I. Project Title:

The Role of Type V Secretion Autotransporters in the Virulence of *Xylella fastidiosa*

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III. Objectives of the Proposed Research:

1) Identify the proteins/molecules that interact with the two adhesin autotransporters.

2) Identify proteins and virulence factors requiring one of the three serine protease autotransporters for their maturation.

3) Identify peptides/small molecules that interfere with the function of the *Xf-PD* autotransporters.

4) Examine the feasibility of exploiting the unique properties of the autotransporters to develop strategies for controlling PD.

Overview: An important feature of the *Xf* infectious cycle is the ability of this pathogen to colonize and interact with the xylem tissue of susceptible plants and with the foregut of insect vectors (Chatterjee et al., 2008). Successful establishment of the infection is dependent on the ability of *Xf* to acquire essential nutrients, to adhere to the host cell surfaces, and to escape any host defense mechanisms. Comparison of the *Xf-PD* genome to other bacterial pathogens has resulted in the identification and characterization of a number of genes that are potential virulence factors. Many of these virulence determinants are proteins that are either secreted to the bacterial cell surface or released into the external environment (Dautin and Bernstein, 2007; Henderson et al., 2004). Our work has focused on one category of virulence determinants, AT-1 autotransporters. AT-1 systems are dedicated to the secretion of a single specific polypeptide, the passenger domain, across the outer membrane. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD.

During the period under review, we have focused on three aspects of this project:

1. Complementation analysis:
   Mutations in genes encoding major outer membrane proteins frequently have pleiotropic effects on membrane permeability and outer membrane protein composition. Therefore, complementation analysis is particularly important for this category of mutations. The presence of multiple restriction systems in *Xf* has made the construction of these strains extremely challenging. To overcome these technical difficulties, we have generated an *Escherichia coli* strain (EAM1), which expresses the Type II methylase encoded by PD1608. When introduced into *Xf* Temecula 1, plasmids isolated from EAM1 are not restricted by the Type II restriction enzyme encoded by PD1607. A description of EAM1 and other strategies designed to overcome restriction by this Type II system are described in a manuscript that we are in the process of revising for Applied and Environmental Microbiology. We are now using these strategies to generate the necessary complementation stains for studying the function of the AT-1 autotransporters.

2. The Autotransporter encoded by XapA (PD0528):
   A major focus during the period under review has been to complete our analysis of the autotransporter encoded by the PD0528 locus. We intend to call this protein XapA for *Xylella* autotransporter protein. We have completed both grapevine experiments and experiments in *E. coli* that have provided insights into the mechanism responsible for XapA secretion. Sherry Huston, a new cooperator on this project, is in the process of repeating our microscopy studies, which show that XapA is localized to the cell surface. Finally, we have just received the results of transmission studies from Nabil Killiny and Rodrigo Almeida at UC Berkeley. Their initial studies indicate that the XapA mutant was transmitted at lower rates than wild type (48% versus 88%). Dr. Ayumi Matsumoto, a former post-doctoral fellow in the laboratory, is currently working on the manuscript describing XapA, which we intend to submit to the Journal of Bacteriology this spring.

3. The Serine Protease Autotransporters encoded by PD0218, PD0313, and PD0950:
   We have initiated experiments to characterize PD0218, PD0313, and PD0950, the three autotransporters predicted to have proteolytic activity. Strains carrying a mutation in either PD0218 or PD0950 exhibit a hypervirulent phenotype in grapevines. In contrast, grapevines infected with a strain missing PD0313 or all three proteases exhibit fewer symptoms than vines infected by wild-type *Xf*. In addition to the grapevine studies, we have also compared the outer membrane protein composition and the secretome of strains carrying a mutation in one of the three proteases. Comparisons of the secreted proteins from these mutants suggest that each protease has a different set of target proteins. Experiments are currently underway to identify the protein targets of the individual proteases and the virulence factors that require these proteases for their maturation. The ultimate goal is to develop methods for interfering with this maturation, thereby reducing the virulence of this important plant pathogen.

Results for each objective:

During the period under review, our research has primarily focused on Objectives 1 and 2. The results from these studies are summarized below. We anticipate initiating studies addressing Objectives 3 and 4 in the next review period.

