# CHARACTERIZATION OF PD0528: A POTENTIAL TYPE V AUTOTRANSPORTER IN THE *XYLELLA FASTIDIOSA* OUTER MEMBRANE

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## ABSTRACT
Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (the passenger domain) across the outer membrane of Gram-negative bacteria. Here, we report our studies on the putative autotransporter protein PD0528. 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 these species-specific tandem repeats in *Xf* virulence, we have begun a detailed characterization of PD0528. Using primer extension analysis, we have located the transcriptional start-site of the PD0528 mRNA. We have also generated a deletion mutation in PD0528 and shown that the PD0528 protein is not present in the outer membrane of this mutant strain. Finally, we have examined the *in vitro* phenotype of the PD0528 deletion mutant. This analysis suggests that the absence of PD0528 in the outer membrane has a profound effect on *Xf* colony morphology and in the ability of this mutant to form cell-to-cell aggregates in liquid culture.

## INTRODUCTION
*Xylella fastidiosa* (*Xf*) is a Gram-negative, endophytic bacterium, which is the causative agent of Pierce's disease (PD) of grapevine, citrus variegated chlorosis (CVC), almond leaf scorch (ALS), and oleander leaf scorch (OLS) (reviewed in Purcell and Hopkins 1996, Hopkins and Purcell 2002). The genomes of four different *Xf* strains isolated from host plants exhibiting disease symptoms have been sequenced (Simpson et al. 2000, Bhattacharyya et al. 2002, Van Sluys et al. 2003). The availability of these sequences has allowed detailed comparisons of these genomes and has greatly facilitated studies aimed at understanding the underlying mechanisms involved in the different diseases. Genes conserved among all four strains are predicted to be responsible for functions central to *Xf* metabolism and cell physiology and for general properties associated with plant and insect colonization and pathogenicity. In contrast, genes exhibiting a higher degree of divergence and strain-specific genes are predicted to be associated with specific interactions between a particular strain of *Xf* and its plant host.

Comparison of the four *Xf* genomes with other bacterial pathogens has also 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 (Meidanis et al. 2002, Smolka et al. 2003). In Gram-negative bacteria, secretion occurs through one of five major secretion pathways, numbered I to V (Pallen et al. 2003, Preston et al. 2005). These pathways are highly conserved and exhibit functionally distinct mechanisms of protein secretion. The current focus of our research is the Type V secretion autotransporters (AT-1) of *Xf*-PD. Type V secretion systems have been divided into three subcategories: the autotransporter system (Type Va or AT-1; TC#1.B.12), the two-partner secretion pathway (Type Vb or TSP; TC#1.B.20), and the Oligomeric Coiled Adhesions family (Type Vc or AT-2; TC#1.B.40) (for recent reviews, see Desvaux et al. 2004, Henderson et al. 2004, Newman and Stathopoulos 2004). The importance of Type V secretion in bacterial pathogenicity was highlighted in a recent review, which stated "To date, all of the functionally characterised autotransporters have been implicated in bacterial virulence (Desvaux et al. 2004)."

The simplest secretion mechanism is exhibited by the AT-1 proteins. AT-1 systems are dedicated to the secretion of a single specific polypeptide across the outer membrane. Proteins secreted by this mechanism have a similar structure, comprised of four functional domains: (1) an unusually long signal sequence; (2) a passenger domain; (3) a linker region; and (4) the β-domain. The autotransported protein is synthesized and targeted for export through the inner membrane by its signal sequence. After export across inner membrane by the general secretory (Sec) machinery, the N-terminal signal sequence is removed and the C-terminal β-domain is inserted into the outer membrane, where it forms a β-barrel channel. The covalently attached N-terminal passenger domain is then translocated through this channel to the cell surface. Once the passenger domain is on the cell surface, it may undergo further maturation. Some passenger domains remain covalently attached to the β-domain and protrude from the bacterial cell surface. Other passenger domains are cleaved and either remain loosely associated with the cell surface or become released into the external environment. Virulence functions associated with

different passenger domains include proteolytic activity, adherence, biofilm formation, intracellular motility, cytotoxic activity, or maturation of another virulence determinant (Henderson et al. 2004, Newman and Stathopoulos 2004).

