

CONTRIBUTION OF *XYLELLA FASTIDIOSA* GENES UNIQUE TO GRAPE STRAINS TO ITS VIRULENCE TO GRAPE AND UTILITY IN SPECIFIC DETECTION OF GRAPE STRAINS BY DNA-BASED METHODS

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ABSTRACT

Xylella fastidiosa (*Xf*) is a group of genetically similar strains that infect a wide range of plants. We hypothesized that discrete genetic factors among the very similar strains determine the ability of a strain to infect a particular host plant. To better understand what makes grape a good host for all grape strains but not for strains such as oleander and almond that cannot colonize grape, we conducted experiments to look for host specific genes of the grape strain. Through our microarray and *in silico* genomic studies, we have identified 20 potential *Xf* grape strain virulence genes. Of these, we have focused on 10 genes. We excluded 10 genes based on criteria such as they are phage related, DNA modification genes, part of a repeated gene complex, or are predicted house keeping genes, and thus not likely to have a role in plant virulence. It was clear from our studies that the microarray studies have produced fewer unique genes (genes present in one strain and lacking in another) to grape strains than expected, indicating that the identity between *Xf* 'Temecula' and other non-grape strains must be closer than expected. Our *in silico* comparisons also revealed a high level of identity between grape and non-grape strains of *Xylella*. Because of this, we are now using dual labeling with our microarray studies to determine even small differences in gene sequence rather than simple lack of a particular gene. This is a more sensitive way to determine qualitative differences between the strains. We have now made knock-out mutants for seven of the 10 genes unique to grape strains that we expect to be most likely involved in virulence to grape. We used constructs that have a Kanamycin gene inserted near the 5' end of the gene for optimum efficiency in knocking out a given gene while preventing partial transcripts to be made in such knockout strains. Inoculation studies with grape have shown that several of these genes confer the ability to move within grape and thus to incite disease at sites away from the point of inoculation. The growth of these mutant strains in grape near the point of inoculation was not usually impaired, suggesting that such traits are involved specifically in other aspects of movement and symptom development in grape.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a group of genetically similar strains that infect a wide range of plants. A particular strain often has a relatively small and distinct host range when compared to other strains. Some strains of *Xf* originating from host plants other than grape do not sustain viable populations or are not virulent in grape. In particular, many of the strains of *Xf* isolated from almond do not infect grape (Almeida and Purcell 2003). This strongly suggests that differing genetic factors among the strains determine the ability of a strain to infect a particular host plant. Other studies provide evidence for host specificity among the *Xf* strains (Chen et al. 1992; Chen et al. 1995; Pooler and Hartung 1995; Henderson et al. 2001; Bhattacharyya et al. 2002a, 2002b; Doddapaneni et al. 2006). Cross inoculations in greenhouse studies showed that the oleander and grape strains of *Xf* were not pathogenic to citrus and that the almond strain was not pathogenic to oleander (Feil et al. *unpublished*). In California, three distinct strains of *Xf* as designated by their host range are recognized; the grape strain, the almond strain, and the oleander strain.

To better understand the underlying genetics of *Xf* as it relates to pathogenesis, several strains have been sequenced. The *Xf* '9a5c', a citrus strain, was fully sequenced in Brazil (Simpson, 2000). The draft-genome sequences of the almond and oleander strains of *Xf*, 'Dixon' and 'Ann1', respectively, are also publicly available. We used this information to identify a list of genes present in the grape strain genome but missing in other strains that do not sustain viable colonies in grape. We also developed a DNA microarray based on the sequence of the Temecula grape strain to interrogate the genomes of other strains by a process of DNA-DNA hybridization. We tested the ability of target DNA from non-grape strains to hybridize to probes designed from the reference strain, *Xf* 'Temecula', which were affixed to epoxy slides. During this process, we determined that most strains are highly identical to each other, having genes that are at least similar in sequence to reference genes in strain Temecula; very few genes were found in the Temecula strain that were lacking in other *Xf* strains. We thus have used a more sensitive approach to identify unique genes of the grape strain that is based on competitive hybridization of mixed DNA samples to the DNA microarray. Using this method, as well as *in silico* and other single strain hybridization results we have now obtained a very short list of genes that were found in all grape strains of *Xf* but are lacking, or substantially divergent in non-grape strains of *Xf*. The goals of this project thus was to determine the role of such genes in the virulence of *Xf* to grape and other plants, and to determine if such genes would be useful in distinguishing grape strains of *Xf* from all other strains in PCR-based detection schemes.

OBJECTIVES

1. Determine the relative contribution of grape strain-specific genes to growth and virulence of *Xf* in grape.
2. Design primers selective for grape-strains of *Xf* to enable the selective detection of grape strains in host plants.

