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

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ABSTRACT
The *Xylella fastidiosa* Temecula1 genome encodes six proteins that have been classified as AT-1 autotransporters. Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (passenger domain) across the outer membrane of Gram-negative bacteria. Here, we describe our genetic characterization of PD0218, PD0313, and PD0950, the three autotransporters predicted to have proteolytic activity. In the laboratory, a strain carrying a mutation in all three protease genes has properties similar to wild type in terms of its growth rate and its ability to aggregate and form a biofilm. When infected into grapevines, the triple mutant shows very few PD symptoms in spite of the fact that its ability to migrate and colonize the xylem is indistinguishable from wild type. We have also compared of the properties of strains missing only one protease versus strains missing two proteases. These studies suggest that PD0313 and PD0950 are both virulence factors and that PD0218 is involved for reducing virulence, possibly through its interaction with PD0950. Finally, comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments are currently underway to examine the secreted protein profiles for the double and triple mutants.

LAYPERSON SUMMARY
Bacteria have developed numerous strategies for infecting and colonizing host organisms; many involve proteins that are either secreted to the cell surface or released into the external environment. Some of these proteins are virulence factors that allow the bacteria to adhere to the host tissue, utilize nutrients available within this tissue, and counteract any defense response launched by the host. However, other proteins are involved in minimizing the damage caused by these virulence factors. Here, we describe our work on three proteins secreted by *Xylella fastidiosa* Temecula1 that are classified as serine protease autotransporters. Mutational analysis indicates that two of the proteins act as virulence factors, whereas the third protein appears to decrease virulence. Understanding the mode of action for each of the proteases will allow us to develop methods for interfering or enhancing their function, thereby reducing the damage caused by this important plant pathogen.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a Gram-negative, endophytic bacterium, which is responsible for a number of economically important plant diseases [reviewed in (1, 7)]. *Xf* is spread from infected plants to uninfected plants by xylem-feeding insects, such as sharpshooters and spittle bugs. 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. Comparison of the *Xf* 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 (4, 6). Another important category of proteins are "anti-virulence factors" or host-protective virulence factors (13). These proteins are not as easy to identify using genomic methods. These pathogen-encoded "anti-virulence" proteins are thought to be important in modulating the activity of the host-damaging virulence factors. It has been suggested that protecting the host cell from too much damage may be an integral part of the strategy adopted by bacterial pathogens to survive and reproduce in their host organisms.

The focus of this project is the Type V secretion AT-1 autotransporters of *Xf* strains that cause Pierce's disease (PD) of grapevines (*Xf*-PD). AT-1 autotransporter proteins have been identified as rational targets for the design of novel vaccines directed against Gram-negative pathogens (15). AT-1 systems are dedicated to the secretion of a single specific polypeptide, the passenger domain, across the outer membrane. What happens to the passenger domain after it reaches the cell surface is dependent upon the specific autotransporter. Some passenger domains are not cleaved and protrude from the cell surface. This is a common feature of the adhesin autotransporters. Other passenger domains are cleaved from their membrane anchor (the β-barrel domain) and either remains loosely associated with the cell surface or are released into the environment. Not surprising, the ultimate location of each passenger domain appears to be integrally associated with its physiological function.

Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD. Functional sequence predictions indicate that three of these secreted proteins have proteolytic activity (PD218, PD0313, PD0950), one protein has lipase/esterase activity (PD1879), and two proteins are thought to act as adhesins (PD0528, PD1379). The goal of this

project is to determine the role of the AT-1 autotransporters in cell physiology and virulence and to examine how we might exploit these proteins to control PD.

OBJECTIVES

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.

RESULTS AND DISCUSSION

Overview of the serine protease autotransporters

Functional sequence predictions indicate that three *Xf*-PD proteins (PD0218, PD0313, and PD0950) are members of the phylogenetic clade containing the S8 subtilisin-like serine protease autotransporters (14). 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* (3). 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. As a first step in this analysis, we have generated strains containing mutations in one, two, or all three of the AT-1 serine proteases. A list of these mutants and some of their phenotypic properties is presented in **Table 1**.

Table 1. The AT-1 serine protease mutants.

Strain	AT-1 Mutation(s)	Biofilm formation	Disease Severity*
Temecula1	Wildtype	100%	3.3
TAM147	PD0218::Cm^R	78%	4.5
TAM152	PD0313::Gm^R	36%	1.2
TAM146	PD0950::Em^R	68%	4.2
TAM148	PD0218::Cm^R, PD0950::Em^R	77%	4.5
TAM150	PD0218::Cm^R, PD0313::Gm^R	70%	4.3
TAM151	PD0313::Gm^R, PD0950::Em^R	84%	1.2
TAM153	PD0218::Cm^R, PD0313::Gm^R, PD0950::Em^R	92%	2.0

* Three plants were inoculated for each mutant. Disease severity was assessed weekly using the visual scale (0-5) described by Guilhabert and Kirkpatrick (5).

