THE ROLE OF TYPE V SECRETION AUTOTRANSPORTERS IN THE VIRULENCE OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted October 1, 2007 to September 30, 2008.

ABSTRACT
Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (passenger domain) across the outer membrane of Gram-negative bacteria. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xylella fastidiosa* (*Xf*) Temecula 1 (*Xf*-PD). Most of our work during the period under review has focused on PD0528 and PD1379, the AT-1 autotransporters whose passenger domains contain tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species. These studies indicated that both PD0528 and PD1379 have a major impact on autoaggregation and biofilm formation in *vitro*. Furthermore, grapevines infected with a strain carrying a mutation in PD0528 do not develop Pierce's disease. We have also initiated experiments to characterize PD0218, PD0313, and PD0950, the three autotransporters predicted to have proteolytic activity. We have generated strains containing single mutations and a strain containing mutations in both PD0218 and PD0950. Experiments are currently underway to generate a strain carrying mutations in all three genes. Characterization of the triple mutant should provide insight into the role of the secreted serine proteases in the *Xf* infection cycle.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a Gram-negative, xylem-limited bacterium and is the causative agent of Pierce's disease (PD), a devastating disease of grapevines (for a recent review, see (Chatterjee et al. 2008)). The ability of *Xf* to colonize grapevines and to incite disease is dependent upon the capacity of this bacterium to produce a diverse set of virulence factors. Many of these virulence factors are proteins that must be secreted to the bacterial cell surface or released into the external environment before they can contribute to pathogenicity. In Gram-negative bacteria, this secretion occurs through one of seven major pathways, Types I to VI and the chaperone-usher pathways (Henderson et al. 2004, Hodak and Jacob-Dubuisson 2007, Cascales 2008). 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 (Henderson et al. 2004, Hodak and Jacob-Dubuisson 2007). 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. Functional sequence predictions of these genes indicate that three of these secreted proteins have proteolytic activity (PD218, PD0313, PD0950), one protein has lipase/esterase activity (PD1879), and two of the proteins encode tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species (PD0528, PD1379). Establishing the role of these secreted proteins in *Xf* cell physiology and virulence will provide new targets for researchers to use in generating tactics that disrupt the ability of *Xf* to colonize plant tissue and to initiate the PD disease cycle in susceptible grapevines.

OBJECTIVES
The primary goal of this project is to determine the role of the six *Xf*-PD autotransporter proteins and their passenger domains in *Xf* cellular physiology and virulence. 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.

1. Generate a mutation in each of the six AT-1 genes and determine their impact on *Xf* cell physiology and virulence. The construction of strains carrying double and triple mutations in the various autotransporters is also part of this objective.
2. Examine the biochemical properties and location of the six AT-1 passenger domains. Priority will be given to any gene identified in Specific Aim 1.

RESULTS AND DISCUSSION
We have successfully generated mutations in five of the six AT-1 genes by the gene replacement method (Feil et al. 2003). This procedure involved generating plasmids, which contain an antibiotic resistance marker flanked on each side by ~750 base pairs of the appropriate chromosomal sequence. To facilitate the construction of double and triple mutants, one of four different antibiotic resistance markers was used: chloramphenicol (Cm^R), erythromycin (Em^R), gentamicin (Gm^R), or kanamycin (Kn^R). The resulting plasmids were then introduced into *Xf* by electroporation. *Xf* cells containing the desired mutations were identified by plating the cells onto antibiotic-containing PD3 plates. Polymerase chain reaction (PCR) was

then used to confirm that a double crossover event had occurred between the disrupted gene carried on the plasmid and the wild-type gene on the *Xf* chromosome.

To date, this series of experiments has resulted in the construction of five strains carrying a mutation in one of the AT-1 autotransporters. We have also generated a strain, which carries a mutation in PD0794. Although PD0794 is not predicted to encode an AT-1 autotransporter or to be localized to the outer member, it is classified as a paralog of PD0528 and PD1379 based on its similarity to their passenger domains. Since PD0794 might be secreted to the cell surface by a different type of secretion system, we decided to include it in our characterization of the PD0528 and PD1379 passenger domains.

Characterization of the AT-1 autotransporters with *Xf*-species specific passenger domains:
The passenger domains of PD0528 and PD1379 contain tandem repeats of a 50-60 amino acid motif. PD0528 contains six copies of this repeat, whereas PD1379 contains three copies. Their paralog PD0794 contains four copies. To investigate the role of this *Xf*-species specific motif in cell physiology and virulence, we first generated strains that carried a single mutation in each of the genes: TAM103 (PD0528::Cm^R), TAM127 (PD1379::Gm^R), and TAM145 (PD0794::Em^R). We then began to construct strains containing mutations in multiple genes. To date, two of these strains have been generated: the double mutant TAM128 (PD0528::Cm^R, PD1379::Gm^R) and the triple mutant TAM140 (PD0528::Cm^R, PD1379::Gm^R, PD0794::Em^R).

Table 1. Properties of strains carrying mutations in genes having the *Xf*-species specific motif.

