THE ROLE OF LIPOPOLYSACCHARIDES IN VIRULENCE, BIOFILM FORMATION, AND HOST SPECIFICITY OF *XYLELLA FASTIDIOSA*

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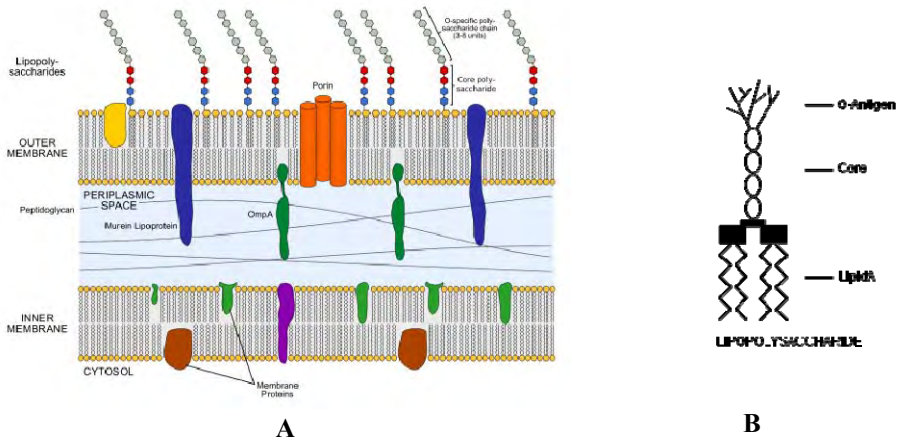
ABSTRACT
This project focuses on the lipopolysaccharide (LPS) component of the outer membrane of *Xylella fastidiosa (Xf)*. In particular, we are investigating if the O-antigen portion of this molecule contributes to *Xf* surface attachment and biofilm formation. More importantly, by targeting genes involved in O-antigen biosynthesis, we will determine if LPS is an important virulence factor for *Xf* infection of grape. Additionally, we will determine if LPS contributes to the high level of host specificity observed for this pathogen.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is a bacterium that has the ability to infect many different plant species. In some plants, this bacterium can cause serious disease. In grapevine, this disease is known as Pierce's disease (PD) and has caused millions of dollars of damage to the California grape industry alone. *Xf* species also infect other economically important crops such as almond, oleander and citrus. Interestingly, while all *Xf* isolates belong to the same group or species, some isolates can cause disease in one host but not another. For example, oleander strains cannot cause disease in grapevine and vice versa. One major goal of this project is to understand the bacterial mechanisms that dictate this high level of host specificity. We are focusing on a key component of the bacterial cell membrane, called lipopolysaccharide (LPS), and how certain parts of this molecule may be important in dictating host specificity. We are also investigating how the LPS molecule is related to bacterial virulence and other key aspects of the disease process, like attachment to the plant cell wall. This molecule makes up more than 70% of the bacterial membrane and if LPS does prove to be an important factor during *Xf* plant infection, its abundance in the bacterial cell membrane makes it a logical target for disease control. Furthermore, antimicrobial compounds that weaken the LPS molecule generally make the bacterium more sensitive to other stresses. Therefore, compounds targeted towards LPS synthesis could increase the efficacy of other anti-*Xylella* compounds currently being developed when both are used in conjunction.

INTRODUCTION
Xylella fastidiosa (Xf) is a gram negative, xylem-limited bacteria with a broad host range encompassing at least 28 families of monocots and dicots, and causes disease in hosts such as grape, almond, peach, plum, alfalfa, elm, sycamore, coffee, oleander, maple, and citrus (Hopkins and Purcell, 2002). The molecular mechanisms that determine this host specificity are poorly understood. This project aims to explore the role of lipopolysaccharides (LPS) as both a virulence factor and host specificity determinant of *Xf*. We will focus on the O-antigen portion of the LPS molecule in three strains of *Xf,* the PD strain (Temecula 1), the almond leaf scorch strain (Dixon-ALS), and oleander leaf scorch strain (Ann-1). These three strains were chosen because either full or draft sequences of the genome are available. This will allow us to easily identify genes that are putatively involved in O-antigen biosynthesis and to construct targeted deletion knockouts in these genes. Additionally, these three strains infect different hosts, grape, almond and oleander. Interestingly, while grape and almond isolates are considered to be separate subspecies or pathovars, grape strains can cross colonize and cause both PD and ALS symptoms. Whereas almond strains do not cause PD symptoms in grape but can sometimes colonize grape, albeit at low titer (Almeida and Purcell, 2003). Furthermore, the oleander strain cannot infect grape or almond and both the almond and grape strains cannot infect oleander (Almeida and Purcell, 2003). This provides an opportunity to study the role of the O-antigen moiety of the LPS molecule as a potential host specificity determinant for *Xf*.

