression of putative pathogenicity-related genes in *Xylella fastidiosa* grown at low and high cell density conditions in vitro. FEMS Microbiol Lett 222:83-92 (2003).

Wolfaardt, G. M., J. R. Lawrence, J. V. Headley, R. D. Robarts, and D. E. Caldwell. 1994. Microbial exopolymers provide a mechanism for bioaccumulation of contaminants. Microbial Ecology. 27:279-291.

Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J. & Salmond, G. P. Quorum-sensing in Gram-negative bacteria. FEMS Microbiology Reviews 25, 365-404 (2001).

FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program.

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

Project Leader:
Steven E. Lindow
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Cooperators:
Subhadeep Chatterjee
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Alexander Purcell
Dept of Env. Sci. Policy & Mgmt.
University of California
Berkeley, CA 94720

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

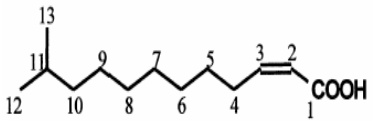
ABSTRACT

Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called DSF that changes its gene expression in cells as they reach high numbers in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease (PD). We have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. *Xf* strains that overproduce DSF cause disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF or by degrading DSF. We have identified the genes needed to degrade DSF and when they were transferred to and over-expressed in other strains they conferred the ability of these strains to degrade DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DFS-degrading strains greatly reduced the incidence and severity of disease in grape. Non-endophytic bacterial species were also established in high numbers inside grape leaves and petioles following spray application to plants with a high concentration of a silicon-based surfactant with a low surface tension. PD was reduced in plants after topical application of a DSF-producing strain of *Erwinia herbicola*. To verify that disease control is due to DSF interference, we have constructed mutants of these strains blocked in their ability to produce or degrade DSF and showed that the mutants are deficient in disease control. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in plants. Initial results indicate that plants produce at least some DSF and are much less susceptible to disease.

INTRODUCTION

Endophytic bacteria such as *Xf* colonize the internal tissues of the host, forming a biofilm inside the plant. A key determinant of success for an endophyte is the ability to move within the plant. We expect activities required for movement to be most successful when carried out by a community of cells since individual cells may be incapable of completing the feat on their own. Cells assess the size of their local population via cell-cell communication and coordinately regulate the expression of genes required for such processes. Our study aims to determine the role of cell-cell communication in *Xf* in colonization and pathogenicity in grapevines and transmission by the insect vector. *Xf* shares sequence similarity with the plant pathogen *Xanthomonas campestris* pathovar *campestris (Xcc).* 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. Two of the Rpf proteins, RpfB and RpfF, work to produce a diffusible signal factor (DSF) which has recently been described as an alpha,beta unsaturated fatty acid.

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. We now have shown that *Xf* makes a molecule that is recognized by *Xcc* but probably slightly different than the DSF of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of Rpf regulation would be genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel and perhaps adhesins that modulate movement.



Given that the DSF signal molecule greatly influences the behavior of *Xf* including both its virulence to grape and ability to be vectored by sharpshooters we have investigated various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. Our preliminary work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*, but until this study we did not know of the manner in which the interaction occurred nor whether such strains had the potential to affect the virulence of *Xf* in grape. In this period we have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-