DIFFERENTIAL PLANT-INDUCIBILITY OF PUTATIVE VIRULENCE GENES BY *XYLELLA FASTIDIOSA* IN SUSCEPTIBLE AND RESISTANT PLANT HOSTS

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ABSTRACT
Although many studies have addressed systemic movement of *Xylella fastidiosa* in the plant (4), we still lack a basic understanding of the mechanism by which *X. fastidiosa* cells, which initially are inoculated into relatively few xylem vessels, are able to spread throughout the xylem network of the host plant. Furthermore, we lack an understanding of why systemic movement by *X. fastidiosa* occurs in some host plant species but not in others. In other plant pathogens, including *Xanthomonas campestris,* which is closely related to *X. fastidiosa,* the expression of virulence genes such as those encoding gum synthesis, endoglucanase and pectinase activities, is required for systemic movement *in planta*. The genomic similarities between *X. fastidiosa* and *Xanthomonas* strongly suggest that similar virulence genes are required by *X. fastidiosa* for symptom formation in the plant (2). In some plant pathogens expression of virulence genes is dependent on certain plant factors. In this study we aim to determine the expression pattern of these virulence genes by *X. fastidiosa* during colonization of grapevines susceptible and resistant to Pierce's disease. Reporter genes will be placed under the control of regulatory regions of the genes to be studied, and these promoter-reporter constructs will be integrated into the *X. fastidiosa* genome to allow their stable expression *in planta*. Reporter gene expression will be monitored during different phases of disease progress to elucidate when expression of each gene occurs during disease progression. These data will enable us to construct a fairly accurate description of the course of virulence events leading to Pierce's disease after infection and to detect plant factors that affect virulence of *X. fastidiosa*.

INTRODUCTION
Traits in bacteria that contribute to virulence are often expressed only in the presence of a susceptible plant. In a variety of bacterial species, genes such as *hrp* genes involved in secreting virulence effector proteins into plant cells as well as those conferring the production of extracellular toxins or enzymes important in the disease process are not expressed in culture, but are rapidly expressed when bacterial cells are introduced into plants (1, 15). Similarly, many genes in the symbiotic nitrogen fixing bacterium *Rhizobium* are not expressed in culture but are expressed during the process of infection of roots, often in response to compounds leaked from the plant itself (12). While in many cases the plant factors that are involved in inducing expression of bacterial genes are unknown (5, 13), in several instances those plant compounds that modulate expression of bacterial virulence genes have been identified. For example flavanol glucosides such as quercetin and kaempferol as well as arbutin induce the expression of the *syrB* gene of *Pseudomonas syringae* that is required for production of the phytotoxin syringomycin (10, 14). Likewise, shikimic and quinic acids were found to induce production of toxin gene expression in *P. syringae* pv. *syringae* (7). Importantly, higher levels of plant-inducible gene expression were observed in those plant species that were most susceptible to these pathogens (6, 7). For example, the highest induction of *syrB* was found in extracts from the bark of the most susceptible cherry trees (6) and coronatine biosynthetic genes were induced to much higher levels when bacteria were introduced into host plants compared to non-host plants (7). Thus it seems clear that virulence genes are not constitutively expressed in plant pathogenic bacteria and that plants often inadvertently induce expression of such genes in the pathogens.

There is strong circumstantial evidence that *X. fastidiosa* expresses many of its virulence genes only when in plants. For example, polysaccharides that surround bacterial cells in xylem vessels are a prominent feature of Pierce's disease infections in grape, and the *X. fastidiosa* genome contains *gum* genes for the production of extracellular polysaccharide. However, such materials are produced in only VERY small amount in culture. This suggests that if, as is commonly thought, the polysaccharide in plants is of bacterial origin, than the *gum* genes must be induced when cells are in the plant. Likewise, there is little evidence of production of cellulases or other extracellular enzymes in X. *fastidiosa* cultures (J. Labovitch, personal communication). Preliminary studies have been made of gene expression in *X. fastidiosa* using DNA microarrays on membrane filters to assay the abundance of mRNAs corresponding to virulence genes (3). These expression arrays revealed that most of the several likely virulence genes such as those conferring production of cellulases, xylanases, pectinases, as well as regulators of other virulence factors were all expressed at a very low level in *X. fastidiosa* cultures (3). These results are consistent with the lack of evidence for virulence factors detected in cultured cells. At this time it is impossible to use such hybridization techniques to assess gene expression of bacteria while in plants. Instead it is possible to assess gene expression *in planta* using powerful and sensitive reporter genes such as *inaZ,* conferring production of ice nucleation protein, which is easily detected in "dirty" biological systems such as within plants, and when bacterial cell numbers are low. The thrust of this proposal will be to obtain direct estimates of virulence gene expression *in planta*.