LPS comprises approximately 70% of the outer membrane of gram-negative bacteria and is therefore essential for growth and viability (**Figure 1A**). Because LPS is what is largely displayed on the cell surface it mediates interactions between the bacterial cell and its surrounding environment. LPS (sometimes called "endotoxin") has been implicated as a major virulence factor in both plant and animal pathogens, such as *Escherichia coli*, *Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* just to name a few (Muhldhorfer and Hacker 1994, Dow *et al*. 1995; Hendrick *et al*. 1984). Because of its location in the outer membrane, LPS can also contribute to the initial adhesion of the bacterial cell to a surface or host cell (Genevaux *et al*. 1999, Nesper *et al*. 2001). Additionally, host perception of LPS is well documented and occurs in both plants and animals (Newman *et al.* 2000). The immune system can recognize several regions of the LPS structure and can mount a defense response in response to bacterial invasion based on this recognition. Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule or by masking it with capsular or exopolysaccharides.



A **B**

Figure 1. **A**. Schematic of a gram-negative bacterial cell wall indicating the location of LPS in the outer membrane. **B**. Schematic of a single LPS molecule composed of lipid A, core oligosaccharide and O-

LPS is composed of three parts: 1) lipid A, 2) core oligosaccharide and 3) O-antigen polysaccharide (**Figure 1 B**). Lipid A is anchored in the membrane and core oligosaccharides are assembled on the preformed lipid A molecule. O-antigen is assembled elsewhere and ligated onto the core oligosaccharide-lipid A complex. Both lipid A and core oligosaccharide are relatively conserved among bacterial species. O-antigen is highly variable even amongst strains of the same species. O-antigen is the immunodominant portion of the LPS molecule and contributes to serotype designation of different strains within the same species. O-antigen is not required for bacterial viability but is often implicated in virulence and host specificity. Even small changes in the type and order of the sugars comprising the O-antigen can result in major changes in virulence.

The lifestyle of *Xf* requires attachment to diverse carbohydrate substrates such as the plant xylem wall and chitin in the mouthparts and foregut of the sharpshooter insect vector. In both environments, *Xf* forms biofilms or biofilm-like structures. Previous studies show that *Xf* produces an extracellular exopolysaccharide (EPS) that is present in minute quantities during initial surface attachment and early biofilm formation. However, in mature biofilms this EPS (termed fastidian gum) is a major component of the three-dimensional *Xf* biofilm both *in vitro* and *in planta* (Roper *et al*. 2008). Other studies have demonstrated that proteinacous adhesins such as type I pili and hemagluttinins contribute to surface adhesion and cell-cell aggregation (Li et al. 2007, Guilhabert and Kirkpatrick, 2005). Because of the location and abundance of LPS in the outer membrane we hypothesize LPS also plays a key role in mediating initial attachment to the carbohydrate substrates *Xf* encounters in the plant and insect.

In this project, we will construct mutants in the O-antigen biosynthesis pathway that will either be completely devoid of O-antigen or produce truncated versions of O-antigen. We will assay these mutants for virulence in their respective host plants as well as their ability to infect non-hosts. Furthermore, because of its abundance in the outer membrane (70%) of *Xf*, LPS is a logical target for developing novel therapeutics against *Xf* for control of PD. In fact, a bacterium with defective LPS is often more susceptible to oxidative stress, antimicrobial peptides, and other stresses bacteria encounter *in planta*. Therefore, treating PD infected grapevines with antimicrobial compounds designed to inhibit or truncate LPS synthesis may be one way to control PD.

OBJECTIVES

1. a. Characterization and comparison of the LPS profiles from the grape, almond and oleander strains of *Xf* grown in PW broth and PW solid media
   b. Investigate the possibility of phase variation in *Xf* LPS
2. Construct *Xf* mutants in O-antigen biosynthetic genes
3. Test virulence and host specificity of the O-antigen mutants *in planta*
4. Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants

PRELIMINARY RESULTS AND FUTURE STUDIES

Objective 1: Characterize the LPS profiles from the grape, almond and oleander strains of *Xf*.