Our examination of the properties of these mutants under laboratory conditions and in grapevines has provided some insights into the roles of the individual proteases. These studies have also revealed that the interactions between these proteases are complex.

Properties of the triple mutant, TAM153

Based on comparison of the growth properties of TAM153 to *Xf* Temecula1, the absence of the three AT-1 serine proteases does not impact the growth rate and has only a modest impact on biofilm formation (92% wild type levels). However, their absence has a profound impact on the properties of TAM153 in grapevines. For this analysis, Thompson seedless grapevines were infected by the pinprick method and disease severity was assessed weekly using the visual scale (0 to 5) described by Guilhabert and Kirkpatrick (5). As shown in **Figure 1A**, by week 21, grapevines infected with wild type were exhibiting marginal leaf scorching and matchsticks characteristic of PD. In contrast, the grapevines infected with TAM153 display very few symptoms and appear similar to the uninfected control plants. We also assessed the bacterial population and movement at week 26 following inoculation. In this experiment, petiole tissues were harvested at different distances above the point of inoculation. The bacterial population within each sample was then determined by plating onto PD3 agar (5). As shown in **Figure 1B**, high populations of TAM153 were recovered within the petiole tissue at the different locations and the colony-forming units per gram (CFU/g) were typically greater than wild type. Based on these results, we conclude that the lack of PD symptom development in TAM153 is not due to the impact of the three mutations on the ability of *Xf* to migrate or colonize the xylem.

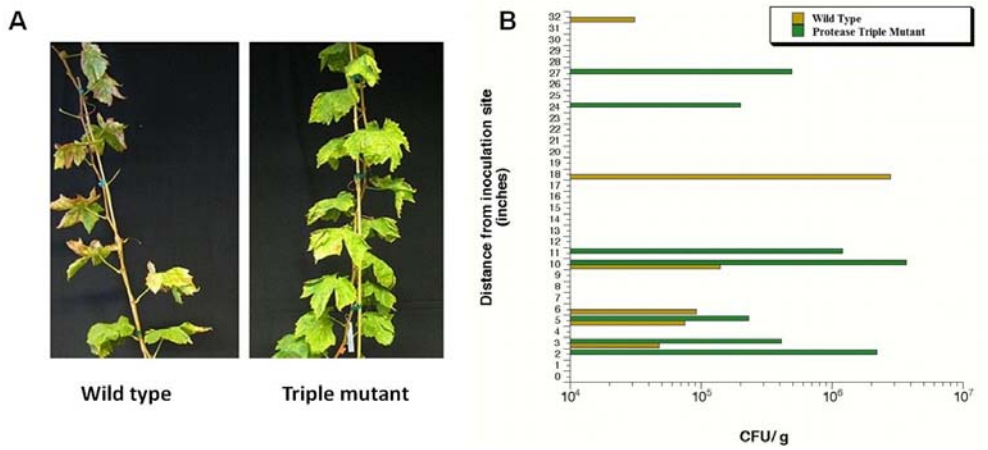

Figure 1. The impact of the triple mutant on *Xf* virulence *in planta*. An *Xf* suspension of wild type or the triple mutant TAM153 was used to inoculate Thompson seedless grapevines using the standard pinprick method. (A)Photographs show a representative vine 21 weeks after infection. (B) Petiole tissues were harvested at 26 weeks and the bacterial populations were determined at different locations above the site of inoculation.

What can we learn about the function of the individual proteases from the single mutants?

The PD0218 mutant TAM147: A strain carrying a mutation in PD0218 (TAM147) was only slightly defective in biofilm formation (80% wild-type levels); its major impact was on virulence in grapevines. Grapevines infected with TAM147 showed earlier symptom development, higher disease scores over a period of 26 weeks, and earlier vine death than wild-type infected plants. Hypervirulent phenotypes have been reported for numerous *Xf* mutants, such as the hemagglutinin HxfA mutant (5) and the cell-cell signaling-deficient RpfF mutant (11). One hypothesis is that proteins like HxfA and RpfF facilitate the attenuation of *Xf* pathogenicity by limiting colonization, thereby reducing the rate of xylem vessel occlusion (1, 5). It is possible that the PD0218 protease plays a similar role, perhaps through the maturation of a target protein that is important for grapevine colonization.

