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

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III. Objectives:

Type V secretion autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (the passenger domain) across the outer membrane. Given the importance of AT-1 proteins in the virulence of other Gram-negative pathogens, it is highly likely that most of the *Xf*-PD AT-1 proteins will play an important role in *Xf* virulence. This project has two objectives:

Specific Objective 1: Determine the role of the six *Xf*-PD autotransporter proteins and their passenger domains in *Xf* cellular physiology and virulence. Highest priority will be given to the two autotransporters that secrete a passenger domain containing tandem repeats of a 50-60 amino acid motif, which is only found in *Xf* species.

Specific Objective 2: Examine the feasibility of using the purified passenger domains of the *Xf*-PD autotransporters as a protein or protein chimera ("anti-Xf protein') capable of inactivating or otherwise interfering with the infectivity of *Xf*.

IV. Summary of major research accomplishments and results for each objective:

Priorities based on the Recommendations of the 2007 PD/GWSS Panel:

Specific Objective 1: The primary focus of this objective is to construct strains containing single mutations in the different autotransporters and to examine the impact of these mutations on *Xf* cell physiology and virulence. To date, we have successfully generated null mutations in four of the six AT-1 genes. Our characterization of one of these genes, PD0528, is almost complete and we plan to publish our results as soon as the grapevine experiments have been completed. We have also generated strains containing single mutations in two of the three AT-1 autotransporters that encode serine proteases and experiments designed to discover the impact of these mutations on *Xf* physiology and biofilm formation are underway. We plan to investigate the role of these AT-1 autotransporters in *Xf* virulence in grapevines this Spring.

The panel recommended that we also include the construction of strains carrying double and triple mutations in the various autotransporters as part of this objective. During the past year, we have focused on the autotransporter PD0528 and its paralogs. We have successfully

generated 3 single mutants, 1 double mutant, and 1 triple mutant. The construction and characterization of these strains is described below. We have also begun to generate mutations in the three autotransporters that encode serine proteases. Single mutants in two of these autotransporters have been constructed and experiments are underway to generate double and triple mutants. Constructing a strain that is missing all three serine proteases may be important for uncovering the role of these proteases in *Xf* virulence.

Specific Objective 2: The panel recommended that objective 2 become a minor part of the total project. Based on this recommendation, we have not focused on this objective during the period under review.

Overview: *Xylella fastidiosa* (*Xf*) is a fastidious, Gram-negative bacterium, which is the causative agent of numerous plant diseases relevant to the California agricultural economy. The ability of *Xf* to colonize the plant and to incite disease is dependent upon the capacity of the bacterium to produce a diverse set of virulence factors. Many of these virulence determinants are proteins that are either secreted to the bacterial cell surface or released into the external environment. In Gram-negative bacteria, secretion occurs through one of six major secretion pathways, numbered I to VI (5, 7, 8). These pathways are highly conserved and exhibit functionally distinct mechanisms of protein secretion.

One of the simplest secretion mechanisms is exhibited by the AT-1 autotransporters, a subcategory of Type V secretion systems (5). AT-1 systems are dedicated to the secretion of a single specific polypeptide called the passenger domain across the outer membrane. Virulence functions associated with passenger domains include proteolytic activity, adherence, biofilm formation, intracellular motility, cytotoxic activity, or maturation of another virulence determinant. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD. Given the importance of AT-1 autotransporters in pathogenicity, the secretion of these passenger domains to the *Xf* cell surface could have important implications in the PD infectious cycle.

Specific Objective 1: Determine the role of the six *Xf*-PD autotransporter proteins and their passenger domains in *Xf* cellular physiology and virulence.

The six *Xf*-PD autotransporter proteins can be divided into three groups based on their passenger domains. The passenger domains of PD0528 and PD1379 contain tandem repeats of a species-specific 50-60 amino acid motif. Since the passenger domains of AT-1 autotransporters are usually involved in virulence, our investigation of PD0528 and PD1379 will uncover the role of these proteins in *Xf* virulence and reveal a potential target for disrupting the interaction of *Xf* with its host. Because the species-specific motif is unique to *Xf*, treatments targeting this motif should not disrupt the interactions of other microbial species with the host. Most of our work during the period under review has focused on the characterization of these two AT-1 autotransporters (Section 1A).