Objective 1: Identify the proteins/molecules that interact with the two adhesin autotransporters**.**

Two of the most interesting *Xf-PD* autotransporters are PD0528 (XapA) and PD1379 (XapB). The passenger domains of these proteins encode tandem repeats of a 50-60 amino acid motif; XapA has six repeats, whereas XapB has three repeats. In addition, both passenger domains contain WD40 repeats. WD40 repeats are predicted to create a specific structure, a β propeller-like platform (Hudson and Cooley, 2008). In other systems, binding partners associate with this platform either stably or reversibly. Usually, the binding partner is a protein that recognizes a specific consensus binding motif within the β-propeller. Therefore, the presence of WD40 repeats makes it highly likely that the passenger domains of XapA and XapB are each interacting with a specific protein or set of proteins. Interestingly, orthologs of XapA have recently been detected in the soil bacterium, *Pseudomonas fluorescens* Pf-5 (4.0 e^{-52}) and in two bacteria isolated as part of the Human Microbiome Project, *Neisseria flavescens* SK114 (2.0 e^{-119}) and *Providencia stuartii* ATCC 25827 (5.0 $^{e-61}$). The function of these orthologs is unknown.

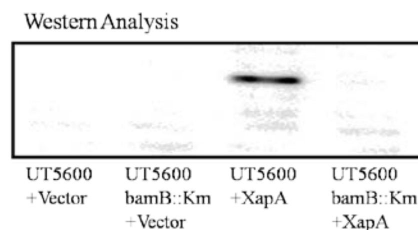
Interaction of XapA with the BAM complex:
To study the interaction of the adhesin autotransporters with other proteins, we have expressed both XapA and XapB on the surface of the *E. coli* strain UT5600. UT5600, which is deficient in the outer membrane proteases OmpT and OmpP, is commonly used for autodisplay (also known as live-cell surface display) (Jose and Meyer, 2007). Although the expression levels were low, both proteins were localized to the *E. coli* outer membrane and conferred the ability to autoaggregate and to form a biofilm.

The ability of XapA and XapB to confer new phenotypic properties to *E. coli* indicates that these proteins are directly responsible for the observed traits. Moreover, the fact that these proteins are functional and present in the *E. coli* outer membrane has allowed us to perform preliminary tests concerning how these autotransporters are secreted. Genetic analysis in *E. coli* has established that the secretion and correct folding of most outer membrane proteins occurs through the BAM (β-barrel assembly machine) complex, which is composed of five proteins BamA-BamE (Knowles et al., 2009). There are orthologs to four of these proteins in *Xf*: PD0326 (BamA), PD1620 (BamB), PD1756 (BamD), and PD1375 (BamE).

Our studies using the *E. coli* system suggest that the translocation and correct insertion of autotransporters into the *Xf* outer membrane is mediated by the *Xf* BAM complex. As shown in Figure 1, the passenger domain of XapA, which is exposed on the surface of wild-type *E. coli* strains, is not present in the outer membrane of strains carrying a mutation that disrupts the BAM complex (*bamB*::Km^R).

Figure 1: XadA is not present in the *E. coli* outer membrane in a BAM complex mutant.

Examination of *E. coli* UT5600 outer membrane proteins by Western blot analysis using an antibody to the XadA passenger domain.



The *bam*::Km^R mutation also interferes with the autoaggregation phenotype conferred to *E. coli* by the XapA protein (Figure 2). A simple explanation for this result is that the XapA requires BamB for its localization to the *E. coli* outer membrane. It also implies that the mechanism for outer membrane protein localization is conserved between *E. coli* and *Xf*.

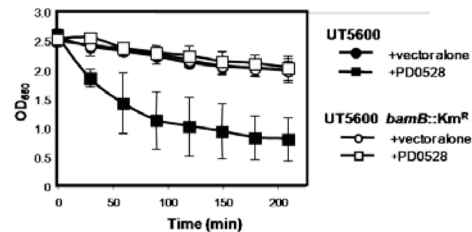


Figure 2: The autoaggregation phenotype conferred by XadA requires the BAM complex.

E. coli cultures expressing XadA (PD0528) were vigorously shaken for 10 s. Samples were then taken ~0.5 cm from the top of the culture tube at the indicated times and the OD_{550} was measured.

Objective 2: Identify proteins and virulence factors requiring one of the three serine protease autotransporters for their maturation.

