CHARACTERIZATION OF *XYLELLA FASTIDIOSA* GENES REQUIRED FOR PATHOGENICITY

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Reporting Period: The results reported here are from work conducted July 2010 through October 2010.

ABSTRACT
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine (Wells et al., 1981). *Xf* is closely related to *Xanthomonas oryzae* pv. *oryzae* (*Zoo).* Recent findings indicate that the sulfated Type 1 secreted protein Ax21 is required for density-dependent gene expression and consequentially pathogenicity of *Xoo*. Two two-component regulatory systems (TCSs) are required for Ax21 mediated immunity. Orthologs for both of the TCSs and Ax21 have been found in *Xf*. In this study, we will investigate the role of Ax21 and the two TCSs that regulate Ax21 in *Xf*.

LAYPERSON SUMMARY
Xylella fastidiosa (Xf) is a plant pathogenic bacterium and the causal agent of disease in a variety of economically important crops, including PD of grapevine. *Xf* causes disease by colonizing the xylem vessels, blocking the flow of water in the grapevine. In many plant pathogenic bacterium's, biofilm formation plays a key role in virulence. A biofilm is a population of microorganisms attached to a solid or liquid interface. The production of biofilm is regulated by quorum sensing system, in which bacteria communicate with one another via small molecular weight compounds. In *Xanthomonas oryzae* pv. *oryzae (Xoo),* a bacterial species related to *Xf,* it has been shown that Ax21, a sulfated peptide, is a quorum sensing compounds that is required for biofilm formation and virulence. Furthermore, two two-component regulatory systems (TCSs) have been identified that are required for Ax21 activity in *Xoo*. In this research, we will investigate the biological function of Ax21 and the two TCSs orthologs that were identified in the *Xf* genome.

INTRODUCTION
Xylella fastidiosa (Xf) is a gram-negative, xylem-limited plant pathogenic bacterium and the causal agent of Pierce's disease (PD) of grapevine (Wells et al., 1981). *Xf* is found embedded in the plant matrix in clumps, which leads to the xylem vessel blockage. The formation of biofilms allows for bacteria to inhabit an area different from the surrounding environment, potentially protecting itself from a hostile environment. Furthermore, biofilm formation is an important factor in the virulence of bacterial pathogens. Biofilm formation is a result of density-dependent gene expression (Morris and Monier, 2003). Density-dependent biofilm formation is triggered by the process of quorum sensing (QS). In QS, bacteria are able to communicate with each other via small signal compounds, generically called "auto-inducers" and the specific case of Xanthomonas and *Xf* the molecules are referred to as diffusible signal factors (DSF). The auto-inducer is a means by which bacteria recognize population size, and mediate the expression of specific genes when bacterial populations reach a threshold concentration. (Fuqua and Winans, 1994; Fuqua et al., 1996).

In *Xanthomonas oryzae* pv. *oryzae (Xoo),* Ax21 is a sulfated, Type 1 secreted protein that is a quorum sensing compound. Ax21 was recently shown to be a requirement for induction of density-dependent gene expression, including biofilm formation (Lee et al., 2006; Lee et al., 2009). In *Xoo*, two two-component regulatory systems (TCSs) required for Ax21-mediated activity have been found and orthologs of the TCSs and Ax21 were identified in the *Xf* genome (Simpson et al., 2000). In order for an active Ax21 gene product to be produced, two TCSs are required: RaxR/H and PhoP/Q (Burdman et al., 2004; Lee et al., 2008). The goal of this research is to investigate the role of Ax21 and the associated two component regulatory genes in *Xf*.

OBJECTIVES
1. Determine the functional role of the Ax21 homolog in *Xf*.
2. Determine the functional role of the PhoP/PhoQ two-component regulatory system in *Xf*.
3. Identify GacA-regulated genes in *Xoo* through microarray analysis and compare with *Xf GacA*-regulated genes.

RESULTS AND DISCUSSION

In the few months that we have worked on this project we made deletion knockout strains of Ax21, *PhoP* and *PhoQ* in *Xf*. For the Ax21 knockout strain, we conducted a variety of assays including pathogenicity on grapevines, biofilm formation, cell-cell aggregation and growth rate. Unfortunately, the grapevine pathogenicity assay did not give us any meaningful data this year because the plants in the greenhouse inoculated with both the wild-type *Xf* and *Xf*Δax21 exhibited foliar symptoms unrelated to PD. We will repeat these pathogenicity assays again next year. We will also inoculate grapevines with *Xf*Δ*PhoP* and *Xf*Δ*PhoQ* mutants.

