CHARACTERIZATION OF *XYLELLA FASTIDIOSA* LIPOPOLYSACCHARIDE AND ITS ROLE IN KEY STEPS OF THE DISEASE CYCLE IN GRAPEVINE.

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Reporting Period: The results reported here are from work conducted July 2011 to October 2011.

ABSTRACT
This project aims to elucidate the molecular mechanisms that *Xylella fastidiosa* (*Xf*) uses in its interaction with host plants. We are focusing on the lipopolysaccharide (LPS) component of the outer membrane. LPS consists of lipid A, core oligosaccharides, and a variable O-antigen moiety. We are specifically investigating the role of O-antigen as it has been implicated as a virulence factor in several other Gram-negative bacterial species. We hypothesize that O-antigen is also involved in virulence of *Xf* on grapevine. Moreover, we are investigating the function of LPS in surface attachment and cell-cell aggregation, two important steps in biofilm formation, a trait necessary for successful colonization of host xylem. We are also determining the role that LPS plays in host specificity observed for this pathogen.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is a bacterium capable of colonizing many different plant hosts. It is the causal agent of Pierce's disease of grapevine, which has caused major losses to the California grape industry. *Xf* also causes disease in other economically important crops, such as almond, citrus, and oleander. While all identified *Xf* strains belong to the same species, some isolates can cause disease in one host, but not the other. For example, oleander isolates do not incite symptom development in grapevine and vice versa. One major goal of this project is to understand the bacterial characteristics that dictate host specificity. This research is particularly focused on elucidating the role of the *Xf* cell surface component, lipopolysaccharide (LPS), in the pathogenic interaction between the grapevine, almond, and oleander hosts. Because LPS plays an important role in several host-pathogen interactions, we are investigating the contribution of LPS in allowing *Xf* to colonize its host. LPS imparts traits that may contribute to pathogenesis such as the ability to attach to host cell walls. Should LPS prove to be important during *Xf* interaction with its host, its abundance on the bacterium's cell surface makes it a logical target for disease control. Antimicrobial compounds that disrupt or retard LPS biosynthesis exist which make bacteria more susceptible to other stresses. Potentially, these compounds could be used in combination with other anti-*Xf* compounds to control disease.

INTRODUCTION
Xylella fastidiosa (Xf) is a Gram-negative bacterium comprised of strains that cause disease on several economically important crops, such as grapevine, almond, and oleander. We are currently exploring the role of lipopolysaccharide (LPS) as both a virulence factor and host specificity determinant for this pathogen.

LPS is primarily displayed on the outer surface of Gram-negative bacteria, thereby mediating interactions between the bacterial cell wall and its environment. LPS is composed of three parts: 1) lipid A, 2) oligosaccharide core, and 3) O-antigen polysaccharide (**Figure 1**) (10). We are focusing on the O-antigen portion of LPS in three *Xf* isolates that colonize different hosts: Temecula1, a causal agent of Pierce's disease (PD); M12, a causal agent of almond leaf scorch (ALS); and Ann-1, the causal agent of oleander leaf scorch (OLS). Recognition, attachment, and biofilm formation are important stages in the interactions between *Xf*, the sharpshooter vector and its plant hosts. We aim to elucidate the role of LPS in the formation of biofilms, the development of PD on grapevine and in host specificity. Based on previously reported investigations of LPS in host-pathogen interactions (5, 9), we have identified two genes, *waaL* (PD0077) and *wzy* (PD0814), that are predicted to encode proteins required for a fully functional O-antigen moiety in *Xf*. Wzy is a polymerase that plays a role in chain length determination of the O-antigen, prior to the O-antigen ligation onto the oligosaccharide core component by WaaL (**Figures 1 and 2**).