PD0218, PD0313, and PD0950 are members of the phylogenetic clade containing the S8 subtilisin-like serine protease autotransporters (Tripathi and Sowdhamini, 2008). Members of this family have been implicated in defense, growth on proteinaceous compounds, and the proteolytic maturation of virulence factors. Although many serine proteases have broad specificities, some are very specialized. One of the best studied members of this clade is the SphB1 autotransporter protein of *Bordetella pertussis* (Coutte et al., 2001). SphB1 serves as a specialized maturation protease, responsible for the timely maturation and extracellular release of the filamentous haemagglutinin FHA. One of the goals of this project is to determine the specificity and targets of PD0218, PD0313 and PD0950.

During the period under review, we have examined the in vitro and in planta properties of the strains containing mutations in one, two, or all three of the AT-1 serine proteases. These strains and some of their properties are listed in following table (Table 1):

Table 1: The properties of the AT-1 serine proteases mutants

Strain	AT-1 Mutation(s)	Biofilm formation in vitro	Week PD symptoms first appear*
Temecula	Wildtype	0.688 ±0.12	11
TAM147	PD0218::Cm^R	0.536 ±0.08	8
TAM152	PD0313::Gm^R	0.248 ±0.02	10
TAM146	PD0950::Em^R	0.469 ±0.02	8
TAM148	PD0218::Cm^R, PD0950::Em^R	0.531 ±0.07	8
TAM150	PD0218::Cm^R, PD0313::Gm^R	0.479 ±0.09	8
TAM151	PD0313::Gm^R, PD0950::Em^R	0.580 ±0.07	10
TAM153	PD0218::Cm^R, PD0313::Gm^R, PD0950::Em^R	0.633 ±0.11	14

* Three plants were inoculated for each mutant on 6/22/09. Disease severity was assessed weekly using the visual scale (0 to 5) described by Guilhabert and Kirkpatrick (2005). On this scale, healthy plants receive a score of 0. The table lists the week when the infected grapevines first receive a score of 1 (only one or two leaves with scorching symptoms starting on the margins of the leaves).

Our characterization of the single mutants suggests that the three serine proteases are involved in different aspects of *Xf* cell physiology and pathogenicity. The mutations in PD0218 and PD0950 result in reduced clumping in liquid and a slight decrease in biofilm formation. In contrast, the mutation in PD0313 eliminates clumping in liquid and has a more severe impact on biofilm formation. The PD0313 mutant also forms a confluent lawn on solid medium. When introduced into grapevines, four mutants (TAM147, TAM146, TAM148, and TAM150) produced symptoms three weeks earlier than Temecula1 and two mutants (TAM 152 and TAM151) exhibited symptoms one week earlier. In contrast, grapevines inoculated with the triple mutant TAM153 exhibited symptoms three weeks later than Temecula1.

We also monitored disease progression every two weeks for a total of 32 weeks after inoculation. Figure 3 shows representative grapevines inoculated with the various mutants.

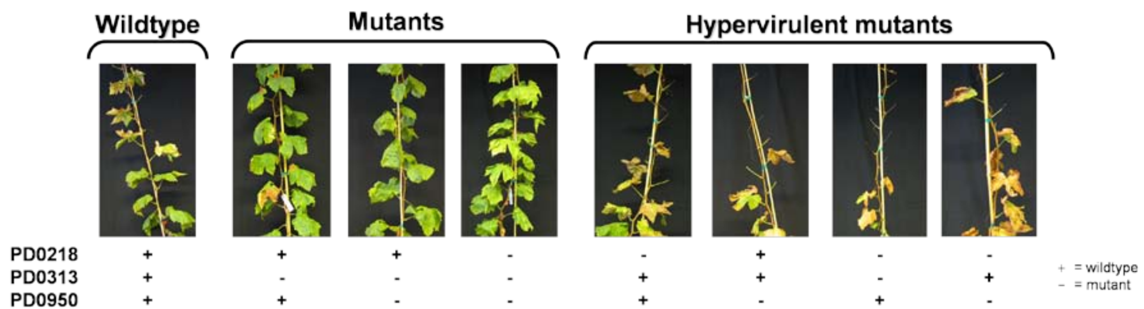


Figure 3: Comparison of grapevines infected with the various AT-1 mutants to wild type. Pictures were taken 22 weeks after inoculation.