Xf has an ortholog of ax21 gene (Lee, et al. 2009). To test if *Xf* has Ax21 activity, we carried out Ax21 activity assay with a previous described method (Lee, et al., 2006). Rice leaves from TP309, susceptible to Xoo PXO99, and TP309-XA21, resistant to PXO99, were cut at the tip and pretreated with supernatants from wild type (*Xf*) and Ax21 knockout (*Xf*Δax21) of *Xf*. Supernatants from *Xoo* PXO99 and PXO99Δax21 were used as positive and negative control, respectively. Five hours later the pretreated leaves were inoculated with the *raxST* knockout strain (PXO99Δ*raxST*), which lacks Ax21 activity. Ax21 activity was evaluated by measuring lesion lengths three weeks after inoculation. If *Xf* had Ax21 activity, leaves of TP309-XA21 pretreated by supernatants from *Xf* would show resistance to PXO99Δ*raxST* strain, but not leaves pretreated by supernatants from *Xf*Δax21. However, both leaves pretreated by supernatants from *Xf* and *Xf*Δax21 were susceptible to PXO99Δ*raxST*. It means *Xf* does not possess Ax21 activity, indicating it is unable to trigger XA21-mediated immunity in our rice plant bioassay (**Figure 1**). A lack of secretion and/or sulfation system in *Xf* may be the cause of the lack of Ax21 activity because *Xf* does not have orthologs of *raxA*, which is required for secretion of Ax21, and *raxST*, which is required for sulfation on Ax21. Further research will be conducted to better understand the role of Ax21 in *Xf* pathogenicity and cell-cell communication.

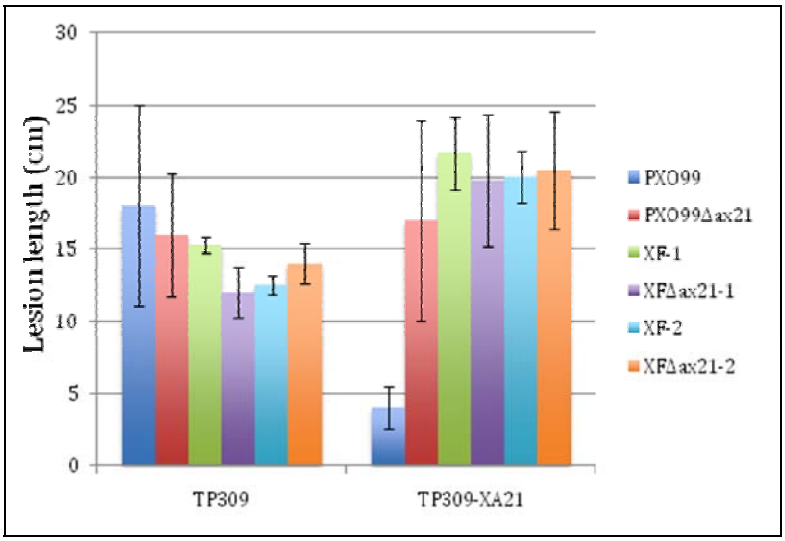

Figure 1: Lesion length on 6 week old TP309, susceptible to *Xoo* PXO99 strain, and TP309-XA21, resistant to PXO99 rice plants inoculated with PXO99Δ*raxST* strain five hours after pretreatments of supernatants. PXO99 and *Xf* indicate wild type of *Xoo* and *Xf* strains, respectively. PXO99Δ*ax21* and *Xf*Δax21 indicates ax21 deletion mutants of *Xoo* and *XF*, respectively. -1 represents supernatants from 8 days incubation culture, -2 represents supernatants from 11 days incubation culture. Each value represents the mean +/- SD.

Based on cell growth, cell-cell aggregation and biofilm production assays, we found some differences between the wild-type *Xf* and *Xf*Δax21. Based on preliminary cell growth results, it appears that the *Xf*Δax21 mutant grows to a lower population density than wild type *Xf* , although it does grow at a similar rate to the wild-type (**Figure 2**). Biofilm production of *Xf*Δax21 is slightly higher than the wild-type (**Figure 3**) when grown statically and measured by the crystal violet method. However, when the mutant and wild type strains were grown in a flask on a shaker, visual inspection showed there was considerably less biofilm formed by the mutant than the wild type strain. This observation needs to be repeated and the amount of biofilm produced will be quantified by the crystal violet method.

Based on the cell-cell aggregation assay, *Xf*Δax21 form less aggregated cells than wild type (**Figure 4**).

