ROLE OF UNIQUE GENES OF *XYLELLA FASTIDIOSA* GRAPE STRAIN IN HOST SPECIFICITY AND VIRULENCE TO GRAPE AND TO INSECT USING MICROARRAY

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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 differing genetic factors among the 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 so far identified 52 potential *Xf* grape strain virulence genes.

We have constructs for knocking out 12 of the 52 identified genes. The genes we chose from our list were greater than 300 bp and were not part of a remnant phage. Our constructs have a Kanamycin gene inserted near the 5' end of the gene for optimum efficiency in knocking out our gene and preventing *Xf* from making partial transcripts. We plan to inoculate plants with our knock-out mutants once they are confirmed.

We noticed that the microarray studies have produced fewer genes than expected, indicating that the similarity between *Xf* 'Temecula' and other non-grape strains must be greater than expected. Our *in silico* comparisons revealed a high level of similarity as well. Because of this, we are now using dual labeling with our microarray studies. This is a more sensitive way to identify differences in gene sequence between the strains.

INTRODUCTION

Xf is a group of genetically similar strains that infect a wide range of plants. A particular strain often has a relatively reduced 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 almond strains of *Xf* 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 Hartung1995; Hendson et al. 2001; Bhattacharyya et al. 2002a, 2002b). For example, 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, we have three identified groups of strains of *Xf* as designated by their host range; the grape strains, the almond strains, and the oleander strains.

To better understand the underlying genetics of *Xf* as it relates to pathogenesis, several strains have been sequenced. Strain *Xf* '9a5c', a citrus pathogen, 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 used target DNA from non-grape strains for hybridizing to probes designed from the sequenced reference strain, *Xf* 'Temecula', which are affixed to epoxy slides. During this process, we determined that most strains are highly similar to each other and require a much more sensitive approach to identify genetic distinctions with the grape strain. That is, few genes are completely missing in non-grape strains compared to grape strains and vice versa. We are thus using a duel labeling approach where the reference strain and the target strain are labeled differentially and co-hybridized on the same array, and sequence differences are reveled by competitive hybridization.

OBJECTIVES

1. Complete our initial work on host-specific gene identification using DNA/DNA microarray studies and to better define the role of 52 genes thus far identified as unique to the grape strain.
2. Produce knock-outs of the unique genes to the grape strain and to test for virulence of these knock-outs in grape**.**

RESULTS

Objective 1

To arrive at a list of 52 genes unique to the grape strain, we have hybridized DNA from six different strains to the DNA on the grape strain array. These strains were isolated from almond (two different isolates), oleander (two different isolates), oak, plum, olive, and maple. We also used two other grape isolates ('STL' and 'Fetzer') in our studies. Eighteen of these 52 genes are presented here (Table 1). There are 34 additional genes not listed that are either less than 300 bp in size or are

phage related. Also, many of the remaining genes are predicted to have only a hypothetical function. Our microarray work has thus far resulted in fewer than expected differences between *Xf* grape strain and other non-grape strains. Because of this we checked our oligonucleotide probes through blast analysis and determined that 93% of the oligos designed for our array have an 86% or higher sequence identity across at least 40 bases with that of the almond or oleander strain (Figure 1). Of the remaining 107, 63 had hits of fewer than 40 bases and only 44 had no hits at all. Many of these did not correspond to missing spots on our array, indicating that the oligos probably fell within regions of the almond or oleander genome that are poorly covered in the draft sequence. We also determined that this identity is too high for single labeling experiments since we would observe hybridization under the low stringent conditions that we use; thus gene variants may be present that would be overlooked in this strategy. Because of this, we are now using a dual labeling approach for our arrays (Figure 2). This should give us more ability to distinguish genes that might vary somewhat (but be present in) *Xf* strains from hosts other than grape. We illustrate this issue in an image of a small part of one microarray in Figure 2, in which *Xf* grape 'Temecula' DNA is labeled with Cy3 (green) and *Xf* oleander 'AnnI' DNA was labeled with Cy5 (red). Bright green spots indicate a potential unique gene for the grape strain, whereas yellow indicates a gene with close sequence identity across the 70 mer oligo is present in both genomes.