A. Comparison of the LPS profiles from *Xf* grown *in vitro*:

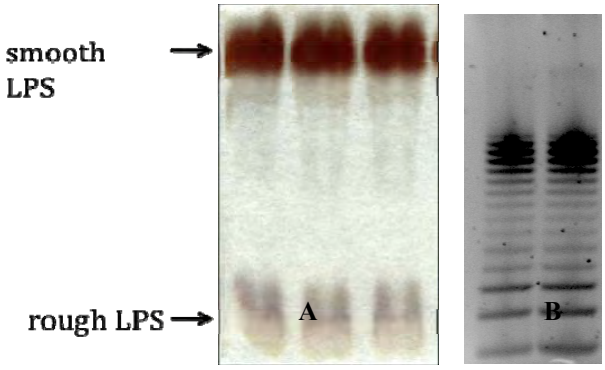


Figure 3. A. Sodium deoxycholate PAGE gel of *Xf* LPS grown on PW medium indicating the presence of both smooth (O-antigen) and rough (core) LPS. All lanes contain LPS from the grape strain of *Xf*. **B.** Tricine-SDS PAGE gel of *P. stewartii* LPS grown on nutrient indicating the presence of both smooth (O-antigen) and rough (core) LPS. All lanes contain LPS from wild type *P. stewartii.* **Note the enhanced resolving power of the Tricine-SDS PAGE gel compared to the Sodium deoxycholate gel.**

A bacterium with only lipid A and core oligosaccharide is said to have "rough" LPS and one with lipid A, core oligosaccharide and O-antigen is said to have "smooth" LPS. There can be both smooth and rough variants of the same bacterial species. Preliminary data indicate that the LPS extracted from *Xf* Temecula 1 grown on solid medium has both rough (core LPS) and smooth (O-antigen) components of the LPS molecule (**Figure 3A**). In this study we will compare the LPS profiles of *Xf* Temecula 1, *Xf* Ann1 and *Xf* Dixon by Sodium deoxycholate PAGE electrophoresis to determine if all three strains have smooth and rough forms of LPS and if there are any variations in the bandings patterns in the O-antigen fraction. Furthermore, we will analyze the *Xf* LPS on Tricine-SDS PAGE gels to provide better resolution of the individual LPS bands. **Figure 3B** is a Tricine SDS PAGE gel of LPS extracted from *Pantoea stewartii* subsp. *stewartii*, another xylem dwelling phytopathogen. This figure is included merely to demonstrate that this technique is capable of resolving individual bands in both the core and O-antigen portions of the LPS molecule. By using this technique we will be able to detect even subtle differences in the LPS banding patterns of the three strains of *Xf* as well as the mutants we will construct in Objective 2.

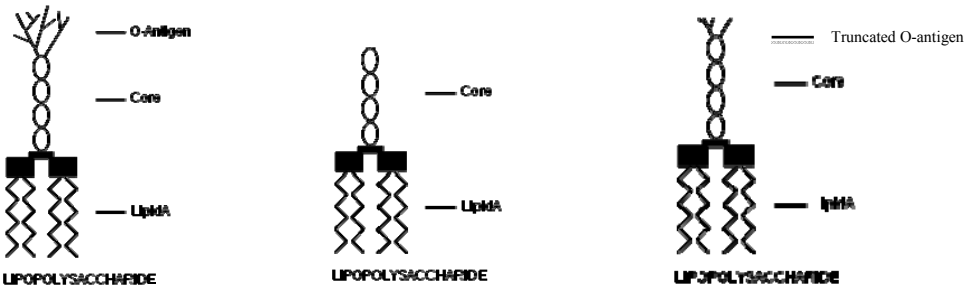
B. Comparison of the *Xf* Temecula 1 (grape strain) LPS profile in PW medium vs. grapevine xylem sap. The outer membrane of a bacterial cell is strongly influenced by its surrounding environment. Gram-negative bacterial pathosystems can undergo a process called phase variation, which is defined as a reversible change in the antigenic determinants in response to environmental conditions. It is well documented that this phenomenon occurs in the extracellular and membrane bound polysaccharide portion of the bacterial cell surface (Bergman *et al*. 2006; Lerouge *et al*. 2002). This includes exopolysaccharide, capsular polysaccharide and LPS. Specifically in the LPS fraction, bacteria can display different O-antigen profiles by varying the degree of polymerization or by completely abolishing synthesis of the O-antigen. The cells can alternate from smooth to rough or semi-rough (truncated O-antigen) LPS variants depending on the developmental phase of the cell. The structure of the O-antigen expressed can be a key factor in how the bacterium interacts with its host or vector. A bacterium may display different O-antigen sugars on its surface that essentially changes its external appearance to the host (Bergman et al. 2006, Guerry *et al*. 2002). This putative masking effect may be important when *Xf* cells are initially introduced into the plant by the sharpshooter vector and presumably not covered or protected in large amounts of EPS.