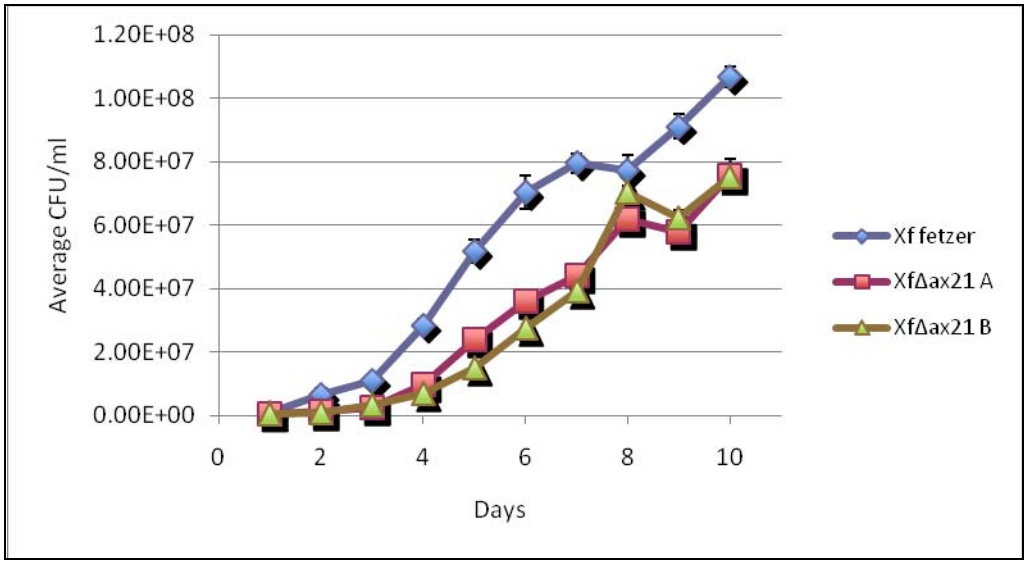

Figure 2. Bacterial growth of wild type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B. . Values shown are the means of 5 samples +/- error.

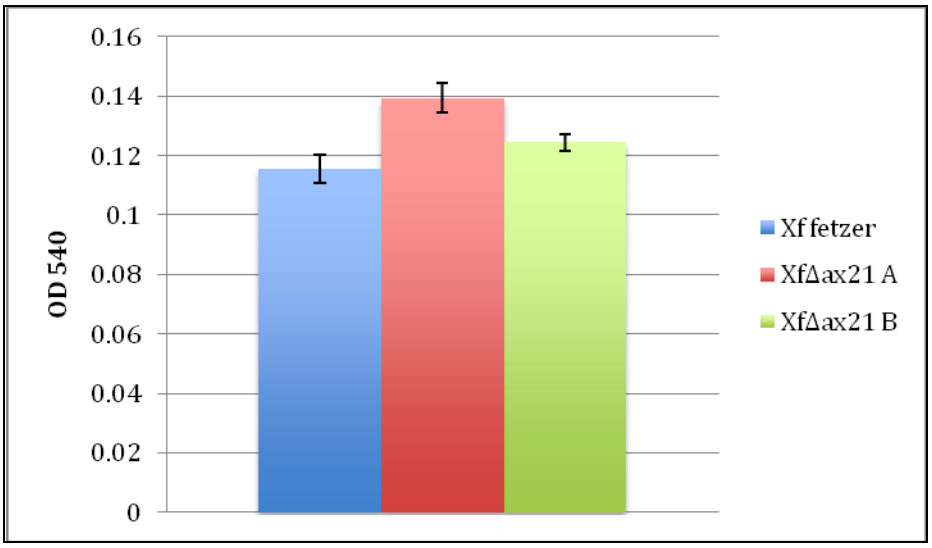

Figure 3. Comparison of biofilm formation in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B in stationary cultures as determined by the crystal violet staining method. Values shown are the means of 10 samples +/- error.

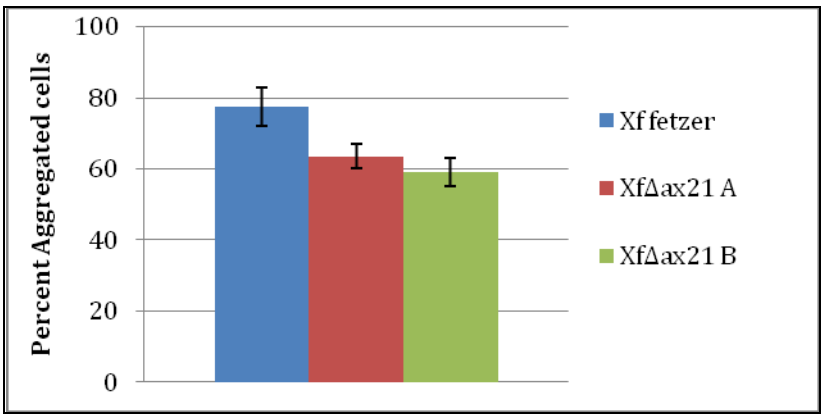

Figure 4. Comparison of percent aggregated cells in wild-type *Xf* fetzer, *XfΔ*ax21-A, and *XfΔ*ax21-B. Percentage of aggregated cells was determined as described by Guilhabert and Kirkpatrick, 2005. Values shown are the means of 10 samples +/- error.

CONCLUSIONS

We have made good initial progress on determining the functional role of Ax21 in *Xf*, although further comparison of wild-type *Xf* and *Xf*Δax21 needs to be done. We have also begun work on objective 2. We are in the process of looking at the differences in cell growth, biofilm formation and cell-cell aggregation of *XfΔPhoP* and *XfΔPhoQ*. We anticipate the combined data from objectives one and two will allow us to better understand the effects of Xa21 and the TCSs the mediate Ax21 activity in *Xf*. Next spring, pathogenicity assays on grapevines will allow us to assess the effects of both Ax21 and the PhoP/Q TCS on the virulence of *Xf*. We will begin work on objective 3 in the coming year.

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FUNDING AGENCIES

Funding for this project was provided by the USDA-funded University of California Pierce's Disease Research Grants Program.

Section 5: Crop Biology and Disease Epidemiology