The mutants fell into two categories based on the severity of the observed PD symptoms. One group exhibited a hypervirulent phenotype. This group includes TAM147, TAM146, TAM148, and TAM150. It has been proposed that mutants resulting in a hypervirulent phenotype identify loci that attenuate *Xf* virulence in susceptible hosts (Guilhabert and Kirkpatrick, 2005). For example, various adhesins on the *Xf* cell surface facilitate autoaggregation and are thought to reduce the rate of xylem vessel occlusion, which in turn decreases the severity of PD symptom development. If one of the AT-1 serine proteases is important for the maturation of this type of adhesin, the absence of the protease would result in a hypervirulent phenotype.

The second group of AT-1 protease mutants results in plants exhibiting fewer symptoms than wild type. This includes the single mutant TAM152, the double mutant TAM151, and the triple mutant TAM153. Of particular interest are the results for the triple protease mutant. Grapevines infected with TAM153 exhibited the first disease symptoms three weeks later than wild type. Moreover, although the infected grapevines showed relatively few symptoms at 6 months, the titer and migration of TM153 from the inoculation site was similar to wild type. This result, together with the ability of the triple mutant to produce a normal biofilm in vitro, support the hypothesis that the three AT-1 proteases are not required for colonization or migration of *Xf* within the xylem and suggest that the proteases play a direct role in pathogenicity, perhaps through the processing or maturation of one or more important virulence factor.

Finally, we have initiated studies to identify potential targets of the proteases. Specifically, we have compared the protein composition of the outer membrane, the membrane vesicles, and the

secretome of the single mutants to wild type on different percentage SDS-PAGE gels stained with Syphro Ruby. Comparisons of the banding patterns of the various samples have allowed us to identify proteins that are affected by the presence or absence of a specific protease. Some of the most interesting results come from our analysis of the secretome of the various mutants. An example of this analysis is shown in Figure 5A. Based on MALD-TOF-MS analysis of the proteins in band indicated by the star, the PD0218 secretome is missing bacteriocin, which is encoded by PD1427. One possible explanation is that the PD1427 is still associated with the bacterial cell surface and that a functional PD0218 protease is required for its release to the extracellular environment. Bacteriocins are known to contribute to the competitiveness of the producing organisms and have been identified as potential targets for alternative approaches for plant disease control (Holtsmark et al., 2008). Furthermore, subtilisin-like serine proteases are known to function as the maturation enzyme for the bacteriocin-like lantibiotics produced by some Gram-positive bacteria (Tripathi and Sowdhamini, 2008). Therefore, although more experiments are needed, the simplest explanation for this result is that the PD0218 protease is required for the maturation of PD1427.

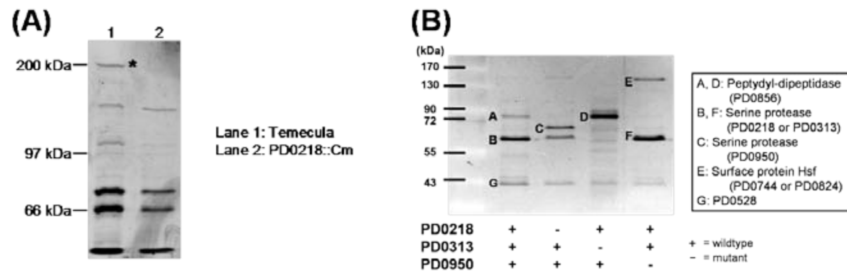


Figure 5: Comparison of the proteins secreted by the protease mutants and wild type. The secreted proteins were concentrated using an Amicon centricon filter and separated on a 6% (A) or 8% (B) SDS-PAGE gel. The gels were stained with Syphro Ruby and the indicated bands were excised and analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility.

Another possible outcome of these comparisons is shown in Figure 5B. In this case, a protein appears in the secretome of the mutant, which is missing from the supernatant fraction prepared from wild-type cells. One example is the *Xf* ortholog to the cell surface protein Hsf, which is found in the secretome of the PD0950 mutant, but not wild type. In *Haemophilus influenza*, Hsf has been shown to mediate adherence to host cells and plays an important role in its pathogenicity (Cotter et al., 2005). Hsf is a large protein that forms fiber-like structures on the bacterial cell surface and is classified as a trimeric autotransporter adhesin (TAA) protein [for a recent review, see (Kline et al., 2009)]. TAA proteins, which belong to AT-2 autotransporter family, form fibers that are attached to the bacterial cell surface through their C-terminal β-barrel domain. Unlike AT-1 autotransporters, the passenger domains of TAA proteins remain covalently linked to β-barrel domain and are not normally released into the extracellular milieu. The presence of the Hsf passenger domain in the supernatant of the PD0950 mutant suggests that the PD0950 protease is involved either directly or indirectly in controlling whether or not the Hsf passenger domain is released from the bacterial cell surface. Experiments are currently underway to test this possibility.