Strain	Mutations	Doubling Time (hr)	Clumping in Liquid	Biofilm formation
Temecula	Wild-type	13.8	++	0.55 ± 0.13
TAM103	PD0528::Cm^R	13.5	+	0.34 ± 0.14
TAM127	PD1379::Gm^R	13.9	+	0.42 ± 0.13
TAM145	PD0794::Em^R	13.4	+	0.29 ± 0.04
TAM128	PD0528::Cm^R PD1379::Gm^R	13.7	+	0.23 ± 0.07
TAM140	PD0528::Cm^R PD1379::Gm^R PD0794::Em^R	13.5	+	0.23 ± 0.11

The next step was to investigate the impact of these mutations on *Xf* cell physiology. As shown in **Table 1**, the growth rate of *Xf* is not affected by the elimination of genes carrying the *Xf*-species specific passenger domain. However, even single mutations had an impact on the ability of *Xf* to form clumps in liquid and a biofilm on a glass surface. This would suggest that all three genes contribute to the ability of *Xf* to autoaggregate and to form a biofilm on a solid surface.

Another method for determining the contribution of the individual *Xf*-species specific passenger domains to cell physiology and virulence is to express the protein in a heterologous system. This strategy has 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 (Yang et al. 2004). In last year's Symposium Report (Igo 2007), we described our successful use of this strategy for analyzing the PD0528 passenger domain. Specifically, we introduced the plasmid pAM61, which carries the gene encoding PD0528 into the *E. coli* strain UT5600. UT5600, which has been used to express other autotransporter proteins, is deficient in the outer membrane proteases OmpT and OmpP. The presence of the PD0528 gene in UT5600 (UT5600/pAM61) results in autoaggregation and the formation of a biofilm, properties not normally demonstrated by the parental *E. coli* strain. Thus, expression of PD0528 in *E. coli* establishes that PD0528 plays a direct role in autoregulation and biofilm formation in this heterologous system and by inference, in *Xf*.

During the period under review, we performed a more detailed analysis of the *E. coli* strain containing the PD0528 gene. To facilitate this analysis, we generated an antibody to the PD0528 passenger domain and then used it to perform Western analysis on UT5600 and UT5600/pAM61. As expected, the antibody did not hybridize to any proteins in UT5600 and recognized a single band in UT5600/pAM61 that corresponds to the predicted size of the PD0528 protein. We next examined the location of PD0528 in *E. coli*. Using a protease accessibility assay (Yen et al. 2007), we established that PD0528 is present on the *E. coli* cell surface. The PD0528 antibody was also used to perform immunofluorescence microscopy. Together, these results indicated that PD0528 is localized to the *E. coli* surface and suggest that the components necessary for secreting autotransporters like PD0258 to the cell surface are conserved between *E. coli* and *Xf*.

We also performed Western analysis on a number of *Xf* strains. In one study, we examined the specificity of the PD0528 antibody by comparing the hybridization pattern obtained for three *Xf* strains: Temecula, TAM103 (PD0528::Cm^R), and TAM127 (PD1379::Gm^R). This study revealed that antibody made with the PD0528 passenger domain does not hybridize to the PD1379 protein. Given the fact that there is only ~50% identity between the two proteins, this is not surprising. Based on this result, we plan to use the PD0528 antibody to examine the localization of PD0528 to the *Xf* cell surface using the protease accessibility assay and immunofluorescence microscopy.

Finally, we examined 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 (Guilhabert and Kirkpatrick 2005). 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.

Temecula TAM103 water

Figure 1. 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 1**, 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 (Guilhabert and Kirkpatrick 2005). Serial dilutions of the samples were made and plated onto PD3. The plates were then incubated at 28°C for 7-10 days and the numbers of *Xf* colonies 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.

Characterization of the AT-1 autotransporters with subtilisin-like serine protease passenger domains:

The passenger domains of three AT-1 autotransporter proteins (PD0218, PD0313, and PD0950) are predicted to encode subtilisin-like serine proteases (Bateman et al. 2004). Extracellular subtilisin-like serine proteases have been implicated in defense, growth on proteinaceous compounds, and the proteolytic maturation of virulence factors (Henderson et al. 2004). In order to investigate the role of these genes in *Xf* pathogenicity, we first generated null mutations in each of these genes and then examined their impact on *Xf* cell physiology. As summarized in **Table 2**, the mutation in PD0218 affects clumping in liquid and biofilm formation, whereas the mutation in PD0950 only affects clumping in liquid. In contrast, the mutation in PD0313 does not appear to affect clumping in liquid, but may result in increased biofilm formation. Although still preliminary, these results suggest that the proteolytic activities of the PD0218, PD0313, and PD0950 passenger domains may make different contributions to *Xf*-PD physiology and virulence.

Table 2. Properties of strains carrying mutations in genes having a serine protease passenger domain.

Strain	Mutations	Appearance of Single Colonies	Clumping In Liquid	Biofilm Formation
Temecula	Wild-type	6-8 days	++	0.55 ± 0.13
TAM148	PD0218::Cm^R	6-8 days	+	0.34 ± 0.09
TAM100	ΔPD0313::Km^R	6-8 days	++	0.79 ± 0.11
TAM147	PD0950::Em^R	6-8 days	+	0.50 ± 0.03
TAM149	PD0218::Cm^R PD0950::Em^R	6-8 days	+	0.44 ± 0.07

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FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program.