During the next year, we will focus on characterizing the remaining four AT-1 autotransporters. The passenger domains of PD0218, PD0313, and PD950 are predicted to encode serine proteases, whereas PD1879 is predicted to encode an esterase/lipase (1). Single mutants in two of these autotransporters have been constructed and experiments are underway to generate double and triple mutants. Although many pathogenic bacteria secrete proteases and lipases, no common role in virulence has been demonstrated. Therefore, constructing strains that are missing these enzymatic activities may be important for uncovering the role of these AT-1 autotransporters in *Xf* virulence (Section 1B).

Section 1A: The AT-1 autotransporters PD0528 and PD1379

Characterization of PD0528:

In order to investigate the role of PD0528 in *Xf* cell physiology and virulence, we used site-directed disruption (3) to generate a null mutation in the PD0528 gene. Briefly, we inserted a chloramphenicol cassette into a *Nhe I* site within the PD0528 open reading frame and then introduced this construct into the *Xf* chromosome. This resulted in the PD0528::Cm^R mutant strain, TAM103. Comparison of the membrane profiles of a wild-type *Xf* Temecula strain and the isogenic PD0528::Cm^R mutant revealed that the band corresponding to the PD0528 protein is missing in the outer membrane of TAM103 (Figure 1). The next step was to perform complementation analysis. For this experiment, we generated the plasmid pAM61, which carries the wild-type PD0528 gene and is a derivative of the plasmid pBBR1MCS-5 (6). Introduction of pAM61 into the PD0528::Cm^R mutant resulted in the strain TAM103/pAM61. We then compared the membrane protein profile of TAM103/pAM61 to the wild-type strain and the TAM103 mutant. As shown in Figure 1, the band that is missing in TAM103 is present in both the wild-type Temecula strain and TAM103/pAM61. Thus, the PD0528 gene carried on the pAM61 plasmid can complement the PD0528 defect in TAM103 and restore the presence of PD0528 in the *Xf* outer membrane.

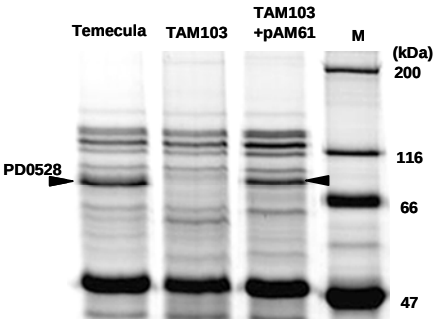


Figure 1: Membrane protein profile of the PD0528 deletion mutant. Outer membrane proteins were isolated from Temecula, TAM103, and TAM103 (pAM61). The position of the missing outer membrane protein in the PD0528 mutant is indicated by the arrow.

Once we had generated the PD0528 null mutation and confirmed that PD0528 is not present on the *Xf* cell surface, we began to investigate the impact of this mutation on *Xf* cell physiology. We discovered that the strain containing the PD0528 null mutation (TAM103) has some growth phenotype in common with the wild-type strain *in vitro*. Specifically, like wild-type, growth of TAM103 in liquid culture requires ~7-10 days and TAM103 is not able to form a confluent lawn on solid media. However, the mutation in PD0528 had a major impact on the ability of *Xf* to form a biofilm formation and to autoaggregate. As shown in Figure 2, TAM103 exhibits a substantial decrease in biofilm formation when compared to the wild-type Temecula strain. Moreover, when a plasmid containing a wild-type copy of PD0528 (pAM61) is introduced into TAM103, biofilm formation is restored. Thus, the absence of PD0528 in the outer membrane has a profound impact on the ability of *Xf* to form a biofilm *in vitro*.

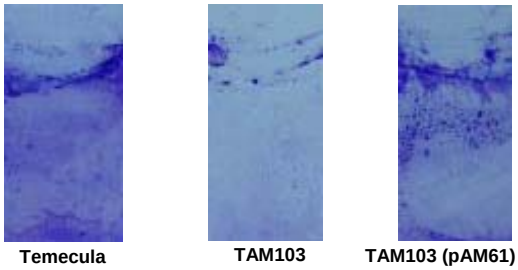


Figure 2: PD0528 impacts biofilm formation. Three *Xf* strains were grown in PD3 liquid medium containing a glass slide: Temecula, TAM103, and TAM103 (pAM61). After 7 days, the slides were removed and the biofilms were stained using 0.1 % crystal violet.