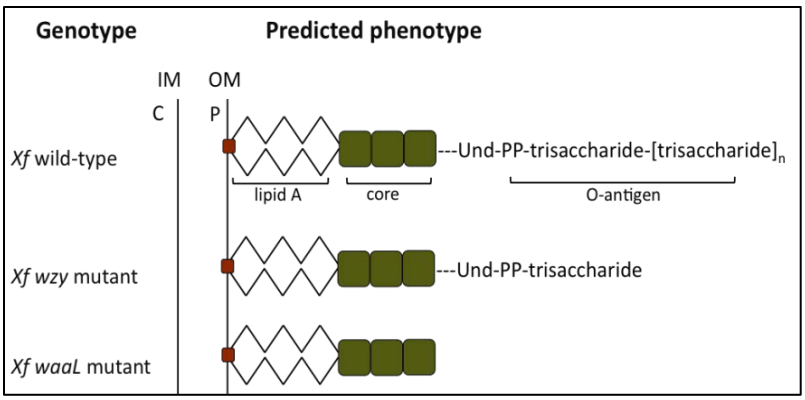

Figure 1. *Xf* strains lacking a functional Wzy (polymerase) are predicted to have truncated O-antigen with only the initiating polysaccharide unit. Strains lacking a functional WaaL (ligase) are predicted to lack the O-antigen. Und-PP = undecaprenylphosphate; IM = inner membrane; OM = outer membrane; C = cytoplasm; P = periplasm.

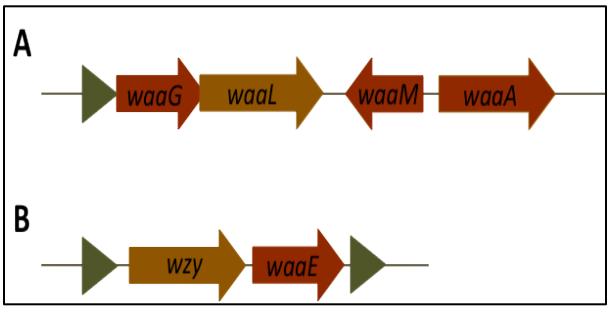

Figure 2. The genomic context of *waaL* **(A)** and *wzy* **(B)** in *Xf* provides evidence for prediction of genes that encode proteins involved in LPS biosynthesis. *waaG* = glycosyl transferase, *waaM* = lipid A biosynthesis lauroyl acyltransferase, *waaA* = 3-deoxy-D-octulosonic acid (KDO) transferase, *waaE* = beta 1,4 glycosyl transferase. Further, WaaL and Wzy are predicted to belong to the Wzy_C protein family of O-antigen ligases. Green triangles represent hypothetical proteins.

OBJECTIVES

1. To determine the contribution of *Xf* Temecula1 WaaL and Wzy in:
   a. LPS biosynthesis
   b surface attachment and biofilm formation
   c. virulence on grapevine
   d. host specificity
2. To determine LPS profiles of wild-type PD, ALS, and OLS strains.

RESULTS AND DISCUSSION

Objective 1a. To determine the contribution of *Xf* Temecula1 WaaL and Wzy in LPS biosynthesis

We identified two genes in the *Xf* LPS biosynthetic pathway, *waaL* and *wzy*, that encode proteins predicted to be important for production of a complete O-antigen component (see above). Mutations of these loci in *Xf* Temecula1 reveal a significant reduction in O-antigen in the *waaL* mutant strain and a lack of O-antigen in the *wzy* mutant strain. Both mutant phenotypes were restored by introducing *waaL* or *wzy* into their respective mutant genomic backgrounds using the chromosomal complementation vector, pAX1Cm (7) (**Figure 3**).

Objective 1b. To determine the role of O-antigen in biofilm formation

Biofilm formation is an important component of the plant-microbe interaction. To test the role of LPS in *Xf* biofilm formation, we are quantifying the ability of the Temecula1 *waaL* and *wzy* mutant strains in surface attachment and cell-cell aggregation. Interestingly, when grown in glass tubes, the Temecula1 *waaL* mutant attached to a greater extent than wild-type, which is contrary to attachment of this strain when grown in polypropylene or polystyrene where it attached less than wild-type (**Figure 4A**). Preliminary results suggest that *wzy* plays little role in *in vitro* surface attachment (**Figure 4B**). We

are currently testing the complemented strains in surface attachment to these same surfaces. As an extension of this work, we are investigating the role of WaaL and Wzy in attachment to biologically relevant surfaces such as chitin and cellulose. The ability of Temecula1 *waaL* mutant to aggregate was diminished in all tested materials, while the Temecula1 *wzy* mutant is reduced in aggregative capability only in polypropylene and polystyrene (**Figure 5**).