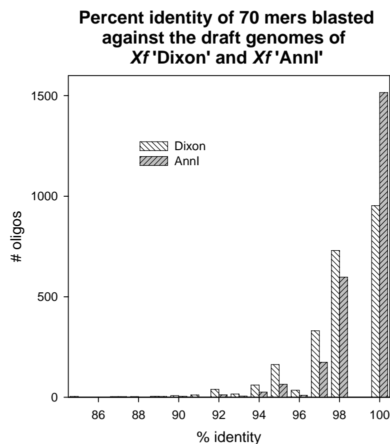

Figure 1

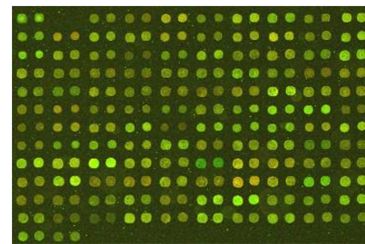

Figure 2

Table 1. Partial list of genes missing in *Xf* oleander 'Ann1' and *Xf* almond 'Dixon' compared to 'Temecula1' using microarray analysis.

RXFZ gene #	gene function
146	Hypothetical protein
105	Hypothetical protein
676	Hypothetical protein
733	No known function
734	No known function
809	Conserved membrane spanning protein
1099	No known function
1116	Hypothetical protein
1174	Iron-sulfur flavoprotein
1216	No known function
1291	No known function
1299	Hypothetical secreted protein
1327	Cell filamentation protein fic
1328	No known function
1613	Hypothetical membrane associated protein
2150	No known function
2737	No known function
2744	No known function

Objective 2
We chose to knock out 12 potential virulence genes noted in Table 1 and have disrupted these genes by inserting a kanamycin resistance gene into them (Table 2). We focused on those genes that were larger than 300 bp in size, reasoning that they are more likely to be functional genes rather than pseudogenes. We also eliminated some genes from Table 1 based on the fact that they were apparently genes associated with a remnant phage or clearly encoded a housekeeping function that could not plausibly be associated with virulence. We then compared the identity of these genes to the genes present in the almond or oleander strains. While some of these identified genes from *Xf* 'Temecula' are shown to have high identity with the almond or oleander strain, these genes were chosen because of differences in the location of the start or stop codon, or there were major differences in small sections within the gene. These differences could produce highly different protein products. Gene knock-out mutants in *Xf* 'Temecula' are being made using the method of marker-exchange mutation developed in our lab. This method is highly efficient and does not require the numerous sub-transfers that are needed with other systems to completely knock-out the gene function. We are now characterizing these knock-outs as they are being made.

Table 2. List of selected genes for knock-out mutants.

Gene ID	Function	identity to grape	
		Almond	Oleander
PD0028	Hypothetical protein	.957	.960
PD0105	Hypothetical protein	.908	.908
PD0515	Hypothetical protein	.667	.538
PD0540	Hypothetical protein	.922	.954
PD0829	Hypothetical protein	.948	No hits
PD0872	Iron-sulfur flavoprotein	No hits	No hits
PD1434	Hypothetical protein	No hits	No hits
PD1510	Hypothetical protein	No hits	.884
PD1511	Hypothetical protein	.750	.789
PD1606	Hypothetical protein	No hits	No hits
PD1607	Modification methylase NspV	No hits	No hits
PD1608	Type II restriction enzyme NspV	No hits	No hits
PD2071	Type I restriction-modification system specificity determinant	.947	.983

The relative contribution of each of these unique genes will be studied by inoculating gene knock-out mutants into a grape host plant. Those genes that affect the growth and virulence of *Xf* more will naturally become a higher priority for further study. Bacterial growth will be analyzed monthly over the four months by removing and grinding a petiole to extract the bacteria. We will select petioles from the region where the initial inoculation occurred to ensure that local growth is not overlooked.

CONCLUSIONS
The identification of the genes unique to the *Xf* grape strain 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 Pierce's disease (PD). These genes could also be studied to find targets for chemical or other forms 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.

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.

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