

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

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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. Here, we report our studies on the putative autotransporter protein PD0528 of *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease of grapevines. The passenger domain of PD0528 contains six tandem repeats of a 50-60 amino acid motif that is found only in *Xf* species. To determine the role of this passenger domain in *Xf* virulence, we have begun a detailed characterization of PD0528. We have generated a mutation in PD0528 by homologous recombination and shown that the PD0528 protein is not present in the outer membrane of this mutant strain. We have also constructed a transcriptional fusion between the PD0528 regulatory region and the luciferase gene (*luc*), which has allowed us to identify bases important for PD0528 expression. Finally, we have expressed PD0528 in the *Escherichia coli* strain UT5600 and discovered that the resulting *E. coli* strain exhibits increased autoaggregation and biofilm formation.

INTRODUCTION

Xf is a fastidious, Gram-negative bacterium, which is the causative agent of numerous plant diseases relevant to the California agricultural economy. Diseases caused by *Xf* strains include Pierce's disease of grapevine (PD), citrus variegated chlorosis (CVC), almond leaf scorch (ALS), and oleander leaf scorch (OLS) (Hopkins and Purcell, 2002). 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 (Henderson *et al.* 2004, Pugsley *et al.* 2004, Pukatzki *et al.* 2006). 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). 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. During the period under review, we have been conducting a detailed characterization of the putative autotransporter protein, PD0528. The passenger domain of PD0528 contains six tandem repeats of a 50-60 amino acid motif. Interestingly, this motif is only found in *Xf* species (Bateman *et al.* 2004). Given the importance of AT-1 autotransporters in pathogenicity, the secretion of this unique motif to the *Xf* cell surface could have important implications in the PD infectious cycle.

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.
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

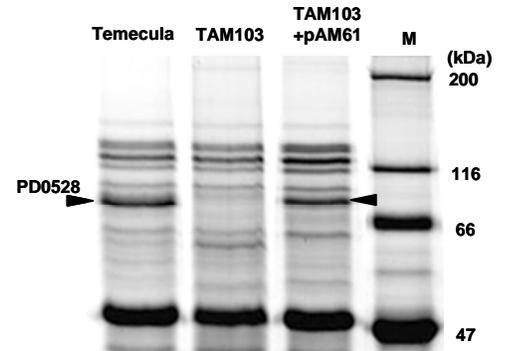
Characterization of TAM103, a strain containing a PD0528 null mutation:

PD0528 is a putative AT-1 autotransporter protein that has a passenger domain containing six tandem repeats of a species-specific 50-60 amino acid motif. In order to investigate the role of PD0528 in *Xf* cell physiology and virulence, we generated a null mutation in the PD0528 gene by inserting a chloramphenicol cassette into a *Nhe I* site within the PD0528 open reading frame. Introduction of this construct into the *Xf* chromosome 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 (Kovach *et al.* 1995). 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 *Xf*-PD Temecula, TAM103, and TAM103/pAM61. The outer membrane proteins were isolated using a sucrose gradient, separated on an 8% SDS-PAGE gel and then stained with Sypro Ruby. The sizes of the molecular weight standards (lane M) are indicated on the right. The position of the missing outer membrane protein in the PD0528 mutant is indicated by the arrow. The identification of the band indicated by the arrow as the PD0528 protein was confirmed by MALDI-TOF mass spectrometry.



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 the same growth phenotype as wild-type *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. Moreover, a variable level of biofilm formation and autoaggregation was observed for TAM103, but not the wild-type strain. Based on these comparisons, the absence of PD0528 in the outer membrane may have an impact on *Xf* cell physiology *in vitro*. Experiments are currently underway to determine if the absence of PD0528 impacts the ability of *Xf* to cause PD in grapevines.

Our inability to detect a definitive phenotype for the PD0528 null mutation might be due to genetic redundancy. The species specific 50-60 amino acid motif found in the PD0528 passenger domain is also observed in two other *Xf*-PD proteins. PD1379, which is an AT-1 autotransporter protein, contains three copies of this motif in its passenger domain. Four copies of this motif are also found in PD0794. Therefore, it is possible that the loss of PD0528 is compensated for by either PD1379 or PD0794. To test this hypothesis, we have inserted a gentamicin cassette into the PD1379 gene and have introduced this construct into both the wild-type strain and into TAM103. The creation of the PD0528/PD1379 double mutant should provide insight into the role of the *Xf* specific 50-60 amino acid motif in *Xf* cell physiology and virulence.

Identifying the sequences important for PD0528 regulation:

In many bacterial pathogens, the production of key virulence proteins is tightly regulated at the transcriptional level, so that the proteins are only produced under certain environmental conditions. Therefore, in order to understand how *Xf* survives in and interacts with its hosts, it is important to discover how different environmental conditions impact gene expression. However, rapid progress in this area is affected by the lack of the genetic and molecular tools necessary to investigate how environmental signals affect *Xf* transcription. During the past year, we have developed a system for examining transcription in *Xf* using the firefly luciferase (*luc*) gene as a reporter gene.