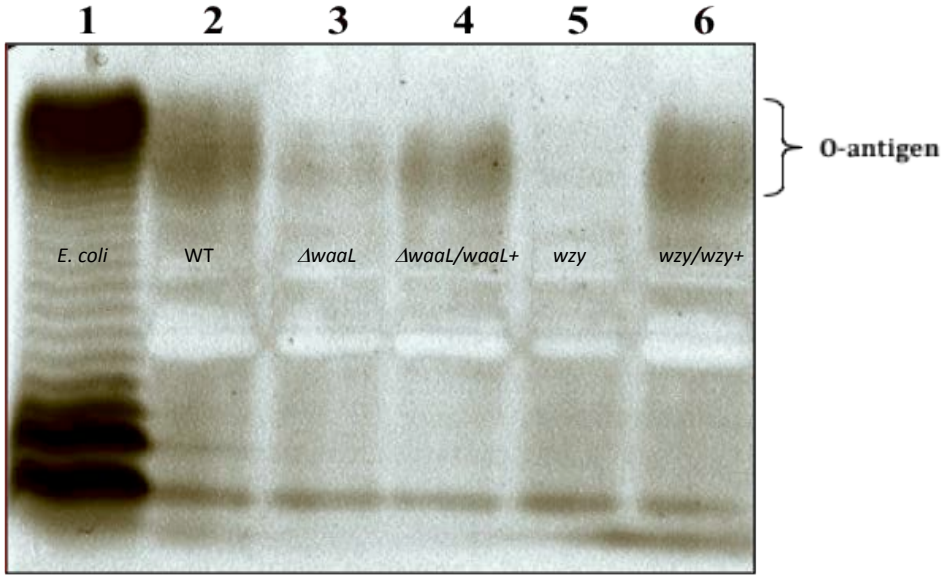

Figure 3. Mutations in key enzymes of the *Xf* LPS biosynthetic pathway result in reduction or abolishment of O-antigen. Temecula1 lacking a functional WaaL (lane 3) had less O-antigen than the wild-type (lane 2) while Temecula1 lacking Wzy (lane 5) appeared to have no distinguishable O-antigen moiety. Complementation of these mutants restored O-antigen quantity to near that of the wild-type parent (lanes 4 and 6). LPS was extracted from cells normalized to cell density using a modified hot phenol method (6). Samples were analyzed on a 4, 12% discontinous Tricine-PAGE gel and silver stained (8). (1) *E. coli* standard, (2) Temecula1 wild-type, (3) Temecula1*waaL*, (4) Temecula1*waaL*/*waaL*+, (5) Temecula1*wzy*, (6) Temecula1*wzy*/*wzy*+.

Objective 1c. To evaluate the contribution of O-antigen in development of Pierce's Disease

Temecula1 O-antigen mutant strains were inoculated into grapevine, cv. "Thompson Seedless' according to the method of Hill and Purcell (1995) (4). Plates were rated weekly on a disease index scale of 0-5 based on PD symptom development (3). PD symptoms were detected four weeks after inoculation, similar to other PD virulence studies conducted in Riverside County. There is no difference in disease progress between the Temecula1 *waaL* mutant and the wild-type strains. In contrast, the Temecula1 *wzy* mutant was delayed in causing PD symptoms on grapevine and, after 11 weeks of incubation, did not cause the extensive PD development observed in plants inoculated with wild-type (**Figure 6**). Plants inoculated with 1X PBS buffer control did not develop any PD symptoms.