One goal of our research is to understand the role of the AT-1 proteins in the virulence of *Xf*-PD. Genomic analysis has identified six members of the AT-1 autotransporter family in *Xf*-PD. Four of these AT-1 proteins were first identified in *Xf*-CVC (Meidanis et al. 2002, Yen et al. 2002) and orthologs are present in all four sequenced *Xf* genomes. The passenger domains of three of these proteins (PD0218, PD0313, PD0950) are predicted to encode subtilisin-like serine proteases (Bateman et al. 2004). The passenger domain of the fourth protein (PD1879) is predicted to encode a member of the GDSL family of esterase/lipases (Bateman et al. 2004). The passenger domains of the last two *Xf*-PD AT-1 proteins, PD1379 and PD0528, contain tandem repeats of a 50-60 amino acid motif. This motif is designated as Pfam-B 3566 (Bateman et al. 2004) and ProDom Family PD25532 (Servant et al. 2002) and is found only in *Xf* species. PD1379 contains three copies of this repeat, whereas PD0528 contains six copies. PD1379 has orthologs in all four sequenced *Xf* strains. In contrast, PD0528 has orthologs in both *Xf*-ALS and *Xf*-OLS. However, in *Xf*-CVC, two adjacent genes exhibit homology to PD0528. The protein encoded by XF1265 shows homology to the PD0528 passenger domain, whereas the protein encoded by XF1264 shows homology to the PD0528 autotransporter domain. The absence of an intact PD0528 ortholog in *Xf*-CVC may be significant and could account for some of the observed differences in the pathogenicity and host ranges of *Xf*-CVC and *Xf*-PD. To address this and other questions concerning the role of these species-specific tandem repeats in *Xf* virulence, we have begun a detailed characterization of the putative autotransporter protein, PD0528.

**OBJECTIVES**

The long-term goal of this project is to analyze the outer membrane protein composition of *Xf* and to determine the role of the major outer membrane proteins in *Xf* cell physiology and virulence. Our specific objectives include:

1. Identifying the major outer membrane proteins of *Xf* and assigning them to a specific gene on the *Xf* chromosome.
2. Generating mutations in the genes encoding these outer membrane proteins and examining their impact on *Xf* cell physiology and virulence.

Here, we report on our characterization of one of these proteins, the putative type V autotransporter PD0528.

**RESULTS**

In the Symposium Proceedings for 2004, we described our assignment of one of the most abundant, integral *Xf* outer membrane proteins to the PD0528 locus (Igo 2004). Based on its predicted amino acid sequence, 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. Our identification of PD0528 as an integral outer membrane protein was based on the characterization of tryptic-digest generated fragments by MALDI-TOF mass spectrometry. This analysis also revealed that at least three of the six repeats in the PD0528 passenger domain are still attached to the C-terminal β-barrel domain. This would suggest that at least a portion of the PD0528 passenger domain is still anchored to the *Xf* cell surface.

In order to investigate the role of PD0528 in *Xf* cell physiology and virulence, we generated a null mutation in the PD0528 gene using the gene replacement method described by Feil et al. (2003). This procedure involved generating a plasmid, which carried a kanamycin resistance marker flanked on each side by ~800 base pairs of chromosomal sequences from immediately upstream and downstream of the PD0528 opening reading frame (ORF). This plasmid was then introduced into *Xf* by electroporation. The resulting kanamycin resistant transformants were screened by PCR to identify a mutant strain in which the PD0528 ORF was completely removed and replaced by the kanamycin resistance marker.

We next examined the impact of the PD0528 deletion mutation on the *Xf* membrane protein profile. In the Symposium Proceedings for 2003, we described our protocol for analyzing the protein profile of the *Xf* outer membrane (Igo 2003). This protocol requires a substantial quantity of *Xf* cells, since it involves rupturing the *Xf* cells with a French pressure cell and isolating the outer membrane fractions by sucrose density gradient centrifugation. During the past year, we have begun to use a new protocol that requires fewer *Xf* cells. Although this method does not allow us to distinguish between outer and inner membrane proteins, it allows us to quickly compare the total membrane profiles of different *Xf* strains. In this procedure, *Xf* membrane proteins were extracted using the BioRad ReadyPrep™ Protein Extraction Kit (Membrane 1) and then treated using the BioRad ReadyPrep 2-D Cleanup Kit. The proteins were then analyzed using SDS-polyacrylamide (PAGE) gel electrophoresis. In Figure 1, we compare the membrane profiles of a wild-type *Xf-PD* strain and an *Xf-PD* strain carrying the PD0528 deletion mutation. As expected, comparison of these profiles indicates that the band corresponding to the PD0528 protein is missing in the strain carrying the PD0528 null mutation.