To establish the usefulness of the *luc* gene for studying transcriptional regulation in *Xf*, we constructed a transcriptional fusion between the PD0528 regulatory region and the *luc* gene. The PD0528-*luc* fusion was inserted into the plasmid pBBR1MCS-5, which carries a gentimicin resistance marker. As a negative control, we also constructed a similar plasmid that carries a promoterless-*luc* gene. The resulting plasmids were then introduced into both *Escherichia coli* strain DH5 α and *Xf*-PD and the relative activity of the PD0528-*luc* fusion was determined by comparing the amount of luminescence produced by the four strains. As shown in Figure 2A, high levels of luciferase were produced in the *Xf*-PD strain containing the PD0528-*luc* fusion. In contrast, the PD0528-*luc* fusion was not expressed in *E. coli*. A simple interpretation of this result is that PD0528 expression involves regulatory elements and/or regulatory proteins, which are only present in *Xf*-PD. To test this hypothesis, we first mapped the PD0528 transcriptional start site by 5' RACE analysis. We then generated mutations in the regulatory region upstream of this start site in the PD0528-*luc* fusion. Our first approach was to generate a series of deletions that removed portions of the PD0528 regulatory region. Our second approach was to introduce base changes into the region immediately upstream of the PD0528 transcription site. We then examined the impact of these mutations on PD0528 transcription by measuring luciferase activity. The results for some of these mutations are presented in Figure 2B.

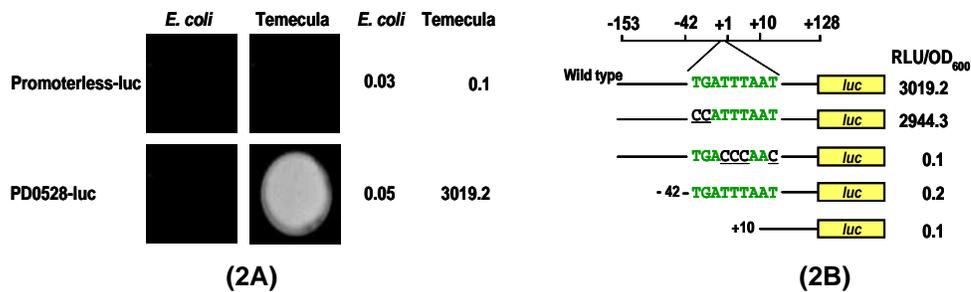


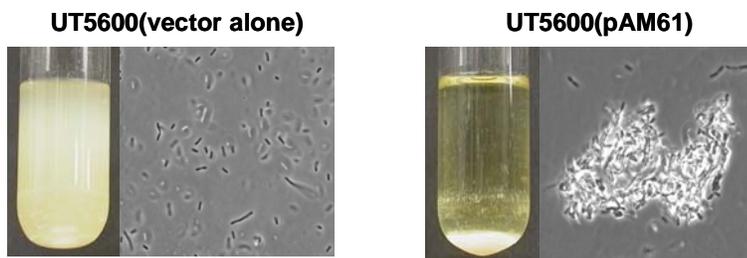
Figure 2: Examination of PD0528 regulation using a *PD0528-luc* fusion. For the luminescence analysis, 1mM D-luciferin was added to cell extracts. Luminescence (RLU) was measured by a Turner TD-2020 luminometer and standardized using OD600. The values are given as RLU/OD600.

We were able to draw two conclusions from these initial experiments. First, deletion analysis indicates that there is a regulatory element in the region between -153 and -42 upstream of the PD0528 transcription start site. Removal of this element is sufficient to eliminate PD0528 transcription. In addition, introduction of three base changes in the -10 region also severely impacts PD0528 promoter activity. It is possible that the two regulatory elements work together as part of the RNA polymerase recognition sequence. However, it is also possible that one of these regulatory elements interacts with a transcriptional activator protein. We are currently generating additional mutations to refine the mapping of these two regulatory elements and to distinguish between these two possibilities.

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 (Yang *et al.* 2004). 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 meningitis* 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 (Capecchi *et al.* 2005), suggesting that NadA may also play an important role in the attachment of *N. meningitis* 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.

Figure 3. Heterologous expression studies reveal a possible role for PD0528 in autoaggregation.



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 3, the presence of the PD0528 gene in *E. coli* (UT5600/pAM61) results in an increase in the autoaggregation of the *E. coli* cells. In addition, UT5600/pAM61 exhibits increased biofilm formation when compared to the UT5600 strain. Finally, membrane profiles 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.

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