At 39 days post-inoculation (5th week), a population study of grapevine petioles was performed in order to quantify any colonization differences between Temecula1 wild-type and O-antigen mutant strains that may be occurring *in planta*. Fifty-three percent of petioles tested from plants inoculated with Temecula1 wild-type were colonized, while 67% of petioles tested from plants inoculated with Temecula1*waaL* were colonized and preliminary statistical analysis indicates that Temecula1 *waaL* strain is colonizing to a slightly higher degree than the wild-type (**Table 1**). No bacteria were recovered from *wzy*-inoculated plants at this early time point. However, a second population study done 95 days post-inoculation (13th week) is currently underway to determine at what level the *wzy* and Δ*waaL* mutants colonize the plants at a later stage of infection. We hypothesize that because PD symptom development has progressed in *wzy*-inoculated plants, albeit at a much slower rate than WT-inoculated plants, the bacterial titer has increased since the first sampling time and we will be able to recover viable *wzy* cells during the second sampling. The colonization studies have been performed and we are awaiting the results of that experiment.

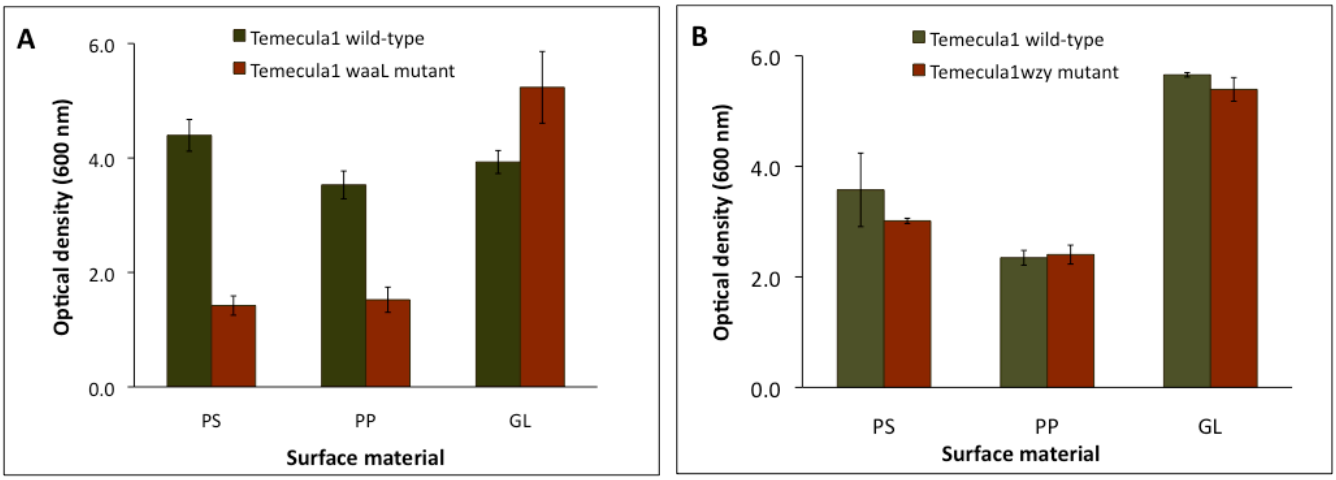

Figure 4. Preliminary results suggest that WaaL plays a role in the ability of *Xf* to attach to various surfaces. **A)** the Temecula1 strain lacking WaaL is reduced in attachment to polystyrene (PS) and polypropylene (PP), but increased in attachment to glass (GL) when compared to wild type. **B)** Wzy appears to play little role in surface attachment on any surface tested. Strains were grown in PD3 medium at 28 °C at 100 rpm for 7 days. Biofilms formed on the surface of the medium/air interface were stained with 1% crystal violet and collected in 95% ethanol (2). Bars represent standard error.