One important question is whether resistance to Pierce's disease by certain plant hosts is due to *X. fastidiosa's* virulence strategy being unsuccessful in resistant plants or is attributable to the plant's ability to modify the behavior of the bacteria via host-specific induction of virulence genes. We hypothesize that in resistant plant hosts, *X. fastidiosa* is not expressing these virulence genes. Alternatively, *X. fastidiosa* may express virulence genes to the same extent in resistant and susceptible hosts but host plant anatomy may differ in its ability to resist the effects of *X. fastidiosa*-generated enzyme activity. This distinction will be important in developing resistant cultivars of grapevines.

To distinguish between these possibilities, we aim to use the genomic sequence of *X. fastidiosa* to identify the regulatory regions of putative virulence genes and to fuse these regions to reporter genes. We will monitor expression of the reporters in tolerant and susceptible grapevine cultivars and resistant alternate hosts as a measure of virulence gene expression. By comparing the level of expression of putative virulence genes by *X. fastidiosa* in resistant and susceptible plants we will learn whether resistance is a function of plant structure or *X. fastidiosa* behavior in different plants. In addition, we will gain an understanding of the pattern of expression of these genes during colonization. This work's contribution to a basic understanding of the mechanisms of *X. fastidiosa* systemic movement and plant resistance will be very useful for researchers attempting to find strategies for controlling disease in important agricultural plants. If, as we hypothesize, plant factors are involved in regulating virulence in a cultivar-specific or species-specific manner, it should be possible to target the production of such "inducers" of virulence in breeding programs or in transgenic plants in order to yield resistant grapevines.

OBJECTIVES

1. Construct *Xylella fastidiosa* strains that will report expression of putative virulence genes. This will be accomplished by fusing regulatory regions of putative virulence genes to the reporter genes *inaZ* and *gfp* and inserting these expression cassettes into a suicide vector, then integrating these constructs into the *X. fastidiosa* genome.
2. Determine putative virulence gene expression *in planta* and determine whether any correlations exist between expression and symptom development and/or population size. This will be accomplished by introducing the reporter strains into tolerant and susceptible grapevines and resistant alternate hosts and monitoring symptom development, *X. fastidiosa* populations and expression of InaZ activity (which will represent expression of putative virulence genes) in plant macerates at various times post-inoculation.
3. Obtain fine scale spatial information about virulence gene expression and determine whether there is a correlation between expression and colony size by using confocal laser scanning microscopy to locate *X. fastidiosa* colonies *in planta,* to measure their size and to monitor Gfp expression (which will represent expression of putative virulence genes) during timepoints determined to be critical in Objective 2.

RESULTS

Objective 1. Regulatory sequences of the putative virulence genes *gumB, engXCA,* and *pg* have been obtained by amplifying the 200-300 bp region upstream of the start codon from the *X. fastidiosa* Temecula genome by polymerase chain reaction. The promoters have been fused to two reporter genes, *InaZ* and *gfp[ASV]*.

The *InaZ* gene encodes a protein that nucleates ice formation in super-cooled water and is an excellent reporter system for monitoring gene expression in plant pathogenic bacteria (8). Advantages of this reporter are its ease of use (plant macerates are directly tested in a quick, simple, inexpensive ice nucleation assay), its extreme sensitivity (gene expression can be measured in as few as 10 cells) and the ability to quantify levels of gene expression. This sensitivity is crucial because virulence genes may be expressed only at very low levels or at specific times during colonization and sensitivity ensures that we will be able to detect even very low expression in a plant macerate. Likewise, we know populations are low in resistant plants and we need to monitor gene expression in such low populations. One drawback to this system is that only macerated tissue can be examined, however it will be ideal for elucidating overall gene expression characteristics, such as when expression occurs in relation to inoculation, what population levels are correlated with expression, and whether expression differs between resistant and susceptible hosts.