RESULTS

Objective 1

Our studies to narrow the list of potential virulence genes unique to grape strains of *Xf* has resulted in a list of 20 genes (Table 1), 10 of which (in **Bold** print) are considered most likely by us to be potential virulence genes. As mentioned above, our microarray studies in which DNA from non-grape strains was hybridized alone to a DNA microarray populated with genes from *Xf* strain Temecula resulted in fewer than the expected differences in gene content between grape strains of *Xf* and other non-grape strains. This suggested that the differences in the strains was not due only to differences in gene content, but also to variations in the genes that might be in common to most, if not all, *Xf* strains. The higher sensitivity of the dual labeled arrays has allowed us to reveal those genes that vary substantially in sequence between *Xf* strains. These genes are included in the list of strain-specific genes listed in Table 1.

Table 1. List of 20 genes unique to grape strains of *Xf*.

Gene ID (a)	Predicted function	Size (bp)	In an operon? (b)	If yes, neighbor(s)
PD0028	Unknown function	354	No	
PD0105	Unknown function	468	Yes	valS, holC, pepA (house keeping genes)
RXFZ00317	Type I restriction-modification system specificity subunit	366	Yes	Type I restriction enzyme subunit hsdR
PD0370	Unknown function (phage)	303	?	Other phage
PD0371	DNA binding protein (phage)	402	?	Other phage
PD0515	Unknown function	399	No	
PD0540	Unknown function	441	No	
PD0829	Unknown function	507	No	
PD0872	Iron-sulfur flavoprotein	654	No	
PD1242	Hemolysin	1140	Yes	Hypothetical cystolic protein
				A series of six hypothetical cystolic proteins
PD1426	Unknown function	618	Yes	interdispersed among six hypothetical proteins followed by a RTX family calcium-binding cytotoxin or bacteriocin (frpC) (PD1415 – PD1427)
PD1434	Unknown function	363	No	
PD1510	Unknown function	417	No	
PD1511	Unknown function	567	No	
RXFZ02076	Unknown function	345	Yes	RXFZ02077 Unknown function
PD1606	Unknown function	795	No	Phage remnants nearby
PD1607	Modification methylase NspV	1455	Yes	PD1608
PD1608	Type II restriction enzyme NspV	663	Yes	PD1607
PD2071	Type I restriction-modification system specificity determinant	1335	Yes	PD2070 – PD2076 Type I restriction system
PD2075	Type I restriction-modification system specificity subunit	1218	Yes	Same as above

(a) Locus tags starting with PD are genes called by FAPESP, Brazil (<http://aeg.lbi.ic.unicamp.br/world/xfpd/>), those starting with RXFZ were called by Integrated Genomics (<http://ergo.integratedgenomics.com/ERGO/>), Chicago, Ill.

(b) A transcriptional operon was determined by the size of the intergenic region(s) (<≈50bp) and the absence of a terminator.

We produced knock-out mutants for 10 potential virulence genes identified in Table 1. These mutants were made by inserting a *kan* gene within the target gene to both disrupt the gene and to enable selection of chromosomal gene replacements. The target genes were chosen because of their size (>300 bp which would indicate that they are sufficiently large to be a functional gene and not a non-transcribed open reading frame) as well as because they had predicted functions that would plausibly be linked to virulence and/or host specificity. We eliminated some genes based on the fact that they resembled remnant phage genes or conferred expression of some other function that could not be logically thought to be associated with virulence. We then compared the identity of these genes to the genes present in the sequenced almond or oleander strains. While some of these identified genes from *Xf* ‘Temecula’ had at least high partial identity with a gene in an

almond or oleander strain, they were chosen because of differences in the location of the start or stop codon which would have yielded proteins of substantially difference size, or there were major differences in regions within the gene which likely would have altered its function. These differences almost certainly would have yielded highly different protein products. We have completed tests on knock-out mutants of seven of the ten grape strain-specific genes so far. The relative contribution of each of these unique genes on growth and virulence were studied by inoculating gene knock-out mutants into grape host plants (Figure 1). The incidence of infection of grape was much lower in several mutant strains of *Xf* (mutations in genes PD0105, PD0540, or PD1434). These mutants also exhibited much less lower levels of disease severity (# of leaves/plant symptomatic) in those plants in which infection occurred (compare Figures 1A and 1B). In addition, mutants with knockouts in genes PD872 and PD1510 also had reduced disease severity compared to wild-type strains, although the incidence of infection with these strains was similar to that attained by the wild-type strain. In all cases, the lower disease severity appeared to be due to a reduced progress of disease in the grapevines distal to the point of inoculation; while disease was noted up to 150 cm away from the point of inoculation in the wild-type strain, disease was restricted to sites much closer to the point of inoculation in many of the mutants (Figure 1B). In contrast, growth, measured as CFUs per gram of petiole tissue at or near the inoculation site was not significantly reduced in any mutant compared to that of the wild-type strain. The population sizes attained in petioles near the point of inoculation was quite high, suggesting that all of the mutants had similar ability to the wild-type strain to multiply in grape at least near where inoculated into grape, and that deficiencies in virulence are associated with reduced abilities to move throughout the plant.