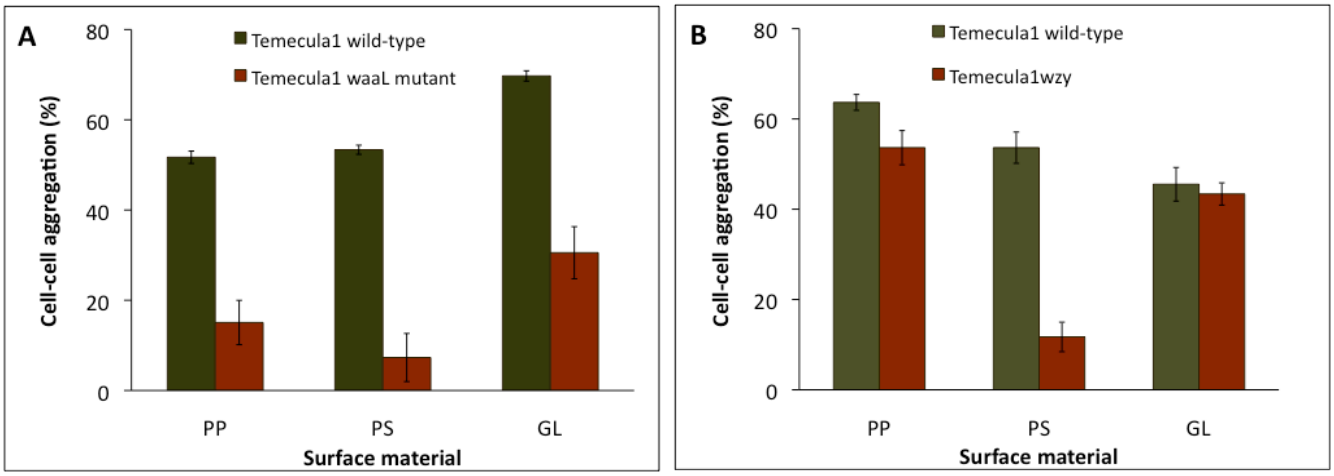

Figure 5. O-antigen plays a role in the ability of *Xf* cells to aggregate. **A)** the Temecula1*waaL* mutant cells are reduced in the ability to adhere to one another when grown in polypropylene (PP), polystyrene (PS), and glass (GL). **B)** the Temecula1*wzy* mutant cells are reduced in cell-cell aggregation in PP and PS, but not GL. Strains were grown in PD3 medium at 28 °C without agitation for 10 days. Percent aggregation is the ratio of unsettled (ie, aggregating) cells in the upper portion of the test tube to the total number of cells (3). Bars represent standard error.

Objective 1d. To investigate the role of O-antigen in host specificity

While there are likely several factors that contribute to host specificity of *Xf*, we would like to investigate if O-antigen is involved. *Xf* Temecula1 can colonize and cause disease in grape and, to some extent, in almond. *Xf* M12, an ALS strain, causes disease in almond and elicits some symptoms in grape (1). Moreover, *Xf* Ann-1 cannot cause disease in grape or almond and neither Temecula 1 nor M12 can cause disease in oleander. We speculated that removal or truncation of the O-antigen would affect the ability of Temecula1 to infect (and elicit scorch symptoms) on grape, almond, and oleander. Further, perhaps changes in O-antigen will allow Ann-1 to become a pathogen of grape or almond while M12 and Temecula1 strains become pathogens of oleander.

We currently have *Xf* Temecula1 and the Temecula O-antigen mutant strains inoculated into almond and oleander. Preliminary results demonstrate that the Temecula1*waaL* mutant strain is more virulent in almond than its Temecula1 wild type parent and is similarly virulent to the ALS wild-type strain M12. This suggests that alterations in the O-antigen moiety of the LPS molecule do affect host specificity. We are currently awaiting the results from host specificity studies conducted in oleander. These plants take longer (approx. 10 months) for OLS symptoms to develop. We are also constructing the corresponding *waaL* and *wzy* mutants in the M12 and Ann-1 strains of *Xf*. Loss of Wzy function (Temecula1 *wzy* mutant)

does not seem to alter the host specificity of the Temecula1 strain. As expected, *Xf* Ann-1, the OLS strain, does not cause scorch symptoms on almond (**Figure 7**).