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V. Publications or reports resulting from this project:

Manuscript:

Matsumoto, A. & Igo, M. M. The Species-Specific Type II Restriction-Modification System of *Xylella fastidiosa* Temecula1. (currently under revision for Applied and Environmental Microbiology)

Symposium Proceedings:

Igo, M. 2009. The role of Type V Secretion Autotransporters in *Xylella fastidiosa. In* T. Esser (eds.), Proceedings, 2009 Pierce's Disease Research Symposium. California Department of Food and Agriculture. p. 97-101.

VI. Presentations of Research:

Poster:

Ayumi Matsumoto and Michele M. Igo. The role of Type V Secretion Autotransporters in *Xylella fastidiosa.* At the 2009 Pierce's Disease Research Symposium. December 9-11, 2009, Sacramento, CA.

Round Table:

Rodrigo Almeida, Alessandra A. de Souza, and Michele Igo. Factors that Mediate Attachment and Biofilm formation by *Xylella fastidiosa* in Plants and Insects. At the 2009 Pierce's Disease Research Symposium. December 11, 2009, Sacramento, CA.

VII. Research Relevance Statement:

One of the research priorities listed in the NAS report entitled "California Agricultural Research Priorities: Pierce's Disease" was to analyze the interactions of the host, pathogen, and insect vector. Recommendation 4.1 states - "A systematic analysis of *Xf* pathogenicity should be accomplished with a combination of biochemical, genetic and genomic analyses."

The focus of this project is to determine the role of *Xf* proteins that are classified as Type V autotransporters in *Xf* virulence. Polypeptides secreted by Type V autotransporters have been shown to play an important role in the virulence of numerous bacterial pathogens. Establishing the importance of these proteins in *Xf* virulence will provide researchers with new targets for developing strategies to control the ability of *Xf* to cause PD.

VIII. Lay summary of current year's results:

Reducing and eliminating the incidence of Pierce's Disease (PD) necessitates the development of control strategies that target the infectious agent, *Xylella fastidiosa* (*Xf*). Our efforts to characterize a specific set of *Xf* virulence factors, called autotransporters, are laying the foundation on which to develop practical strategies to kill this bacterium or to interfere with its ability to cause disease. Importantly, our studies suggest that these autotransporter proteins

likely contribute to key aspects of pathogen survival in plants by promoting adhesion to the xylem wall, formation of biofilms and enhancing transmission by sharpshooters. Four of the autotransporter proteins are predicted to have enzymatic activity. It seems likely that previously developed strategies to disrupt the function of the equivalent enzymes in other bacteria will also be effective against the *Xf* proteins. The remaining two proteins are unique to *Xf* and appear to be involved in the attachment of the bacterium to solid surfaces. Treatments designed to prevent this attachment could have a profound impact on the ability of *Xf* to cause Pierce's Disease.

IX. Status of Funds:

The post-doctoral fellow Ayumi Matsumoto left the laboratory in August, which was much earlier than we had anticipated. Therefore, there was a short period during which the scientist position on the project was vacant. After a careful search, this position was offered to Dr. Sherry Huston, who has been working at UC Davis for a number of years and is currently classified as staff research associate III (SRA III). As a result of the delay in filling this position, there will be funds remaining in the 2009-2010 budget. However, in the original budget for 2010-2011, we requested the salary for a post-doctoral fellow rather than the higher salary required for an SRA III. By transferring the funds remaining in 2009-2010 budget to the 2010-2011 budget, it should be possible to cover this difference.

X. Summary and Status of Intellectual Property: Not applicable

The goal of this project is to produce materials and procedures that will help uncover the underlying mechanisms of *Xf* virulence. This information will be made available to other researchers interested in finding a solution to PD. As anticipated, during the period under review, this research did not lead to the development of materials or procedures that were subject to intellectual property restrictions.