The next step in our analysis was to examine the impact of the absence of PD0528 on *Xf* virulence *in planta.* In this experiment, grapevines were infected by pinprick using the protocol

provided by our cooperator Dr. Bruce Kirkpatrick (4). Briefly, *Xf*-Temecula and TAM103 were grown at 28°C on PD3 plates. The cells were harvested after 7-10 days and the suspension was adjusted to a concentration of 10^9cells/ml. Then, 20 µl of the adjusted suspension was used to inoculate three Thompson seedless grapevines by the standard pinprick method. The parental Temecula wild strain served as a positive control, whereas a water inoculation served as a negative control. The vines were then monitored for symptom development. Figure 3 shows a photograph of a representative vine 16 weeks after inoculation for each infection.

Temecula TAM103 water

Figure 3: PD0528 impacts *Xf* virulence *in planta*. An *Xf* suspension of Temecula or TAM103 was used to inoculate Thompson seedless grapevines using the standard pinprick method. These photographs show a representative vine 16 weeks after infection.

As shown in Figure 3, the vine inoculated with TAM103 does not exhibit the symptoms associated with Pierce's Disease, suggesting that PD0528 plays an important role in *Xf* virulence. As expected, symptoms were observed in the grapevines inoculated with *Xf-PD* Temecula and were not observed in the grapevines inoculated with water. To confirm the presence of *Xf* in both the Temecula-infected and TAM103-infected vines, petiole tissues from each vine were harvested one inch and six inches above the inoculation sites. The samples were then processed using published protocols (4). Serial dilutions of the samples were made and plated onto PD3 plates. The plates were then incubated at 28°C for 7-10 days and the numbers of *Xf* colonies on the plates were compared. *Xf* colonies were observed on both sets of PD3 plates, confirming the presence of *Xf* in the Temecula-infected and TAM103-infected vines. Interestingly, the TAM103-infected vines appeared to have approximately 10-fold fewer *Xf* cells. However, further experiments are needed to determine whether or not this 10-fold difference is significant.

Although the simplest explanation for the phenotype of TAM103 is that the disrupted PD0528 gene is required for the normal development of PD, it is also possible that the gene disruption is affecting the expression of neighboring genes or that a secondary mutation was acquired during the construction of the original mutation and that the secondary mutation is responsible for the phenotype. The classical approach for conclusively establishing that a specific gene is responsible for a specific physiological and virulence function is to perform complementation analysis. Thus, if the reintroduction of a wild-type copy of the PD0528 gene into TAM103 restores the normal PD infection cycle *in planta*, we can conclude that the PD0528 is important for the development of PD. Due to plasmid stability issues, we cannot use the multicopy plasmid pAM61 that we used in our *in vitro* complementation studies *in planta*.

However, we should be able to overcome the stability problem using a series of integration vectors that we developed in collaboration with Dr. Glenn Young (10). These plasmids carry an antibiotic resistance marker and a multiple cloning site flanked by sequences homologous to the intergenic region between PD702 and PD703. We have designated this location in the *Xf* chromosome as NS1 (neutral site 1). These plasmids have been introduced into *Xf* and antibiotic resistant transformants have been obtained. Because these vectors are

unable to replicate in *Xf*, the antibiotic resistant transformants must have arisen as the result of a recombination event between the integration vector and the *Xf* chromosome. Our analysis of these transformants by PCR indicates that the antibiotic resistant marker is located in the chromosome between PD702 and PD703. We have also analyzed both the *in vitro* and *in planta* properties of the antibiotic resistant transformants. The introduction of an antibiotic resistance marker into the NS1 site did not impact *Xf* cell physiology *in vitro* or *Xf* virulence *in planta* (data not shown). The next step will be to introduce a wild type copy of PD0528 into NS1 in the strain TAM103. Experiments are currently underway to construct this strain.

In January 2008, we purchased grapevines in order to perform the PD0528 complementation analysis and plan to infect these grapevines in late Spring. In addition, we have set up a collaboration with Dr. Rodrigo Almeida and Dr. Nabil Killiny in the Department of ESPM at UC Berkeley to examine the impact of PD0528 on the transmission of *Xf* by sharpshooters. These experiments are also scheduled to begin in the Spring.