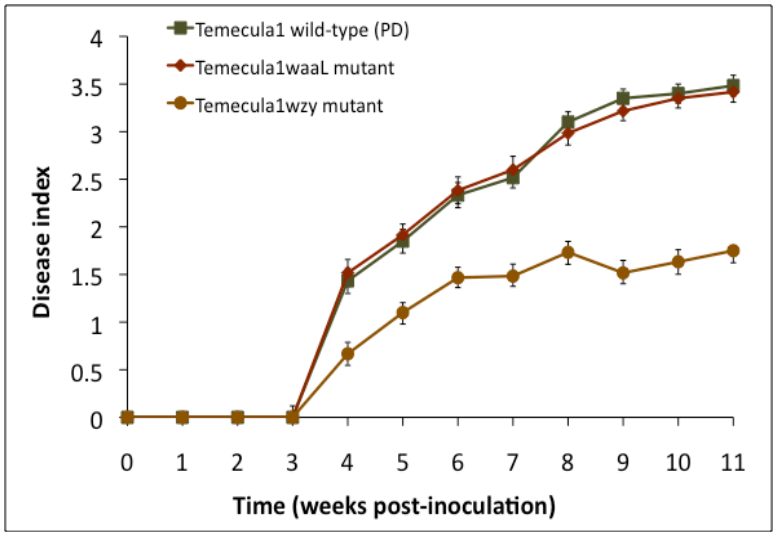

Figure 6. Disease progress of *Xf* Temecula1 wild-type and O-antigen mutant strains on grapevine cv. 'Thompson Seedless'. The *wzy* mutant strain lags behind the wild-type in causing scorching symptoms and does not cause wild-type levels of PD. The *waaL* mutant appears to be as virulent as wild-type. Data are means of three independent assays with 10 replications each. Bars represent standard error.

Table 1. Mean populations in tissue of colonized grapevine at 39 days post-inoculation.

Strain	**Xylem population (per g tissue)[a]**
Temecula1 wild-type	5.09 (± .21)
Temecula1*waaL*	5.69 (± .16)
Temecula1*wzy*	Not detected[b]

[a] mean population values are log transformed; parenthetical values represent standard error
[b] populations below detection threshold (<500 CFU)

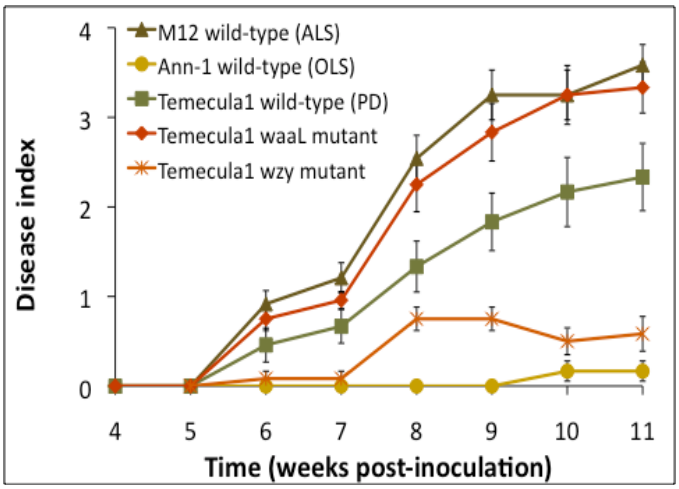

Figure 7. Disease progress of ALS strain M12, OLS strain Ann-1, PD strain Temecula1, and Temecula1 O-antigen mutants on almond cv. 'Sonora.' Loss of WaaL may render a PD strain as virulent on almond as the ALS wild-type strain M12. Data are means of two independent assays with 6 replications each. Bars represent standard error.

Objective 2. LPS profile of *Xf* Temecula1 (grape), M12 (almond), and Ann-1 (oleander)

Variation in LPS can be classified as "smooth" (those with O-antigen) and "rough" (those without O-antigen). Bacteria can possess both variants simultaneously which can be readily visualized by electrophoretic analysis. The three isolates used in this study were grown on PD3 (for Temecula1 and Ann-1 strains) or PW (for M12 strain) solid medium. Cells were harvested and normalized to cell density. LPS was extracted as described above. There are slight differences among the strains with regard to the high MW O-antigen and it appears that the O-antigen population of the Ann-1 strain may be comprised of trisaccharide repeats of slightly greater length (**Figure 8**). We are further characterizing the LPS molecule for all three strains using more sensitive biochemical techniques to identify the carbohydrate composition and linkage of the sugars in the *Xf* LPS molecule.