The PD0313 mutant TAM152: The strain carrying a mutation in the PD0313 locus (TAM152) had a number of characteristics that distinguished it from wild-type *Xf*. First, TAM152 cells did not aggregate (clump) when grown in PD3 broth. Second, there was a significant decrease in biofilm formation under laboratory conditions (36% wild-type levels; **Table 1**). Finally, grapevines infected with TAM152 exhibited very few PD symptoms and we were unable to recover TAM152 from petiole tissue at 26 weeks. This would suggest that PD0313 is required for *Xf* to survive in grapevines, a phenotype previously reported for mutations in *tolC* (12). Interestingly, both double and triple mutants containing the PD0313 mutation can be recovered from infected grapevines. This suggests that the PD0313 protease may only be important for grapevine survival when one of the other AT-1 proteases is present.

The PD0950 mutant TAM146: Strains carrying a mutation in PD0950 (TAM146) exhibited a phenotype very similar to strains carrying a mutation in PD0218 (TAM147). Both mutants were only slightly defective in biofilm formation and exhibited a hypervirulent phenotype in grapevines. Based on these properties, we initially assumed that PD0950 would facilitate the attenuation of *Xf* pathogenicity. However, as described below, studies of the double mutants revealed a more complex role for PD0950 in *Xf* virulence.

What can we learn about the function of the individual proteases and their interactions from the double mutants?

Comparison of triple mutant TAM153 to the three double mutants allowed us to examine how the presence of a single AT-1 protease impacted *Xf* cell physiology and virulence. The data concerning the contributions of the three proteases are presented in **Figure 2**.

PD0218: The double mutant TAM151, which is missing both PD0313 and PD0950, exhibited very few PD symptoms and its ability to colonize and migrate within the xylem was similar to wild type. The reduction of virulence in the presence of PD0218 is consistent with the hypervirulent phenotype observed for strains missing PD0218. Together, these results imply that the PD0218 protease actually reduces the virulence of *Xf*, making it an "anti-virulence" factor.

PD0313: The double mutant TAM148, which is missing both PD0218 and PD0950, exhibited a hypervirulent phenotype. Since PD0313 is the only AT-1 protease present in the strain, this would suggest that PD0313 is responsible for the observed increase in PD symptoms. This result, together with the results obtained for the PD0313 single mutant (TAM152), support the hypothesis that PD0313 is a virulence factor.

PD0950: Based on the hypervirulent phenotype exhibited by strains missing PD0950, we initially classified PD09050 as an "anti-virulence" factor. However, the double mutant TAM150, which has only the PD0950 protease, also exhibited a hypervirulent phenotype. How might the presence and absence of PD0950 produce the same phenotype in grapevines? One possible explanation was revealed by TAM152, which carries the wild-type genes for both PD0950 and PD0218. The low level of disease in TAM152 infected grapevines suggests that the virulence-reducing PD0218 protease is masking the activity of PD0950.

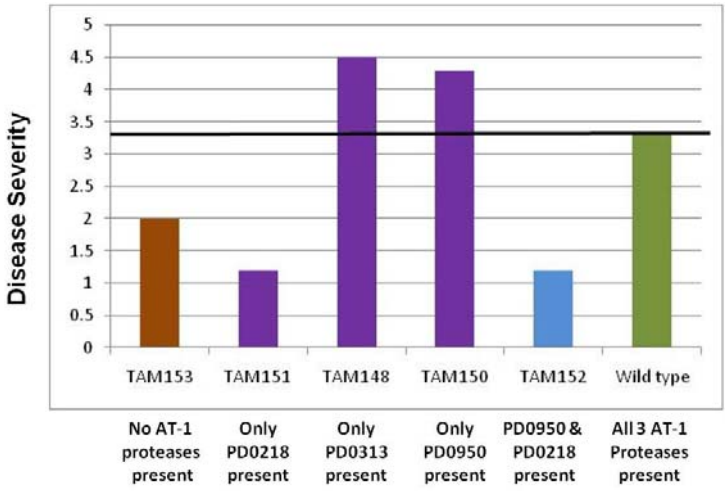

Figure 2. Contributions of the AT-1 proteases to disease severity. Grapevines were inoculated with different *Xf* strains and their impact on disease severity was assessed at week 21. The colors of the bars indicate the number of mutations present in the strain: gold (three), purple (two), blue (one), and green (none).

What are the potential targets of the AT-1 serine proteases?

To address this question, we compared the protein composition of the outer membrane, the membrane vesicles, and the secretome of the various mutants to wild type on different percentage SDS-PAGE gels stained with Syphro Ruby. Comparisons of the banding patterns of the various samples allowed us to identify proteins that are affected by the presence or absence of a specific protease. Some of the most interesting results came from our analysis of the secretome of the single mutants.