Characterization of AT-1 autotransporter PD1379 and PD0794, paralogs of PD0528:

The passenger domains of PD0528 and PD1379 contain tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species (1). Although it is not an AT-1 autotransporter, this tandem repeat is also found in PD0794. Therefore, we decided to include PD0794 in our study of this passenger domain. For this analysis, we generated a null mutation in both of these genes by site-directed disruption (3). Introduction of these constructs into the *Xf* chromosome resulted in the PD1379::Gm^R mutant and the PD0794::Em^R mutant. Characterization of the PD1379::Gm^R mutant revealed that its *in vitro* properties are similar to the PD0528::Cm^R. Experiments are currently underway to examine the phenotype of the PD0794::Em^R mutant.

We are also using the disruption plasmids to construct double and triple mutants. To date, we have constructed the double mutant TAM129, which contains both the PD0528::Cm^R and the PD1379::Gm^R mutations. We have also constructed the triple mutant TAM131, which contains three mutations: PD0528::Cm^R, PD1379::Gm^R and PD0794::Em^R. Examining the phenotypic properties of the double and triple mutants should provide important insights into the role of this species-specific 50-60 amino acid motif in *Xf* cell physiology and virulence,

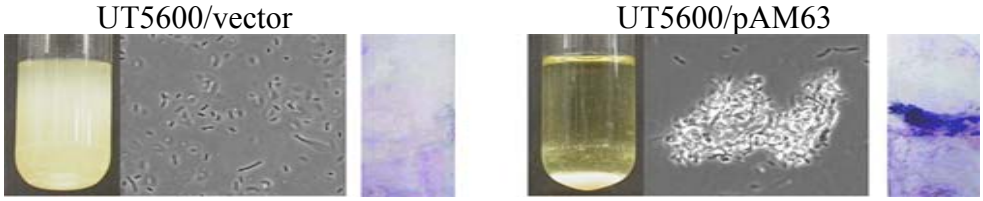
Expressing the PD0528 autotransporter protein in a heterologous system:

Another method for determining the role of the PD0528 passenger domain in *Xf* cellular physiology and virulence is to express the protein in a heterologous system. This strategy has successfully been used to generate *E. coli* strains that display the passenger domain of heterologous autotransporter proteins on their cell surface. These recombinant strains have been employed for binding assays, for developing antibody specificity tests, and for exposing antigenic determinants for vaccine development (9). In addition, these strains have provided important insights into the biological properties of the heterologous protein. For example, when expressed in *E. coli*, the *Neisseria meningitidis* NadA protein is exported to the surface and assembled in oligomers anchored to the outer membrane. The resulting recombinant *E. coli* strain is able to adhere and invade epithelial cells (2), suggesting that NadA may also play an important role in the attachment of *N. meningitidis* to epithelial cells. Therefore, given the success in other systems, we predict that our use of a similar strategy will uncover important information concerning the role of PD0528 in *Xf* virulence.

Although we would eventually like to introduce PD0528 into endophytic bacteria, we decided to test the feasibility of this approach using *E. coli* in our initial studies. Specifically, we

introduced the plasmid pAM61, which carries the gene encoding PD0528 into the *E. coli* strain UT5600. UT5600, which has been successfully used for the heterologous expression of other autotransporter proteins, is deficient in the outer membrane proteases OmpT and OmpP. As shown in Figure 4, the presence of the PD0528 gene in *E. coli* (UT5600/pAM61) results in an increase in the autoaggregation and biofilm formation when compared to the UT5600 strain. Moreover, membrane profiles and Western blots have established that the PD0528 protein has been localized to the *E. coli* outer membrane. Although more experiments need to be done to establish conclusively that the PD0528 protein is responsible for the new physiological properties of the UT5600/pAM61 strain, these initial experiments are extremely promising and suggest that expression of the *Xf* autotransporter proteins in a heterologous system is a powerful approach for uncovering the role of these proteins in *Xf* cell physiology and virulence.

Figure 4. Heterologous expression studies reveal a possible role for PD0528 in autoaggregatation and biofilm formation.