The aim of this objective is to determine if *Xf* LPS undergoes phase variation in different growth conditions. We will assay this by growing the *Xf* Temecula 1 grape strain of *Xf* in PW broth vs. PW broth amended with increasing concentrations of grapevine xylem sap vs. pure xylem sap.

Objective 2: Construct *Xf* mutants with altered LPS profiles. The goal of this objective is to construct *Xf* mutants that either 1) completely lack O-antigen ("rough" LPS mutants) or 2) produce truncated forms of O-antigen ("semi-rough" mutants). Genomic analyses reveal approximately 30 *Xf* genes involved in LPS biosynthesis. In this objective we will focus on two of these genes that are involved in the biosynthesis of O-antigen as well as the attachment of O-antigen to core LPS. While the mechanism of assembly is likely the same for O-antigen assembly and ligation for all *Xf* strains, the composition of the O-antigen could be markedly different depending what sugars are incorporated into the O-antigen chain.

The first gene of interest is a *waaL* ortholog, designated PD0077 in the genome of the Temecula 1 grape strain. All three strains (oleander, grape and almond) have a single copy of this gene. We will construct deletion mutants in this gene in all three strains by site-directed mutagenesis. In other bacterial systems, mutations in *waaL* prevent the ligation of O-antigen (Perez *et al*. 2008). Therefore, a *Xf* mutant in *waaL* would produce rough LPS composed only of lipid A + core and be completely devoid of O-antigen as depicted in **Figure 4B**. The LPS phenotype of the *waaL* mutants will be confirmed by Tricine-SDS PAGE gel electrophoresis described in Objective 1. As documented in other bacterial species, we expect *Xf* Δ*waaL* mutants to be decreased in virulence if the O-antigen expressed *in planta* is indeed providing some kind of masking effect from the host defense responses (Berry *et al*. 2009; Carroll *et al*. 2004; Moran 2008). However, a mutation in *waaL*

could very well have the opposite effect. These mutants could potentially be hypervirulent if the host has evolved to recognize *Xf* O-antigen and absence of the molecule leaves the plant partially blind to *Xf* invasion.



A. wild type *Xf* LPS **B**. putative *Xf* Δ*waaL* LPS **C**. putative *Xf* Δ*wzy* LPS

Figure 4. A. Model of wild type *Xf* LPS molecule containing all three components: lipid A, core polysaccharide, and O-antigen **B**. Model of hypothetical Δ*waaL Xf* LPS molecule containing no O-antigen. **C.** Model of hypothetical Δ*wzy Xf* LPS molecule with truncated O-antigen. Images adapted from http://www.wikipedia.org/

The second gene we will target is the *wzy* ortholog, designated PD0814 in the Temecula 1 grape genome. This gene is also present in single copy in all three strains. Wzy is an O-antigen polymerase responsible for the assembly of O-antigen prior to its ligation to the core. Wzy acts in concert with Wzz, a chain length regulator or "molecular ruler" to polymerize LPS O-antigen subunits to a certain chain length. We predict that *Xf* mutants in *wzy* will produce an O-antigen that look likes Figure 4C.

*All mutants constructed in this study will be complemented with the wild type copy of the gene. We will use the complementation vectors now available for *Xf* or introduce a wild type copy of the gene into a neutral part of the chromosome as reported by the Igo lab at UC-Davis.

Objective 3: **Test *Xf* O-antigen mutants for virulence and host specificity**. For all experiments described below, we will mechanically inoculate plants using the pin-prick method originally described by Hill and Purcell 1995. All plants will be rated on a disease scale of 0-5 with 0 being healthy and 5 being dead (Guilhabert and Kirkpatrick, 2005). First, for the virulence assays, each of the LPS mutants we constructed for each strain will be tested for virulence in their respective susceptible host plants. If a particular mutant is no longer pathogenic or hypervirulent, we will quantify *Xf* populations/gram of plant tissue by performing isolations on petioles harvested nearest the point of inoculation. The results of these experiments will indicate if the O-antigen portion of the LPS molecule is indeed a virulence factor for *Xf*. Secondly, for the host specificity assays, we will inoculate all strains into "non-host" plants and determine if an alteration in O-antigen correlates with a shift in host range or specificity. It is well documented that different pathovars or serotype groups display different O-antigens on their surface (Benedict et al., 1990; Lerouge et al. 2002). It is assumed that presence/absence and carbohydrate composition of the O-antigen correlates with the ability of a particular bacterial strain to infect one host but not another. However, there are very few studies actually documenting this. In one study, a spontaneous mutant of *Xanthomonas campestris* pv. *citrumelo* was altered in core LPS and lost O-antigen displayed an altered host range (Kingsley et al. 1993). Normally, this pathogen has a wide host range that includes citrus and bean. The spontaneous mutant lost the ability to infect citrus but retained its ability to infect bean. The authors attributed this difference to the change in the LPS profile although other polysaccharides were affected. It will be interesting to see if by removing O-antigen or altering its chain length if the Temecula 1 strain will retain its ability to infect both grape and almond and if Dixon-ALS can gain the ability to cause disease in grape. Furthermore, can these changes in O-antigen allow the oleander *Xf* Ann1 strain to become a pathogen of grape or almond and the *Xf* Dixon and *Xf* Temecula1 strains become pathogens of oleander?