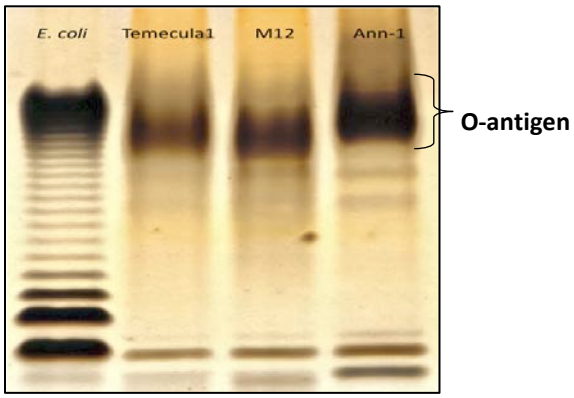

Figure 8. There are minor differences between *Xf* wild-type strains used in this study (Temecula 1, M12 and Ann-1). These differences are primarily in the polysaccharide length that comprises the majority of the O-antigen. LPS was extracted from cells normalized to cell density, run on a discontinuous 14% Tricine-SDS PAGE gel, and silver stained. LPS extracted from *Escherichia coli* (Sigma) was used as the standard.

CONCLUSIONS

The main focus of this project is to further understand the molecular mechanisms governing *Xf* virulence. We are working toward understanding the role of the O-antigen component of LPS in contributing toward virulence and those behaviors associated with xylem colonization, such as cell wall attachment and cell-cell aggregation required for biofilm formation. The broad host range, but stringent host specificity, of *Xf* provides an opportunity to study the molecular mechanisms underlying the essential traits that lead to host specificity observed for this pathogen. There are likely numerous bacterial traits that contribute to host specificity of *Xf*, and we hypothesize that LPS plays a role. Because of its abundance in the outer membrane, LPS may provide a target for disease control, as it appears to be implicated as an important factor in disease development.

REFERENCES CITED

1. Almeida, R. P. P., and A. H. Purcell. 2003. Biological traits of *Xylella fastidiosa* strains from grapes and almonds. Appl Environ Microbiol 69: 7447-7452.
2. Espinosa-Urgel, M., A. Salido, and J.-L. Ramos. 2000. Genetic analysis of fnctions involved in adhesion of *Pseudomonas putida* to seeds. J Bacteriol 182:2363-2369.
3. Guilhabert, M. R., and B. C. Kirkpatrick. 2005. Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. Mol Plant-Microbe Interact 18:856-868.
4. Hill, B. L., and A. H. Purcell. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. Phytopathology 85:209-212.
5. Kao, C. C., and L. Sequeira. 1991. A gene cluster required for coordinated biosynthesis of lipopolysaccharide and extracellular polysaccharide also affects virulence of *Pseudomonas solanacearum*. J Bacteriol 173:7841-7847.
6. Marolda, C. L., P. Lahiry, E. Vinés, S. Salidías, and M. A. Valvano. 2006. Micromethods for the characterization of lipid A-core and O-antigen lipopolysaccharide. *In* I. Brockhausen (ed.), Methods in Molecular Biology, vol. 347. Humana Press, Inc., Totowa, NJ.
7. Matsumoto, A., G. M. Young, and M. M. Igo. 2009. Chromosome-based complementation system for *Xylella fastidiosa*. Appl Environ Microbiol 75:1679-1687.
8. Schägger, H. 2006. Tricine-SDS-PAGE. Nature Prot 1:16-22.
9. Whitfield, C. 1995. Biosynthesis of lipopolysaccharide O antigens. Trends Microbiol 3:178-185.

FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

ACKNOWLEDGEMENTS
We thank Dr. Bruce Kirkpatrick (UCD) and Dr. Donald Cooksey (UCR) for providing us with the M12 and Ann-1 isolates. We also thank Dr. Michelle Igo (UCD) for providing us with the pAXCm1 vector.