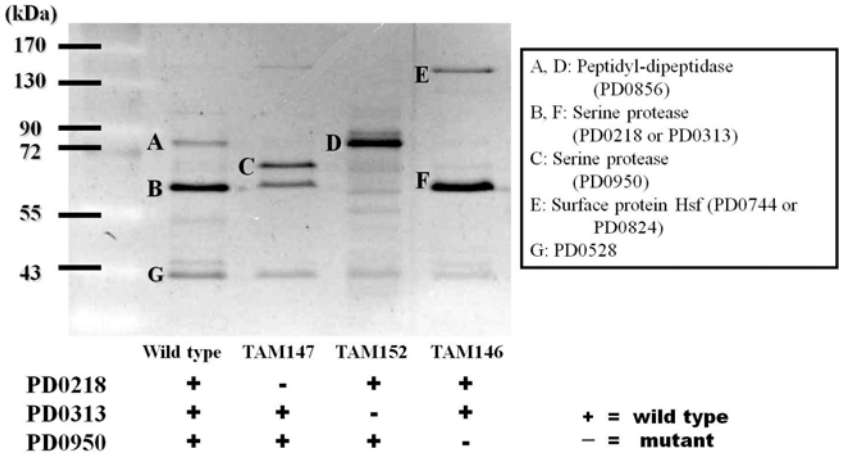

Figure 3. Comparison of the proteins secreted by the protease mutants and wild type. The secreted proteins were concentrated using an Amicon centricon filter and separated on a 8% SDS-PAGE gel. The gels were stained with Syphro Ruby and the indicated bands were excised and analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility.

As shown in **Figure 3**, each mutant exhibits a unique protein profile. In some cases, a specific protein is missing, such as the absence of peptidyl-dipeptidase in TAM147 and TAM 146. In other cases, a protein appears in the secretome of a specific mutant that 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 (2). Hsf is a large protein that has been classified as a trimeric autotransporter adhesin (TAA) protein [for a review, see (9)]. 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.

What can we learn from the heterologous expression studies in E. coli?

Another way in which we have studied the function of the individual AT-1 proteases is to express each of these proteins on the surface of the *E. coli* strain UT5600. UT5600, which is deficient in the outer membrane protease OmpT, is commonly used for autodisplay (also known as live-cell surface display) (8). These studies support the hypothesis that each protease has a different set of target proteins. For example, when plated on a solid medium containing Tween 20 and $CaCl_2$ (10), *E. coli* strains expressing the PD0218 protein are surrounded by a halo, which is indicative of lipase activity (**Figure 4**). Halos are not found around strains expressing either PD0313 or PD0950 (data not shown). The simplest explanation for this result is that PD0218 acts on one or more of the inactive lipases of *E. coli* and the resulting maturation of a lipase is responsible for the observed phenotype. We are currently trying to determine the identity of this lipase(s) in order to help identify PD0218 substrates in *Xf*.

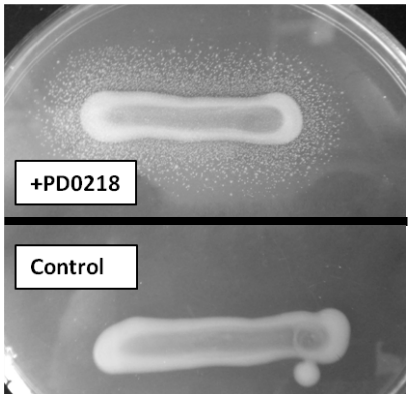

Figure 4. Phenotype of an *E. coli* strain expressing PD0218.
E. coli strain UT5600 containing either the vector pBBR1MCS-5 (control) or plasmid pAM216 (+PD0218) were grown on solid medium containing Tween 20 and $CaCl_2$. The presence of a halo on this medium is indicative of lipase activity.

CONCLUSIONS

Autotransporters have been identified as rational targets for the design of novel vaccines and control strategies. The goal of this project is to characterize the six autotransporters of *Xf*. During the period under review, we have been examining how the absence of one or more autotransporters affects *Xf* cell physiology and virulence. Here, we described our characterization of strains carrying mutations in the three autotransporters predicted to have proteolytic activity. Specifically, we compared the phenotypic properties of strains carrying mutations in one, two, or all three serine protease autotransporters. These studies suggest that PD0313 and PD0950 are both virulence factors and that PD0218 is involved for reducing virulence, possibly through its interaction with PD0950. Furthermore, comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments designed to probe the interactions between the individual proteases and to investigate the mechanism underlying their different role in *Xf* virulence are currently underway.

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