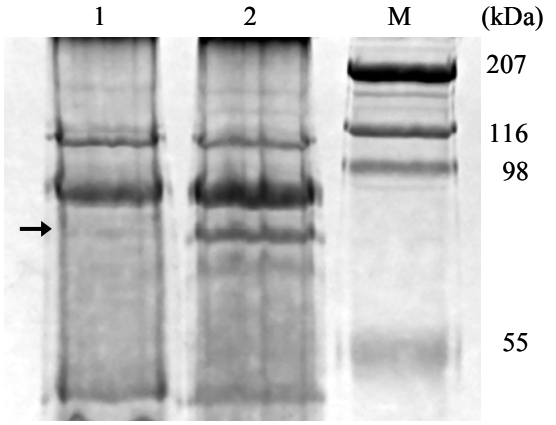


**Figure 1.** Membrane protein profile of the PD0528 deletion mutant.
Membrane proteins were isolated from both the PD0528 deletion mutant (Lane 1) and from the isogenic wild-type *Xf* Travers strain (Lane 2). The membrane proteins were separated on a 10% SDS-PAGE gel and stained with Coomassie blue. 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 deletion 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. Our preliminary characterization indicates that strains carrying the PD0528 deletion have a number of distinctive phenotypic properties. First, PD0528 null mutants grow faster than wild-type strains. Second, PD0528 null mutants are impaired in their ability to form cell-to-cell aggregates in liquid culture. Third, PD0528 null mutants are able to form a continuous lawn on solid medium. These *in vitro* properties are similar to those reported by Guilhabert and Kirkpatrick (2005) for a Tn*5* insertion in PD2118, which encodes the putative hemagglutinin adhesion HxfA. The next step in our analysis will be to determine the impact of the PD0528 deletion mutation on adhesion to various substrates and on the progression of PD symptoms *en planta*.

Finally, we have begun to examine the genetic organization and the regulatory region of the PD0528 gene (Figure 2B).

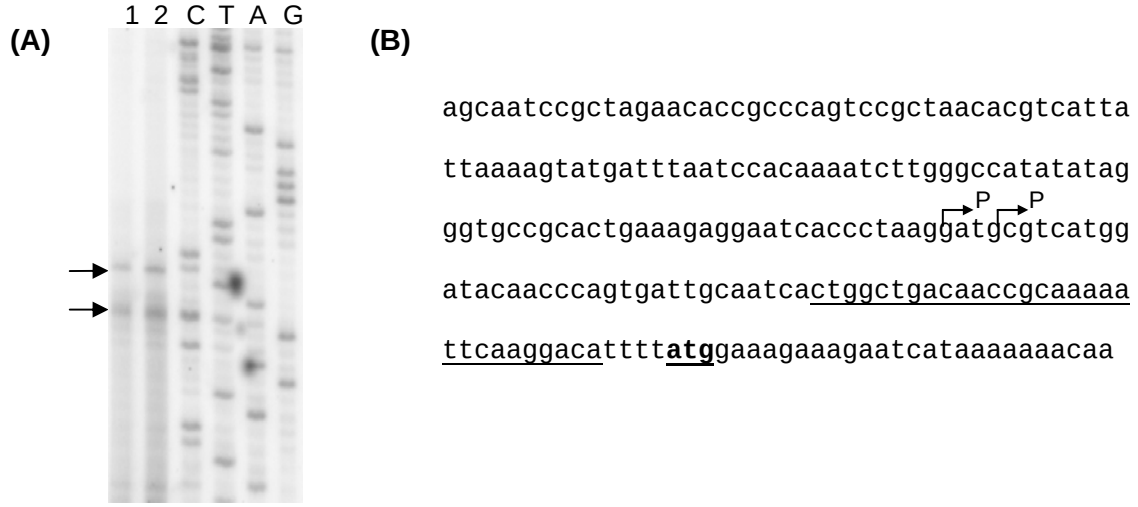


**Figure 2.** Identification of the PD0528 transcription startsite: **(A)** Primer extension analyses were carried out for RNA extracted from the exponentially growing *Xf* cells. Primer extension products from 12 µg (lane 1) and from 24 µg (lane 2) total RNA were subjected to electrophoresis on 8% DNA sequencing gel. The arrows indicate the positions of primer extension products. Lanes C, T, A and G show the results of a dideoxy sequencing reaction using the same primer on a plasmid carrying the PD0528 regulatory region. **(B)** The nucleotide sequence of the region immediately upstream of the PD0528 open reading frame is shown. The arrows indicate the location of the two major start site of transcription. The putative ATG start codon of the PD0528 gene is indicated in bold and underlined. The primer for primer extension experiment and the DNA sequencing reaction is underlined.

To determine the location of the PD0528 promoter, we have performed primer extension analysis using a primer that mapped slightly upstream of the PD0528 translation initiation codon. This primer was labeled with $\gamma$-$^{32}$P and hybridized to total RNA extracted from an exponentially growing culture of *Xf* Temecula 1. The resulting products of the primer extension reactions were then analyzed on a standard 8% DNA sequencing gel. As shown in Figure 2A, two major bands were observed in our primer extension reactions. The sizes of these bands allowed us to map the PD0528 mRNA start site to approximately 70 bases upstream of the PD0528 translational initiation codon (ATG). Experiments are currently underway to identify the bases important for PD0528 promoter activity and to determine the form of RNA polymerase responsible for generating the PD0528 mRNA.

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