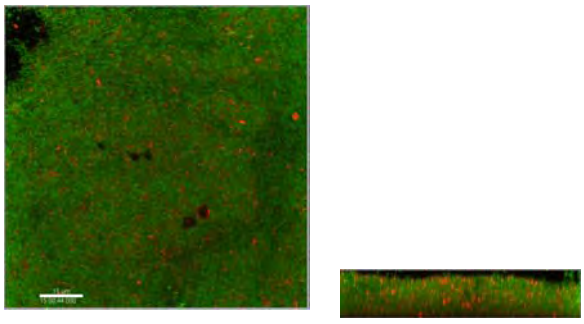
For these experiments, *Xf* Temecula 1 grape strain mutants will be inoculated into almond and oleander. The *Xf* Ann1 oleander strain mutants will be inoculated into grape and almond and the *Xf* Dixon almond strain mutants will be inoculated into grape and oleander. This is outlined in **Table 1**. All mutants and wild type will also be inoculated in their respective host plants in order to compare symptoms; *WT=wild type

Objective 4: **Test surface attachment and biofilm formation of *Xf* O-antigen mutants.** *Xf* is known to attach to glass surfaces and form a biofilm at the air/liquid interface when grown in liquid culture (**Figure 5**). In this objective we will further characterize the *Xf* Temecula 1 Δ*waaL* and Δ*wzy* mutants by assaying 2 different behaviors: surface attachment and biofilm formation. In order to quantify surface attachment, *Xf* wild type Temecula 1, Δ*waaL* and Δ*wzy* mutants will be grown in PD3 medium in 10 ml borosilicate glass tubes and attachment on the surface walls of the tubes will be assessed by a

crystal violet staining method (Espinosa-Urgel *et al*. 2000). We will assess the capability of the *Xf* Temecula 1 Δ*waaL* and Δ*wzy* O-antigen mutants to form biofilms on glass surfaces. Biofilms will be imaged using a confocal laser scanning microscope available in the UCR core microcopy facility.

Table 1. Inoculations for *Xf* host specificity tests. *Xf* Temecula 1 grape strain mutants will be inoculated into almond and oleander. The *Xf* Ann1 oleander strain mutants will be inoculated into grape and almond and the *Xf* Dixon almond strain mutants will be inoculated into grape and oleander. All mutants and wild type (WT) will also be inoculated in their respective host plants in order to compare symptoms.

	Host Inoculations		
***Xf* mutant**	**Grape**	**Almond**	**Oleander**
WT Temecula 1		x	x
Δ*waaL* Temecula 1		x	x
Δ*wzy* Temecula 1		x	x
WT Dixon	x		x
Δ*waaL* Dixon	x		x
Δwzy Dixon	x		x
WT Ann1	x	x	
Δ*waaL* Ann1	x	x	
Δ*wzy* Ann1	x	x	



A B

Figure 5. Confocal laser scanning microscope images of *Xf* biofilms formed at the air/liquid interface after 10 days growth in PD3 medium. *Xf* cells are depicted in green and *Xf* EPS is depicted in red. **A**. Overhead view of the *Xf* biofilm. **B**. Sagittal view of the *Xf* biofilm.

CONCLUSIONS

This project aims to further elucidate the molecular mechanisms of *Xf* virulence. At the same time, we will investigate the possibility of O-antigen as a host specificity determinant for this pathogen. While there are likely several factors that contribute to host specificity of *Xf,* we are investigating if O-antigen presence and composition is involved. We feel that the wide host range and stringent host specificity of different *Xf* strains affords a unique opportunity to study the molecular mechanisms underlying the host specificity observed for this pathogen.

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