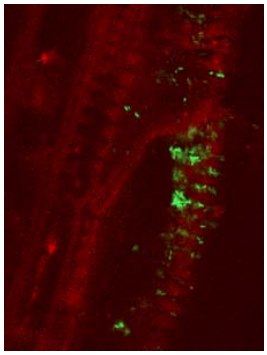

Figure 1. *X. fastidiosa* expressing green fluorescent protein observed in an unperturbed xylem vessel by CSLM.

The *gfp[ASV]* gene encodes an unstable variant of the green fluorescent protein. The unstable nature of the gfp will enable us to achieve the most accurate data on promoter activation conditions (9). *X. fastidiosa* has been successfully engineered to

express a green fluorescent protein constitutively (11). Fluorescent *X. fastidiosa* cells can be directly visualized in the plant using confocal laser scanning microscopy (CSLM). This type of microscopy captures images from deep within a sample, allowing visualization of unperturbed *X. fastidiosa* cells in intact xylem vessels (Figure 1). In addition, no fixation, washing or staining are needed and sample dissection is minimal, eliminating the potential for artifacts that plagues other types of microscopy used for *in planta* analysis of *X. fastidiosa*. This gfp expression is stable *in planta* and disease symptom formation and growth in the plant are the same for the gfp-expressing *X. fastidiosa* as for the wild type (11). In this study, *X. fastidiosa* will be engineered to express a green fluorescent protein under the control of virulence gene regulatory sequences. CSLM will be used to visualize *X. fastidiosa* cells in the plant and determine the pattern of virulence gene expression (which will correspond to green fluorescence). We will be able to visualize directly any cultivar-specific differences that were found using the InaZ system. This will be important for verifying whether differences detected are correlated with different patterns of growth in those plants or are due to a difference in host-specific gene induction.

These promoter-reporter cassettes have been introduced into plasmids designed to integrate into the *X. fastidiosa* chromosome (Figure 2). These plasmids have been electroporated into *X. fastidiosa* and we are currently screening

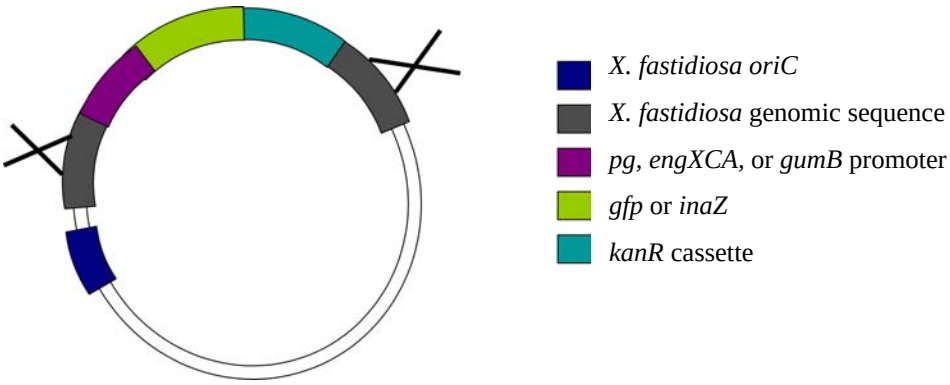

Figure 2. Design of suicide plasmids for integration of promoter-reporter and marker genes into the *X. fastidiosa* genome. Black "x" marks site of homologous recombination with the *X. fastidiosa* chromosome.

transformants for correct insertions. Since the project has only just recently started, we are performing initial aspects of the proposed work. The studies are proceeding according to schedule. The completion of this objective will result in the strains listed in Table 1.

Table 1. *X. fastidiosa* strains to be constructed

Strain	*Promoter*	*Reporter*	*Markers*	*Putative virulence activity*
Pg-ice	*pg*	Ice nucleation	Kan-2	pectinase
Pg-gfp	*pg*	Green fluorescence	Kan-2	pectinase
Eng-ice	***engXCA***	Ice nucleation	Kan-2	cellulase
Eng-gfp	***engXCA***	Green fluorescence	Kan-2	cellulase
Gum-ice	*gumB*	Ice nucleation	Kan-2	extracellular polysaccharides
Gum-gfp	*gumB*	Green fluorescence	Kan-2	extracellular polysaccharides

Objectives 2 and 3. These objectives will be addressed after the completion of Objective 1.

CONCLUSIONS

Conclusions will be drawn from future data that will be obtained during fulfillment of Objectives 2 and 3.

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