Section B: The AT-1 autotransporters PD0218, PD0313, PD0950, and PD1879:

The passenger domains of three AT-1 autotransporter proteins (PD0218, PD0313, and PD0950) are predicted to encode subtilisin-like serine proteases (1). Extracellular subtilisin-like serine proteases have been implicated in defense, growth on proteinaceous compounds, and the proteolytic maturation of virulence factors (5). The passenger domain of the final protein (PD1879) is predicted to encode a member of the GDSL family of esterase/lipases (1). Members of GDSL family have been implicated in bacterial adhesion (5). In order to investigate the role of these genes in *Xf* cell physiology and virulence, we have begun to generate null mutations in each of these genes by site-directed disruption (3). We have already generated null mutations in two of these genes and examined their *in vitro* phenotypes. TAM126, which carries the PD0218::Kn^R mutation, shows a severe defect in biofilm formation. In contrast, TAM100, which carries the PD0313::Kn^R mutation, exhibits a wild-type phenotype. Although preliminary, the properties of these two mutants suggest that PD0218 and PD0313 may play different roles in *Xf* cell physiology and virulence.

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V. Publications or reports resulting from this project:

Symposium Proceedings:

Igo, M. 2007. The role of Type V Secretion Autotransporters in *Xylella fastidiosa.* *In* T. Esser (eds.), Proceedings of the 2007 Pierce's Disease Research Symposium. December 12-14, 2007, San Diego, CA p. 140-143.

VI. Presentations of Research:

Posters:

Ayumi Matsumoto, Ee-Been Goh, Glenn M. Young, and Michele M. Igo. Characterization of PD0528: A potential Type V autotransporter in the *Xylella fastidiosa* outer membrane. At the APS/SON Joint Meeting. July 28-August 1 2007, San Diego, CA.

Ayumi Matsumoto, Glenn M. Young, and Michele M. Igo. 2007. The role of Type V Secretion Autotransporters in *Xylella fastidiosa.* At the 2007 Pierce's Disease Research Symposium. December 12-14, 2007, San Diego, 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 was 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.

During the period under review, we generated mutations in four of the *Xf-PD* Type V autotransporters. We have also examine the genetic, molecular, and biochemical properties of one of these proteins, PD0528, in detail. PD0528 is located on the *Xf* cell surface and secretes a polypeptide containing tandem repeats of a 50-60 amino acid sequence that is only found in *Xf* species. Our characterization of PD0528 has established that this cell surface protein plays an important role in biofilm formation and autoaggregation *in vitro*. Moreover, grapevines infected with a mutant *Xf* strain that is missing the PD0528 protein do not develop Pierce's Disease. Finally, we have set up a collaboration with Dr. Rodrigo Almeida and Dr. Nabil Killiny in the Department of ESPM at UC Berkeley to examine the impact of PD0528 on the transmission of *Xf* by sharpshooters. Establishing the importance of this polypeptide in *Xf* virulence will provide researchers with a new target 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, is laying the foundation on which to develop practical strategies to specifically kill this bacterium or 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.

During the past year, we focused our efforts on a particular autotransporter called PD0528. PD0528 is a multi-domain protein that is responsible for secreting a single specific polypeptide across the bacterial cell surface. This secreted polypeptide contains six tandem 50-60 amino acid architectural repeats, a structural property that is uniquely found in *Xf* species. We hypothesized PD0528 plays an essential role in *Xf* virulence. To test this idea, we developed a mutant *Xf* strain that is missing the PD0528 protein and compared its properties to a wild-type strain. This comparison revealed that PD0528 plays an important role in the ability of *Xf* to form clumps in liquid and biofilms on solid surfaces. Perhaps even more important, grapevines infected with the PD0528 mutant strain did not develop Pierce's Disease. Experiments are currently underway to determine the role of PD0528 in the transmission of *Xf* by sharpshooters. Establishing the importance of PD0528 in *Xf* virulence will provide researchers with a new target gene for developing strategies to control the ability of *Xf* to cause PD. Because the PD0528 secreted polypeptide is unique to *Xf*, treatments targeting this protein should not impact the growth or the physiological properties of other microbial species within the plant or insect host.

IX. Status of Funds:

The laboratory assistant originally assigned to this project has left the laboratory and we are currently looking for a skilled researcher to replace her. If this search is successful, we anticipate using all of the funds budgeted for 2007-2008. However, if this search is not successful, we may request permission to rebudget these funds for 2008-2009.

X. Summary and Status of Intellectual Property: Not applicable

The goal of this project was to produce materials and procedures that would uncover the underlying mechanisms of *Xf* virulence. This information was made available to other researchers interested in finding a solution to PD. As anticipated, this research did not lead to the development of materials or procedures that are subject to intellectual property restrictions.