Genes PD1007 and PD1608, genes unique to grape strains but not selected by us as among the 10 most likely genes involved in grape virulence, were included in these tests as “negative controls”. These genes are apparently genes introduced by phage infection and associated with phage biology and thus we did not expect them to be involved in grape virulence. As expected, mutants of these two genes did not exhibit any significant different in the ability to infect or multiply in grape compared to wild-type strains (Figure 1). While a limited sample size of genes, it appears that our decision to disregard phage genes as virulence determinants in *Xf* was justified.

Objective 2

As this was a new objective for this project, work has only recently begun on developing and testing DNA sequences as primers for selective detection of grape strains of *Xf*. In silico studies to test the specificity of oligonucleotides based on the novel genes noted in Table 1 are being assessed prior to actual testing of them as PCR primers against a collection of *Xf* strains of different origins and host ranges.

CONCLUSIONS

The results of the tests of our knockout mutants provide us with optimism that the novel genes associated with grape strains of *Xf* are in fact contributing to the unique behaviors of such strains to cause Pierce’s disease (PD). The identification of the unique genes to grape strains of *Xf* and the understanding of how these unique genes confer host specificity and virulence to grape will help researchers with their breeding programs for resistance to PD. These genes could also be studied to find targets for chemical or other types of control. Knowing those unique genes necessary for grape virulence should also prove valuable for the design of specific primers for the detection of all *Xf* grape strains. We are in the process of design such primers now.

Since there are only a few sequenced strains available for a direct comparison, finding the unique genes in grape required us to examine hybridization profiles from other non-sequenced strains and determine the absences of genes in those genomes. All grape strains of *Xf* should carry the same suite of genes for growth and virulence in grape. However, the grape strain has other hosts than grape. Some of the unique genes we find may be used for other reasons than just grape related virulence. If we determine those genes uniquely needed for virulence in grape, we will also determine what constitutes a grape strain. Knowing what every grape strain processes genetically will allow us to develop better molecular screens, especially for strains collected from non-grape hosts, and may allow us to work towards the discovery of more specific remedies to PD.

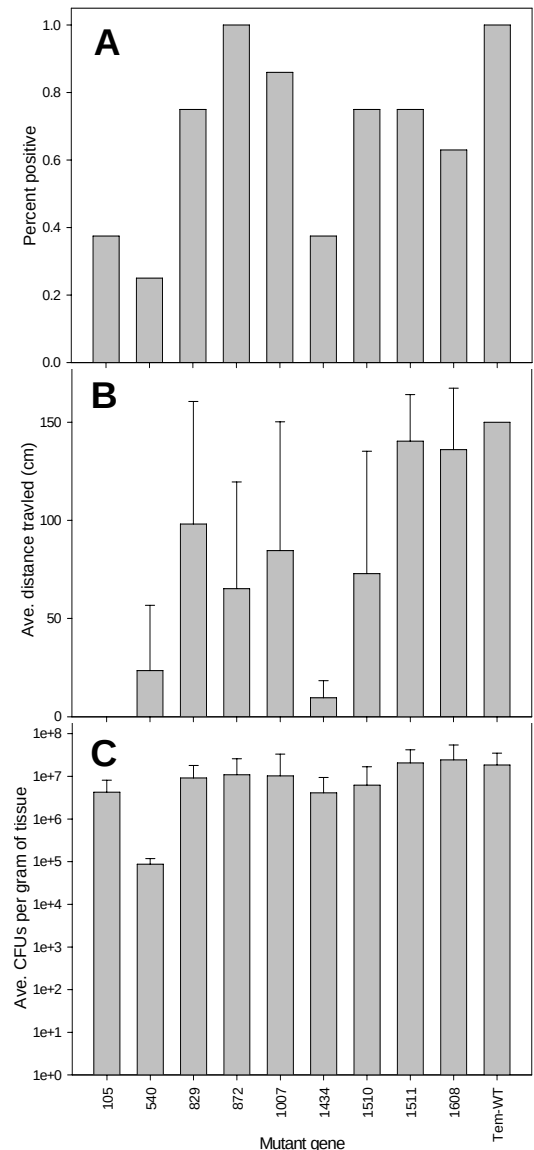


Figure 1. Incidence (A), movement (B), and growth (C) of *Xf* following inoculation with either mutant or wildtype *Xf* strains. Observations were made 12 weeks after inoculation.

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

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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 *rpffC* 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.