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

Project Leader:
Michele M. Igo
Section of Microbiology
University of California
Davis, CA 95616
mmigo@ucdavis.edu

Cooperators:
Ayumi Matsumoto
Dept. of Food Science & Technology
University of California
Davis, CA 95616
amatsumoto@ucdavis.edu

Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 95616
bckirkpatrick@ucdavis.edu

Reporting Period: The results reported here are from work conducted October 1, 2005 to September 30, 2006.

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. At last year's Symposium, we reported our studies characterizing a strain containing a kanamycin insertion in the putative autotransporter protein PD0528. Here, we report the results of our complementation analysis with the PD0528::Km mutant strain. Reintroduction of the wild-type PD0528 gene into the mutant strain restored the presence of PD0528 and a number of other proteins to the *Xf* outer membrane. However, reintroduction of PD0528 did not correct the phenotypic properties of the mutant strain or restore a wild-type phenotype. This would suggest that a second mutation(s) was acquired during the initial construction of the PD0528::Km mutant strain. Therefore, we decided to regenerate a null mutation in PD0528 using a variety of different strategies. None of these strategies were successful, which suggests that PD0528 might be an essential gene. During the course of these experiments, we also discovered a technical problem with the selection method we used for gene replacement in *Xf*. Although this may be a problem unique to our laboratory, we decided to include these data in case other researchers in the field are running into similar technical difficulties.

INTRODUCTION
The Gram-negative bacterium *Xylella fastidiosa* (*Xf*) is the causative agent of Pierce's disease (PD) of grapevine (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 (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 (Preston *et al.* 2005). 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 (for a review, see 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. Three of these proteins (PD0218, PD0313, PD0950) are predicted to encode subtilisin-like serine proteases (Bateman *et al.* 2004). The fourth protein (PD1879) is predicted to encode a member of the GDSL family of esterase/lipases (Bateman *et al.* 2004). The last two AT-1 proteins, PD1379 and PD0528, contain tandem repeats of a 50-60 amino acid motif within their passenger domains. PD1379 contains three copies of this repeat, whereas PD0528 contains six copies. 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. To address this and other questions concerning the role of these species-specific tandem repeats in *Xf* virulence, we have been conducting a detailed characterization of the putative autotransporter protein, PD0528. Our studies of PD0528 were also designed to develop the protocols and genetic tools necessary for characterizing all six *Xf*-PD AT-1 autotransporters.

OBJECTIVES
1. 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.
2. Generate a mutation in each of the six AT-1 genes and determine their impact on *Xf* cell physiology and virulence.]
3. 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
One of the most abundant, integral *Xf-PD* outer membrane proteins is the gene product encoded by the PD0528 locus. 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. In order to investigate the role of PD0528 in *Xf-PD* 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 the plasmid pAM12, which carried a kanamycin resistance marker flanked on each side by 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 selected on PD3 containing 5 μg/ml kanamycin and screened by PCR to identify a mutant strain in which the PD0528 ORF was completely removed and replaced by the kanamycin resistance marker.

Our initial characterization of the PD0528::Km deletion mutation was reported in the Symposium Proceedings for 2005. In these experiments, *Xf* membrane proteins were extracted using the BioRad ReadyPrep™ Protein Extraction Kit (Membrane 1). Although this method did not allow us to distinguish between outer and inner membrane proteins, it allowed us to quickly compare the total membrane profiles of different *Xf* strains. As expected, comparison of the membrane profiles of a wild-type *Xf-PD* strain and an *Xf-PD* strain carrying the PD0528::Km mutation revealed that the band corresponding to the PD0528 protein is missing in the PD0528::Km mutant. However, it was not possible to use membranes prepared with the protein extraction kit to obtain more detailed information concerning the impact of the PD0528::Km mutation on the *Xf* outer membrane protein profile. Therefore, we repeated this analysis using membranes prepared by a different method. In these experiments, the *Xf* strains were grown in 1 liter of PD3 medium and the harvested cells were then rupture with a French pressure cell as described previously (Igo 2003). The outer membrane fractions were isolated by sucrose density gradient centrifugation. The proteins were then analyzed using SDS-polyacrylamide (PAGE) gel electrophoresis.

Comparison of the membrane profiles of a wild-type *Xf* Temecula strain and an isogenic PD0528::Km mutant revealed that there are many differences in the outer membrane protein profiles of the two strains (Figure 1). Similar results were obtained with an *Xf* Travers strain containing the PD0528::Km mutation (data not shown). In addition to the absence of the PD0528 protein, the outer membrane of the PD0528::Km mutant is missing a number of other outer membrane proteins. One simple explanation for this result is that the absence of the PD0528 protein is causing a major perturbation in the protein composition of the outer membrane and of the *Xf* cell surface. However, it is also possible that a second mutation(s) was acquired during the construction of the original PD0528::Km mutation and that this second mutation(s) is responsible for the phenotype.

To distinguish between these possibilities, we performed complementation analysis using the plasmid pAM61. pAM61 carries the wild-type PD0528 gene and is a derivative of the plasmid pBBR1MCS-5 (Kovach *et al.* 1995). We chose this plasmid vector because a pBBR1MCS-5 derived plasmid was successfully used by Gabriel and his colleagues for complementation of the *Xf tolC* gene *en planta* (Gabriel 2005). We began our complementation analysis by introducing the plasmid pAM61 into the PD0528::Km mutant and then compared the membrane protein profile of the resulting strain to the wild-type strain and the PD0528::Km mutant. As shown in Figure 1, the PD0528 protein is present in both the wild-type strain (Lane 1) and the PD0528::Km mutant strain containing plasmid pAM61 (Lane 3). The identification of the band indicated by the arrow as the PD0528 protein was confirmed by MALDI-TOF mass spectrometry. Moreover, the reintroduction of the PD0528 gene into the mutant strain also restores many proteins to the outer membrane that were missing in the outer membrane of the PD0528::Km mutant. This would suggest that the absence of the PD0528 protein is having a profound impact on the protein composition of the outer membrane. However, it is worth noting that the reintroduction of PD0528 does not completely restore the wild-type outer membrane profile, suggesting that there might be a second mutation(s) in the PD0528::Km mutant strain.

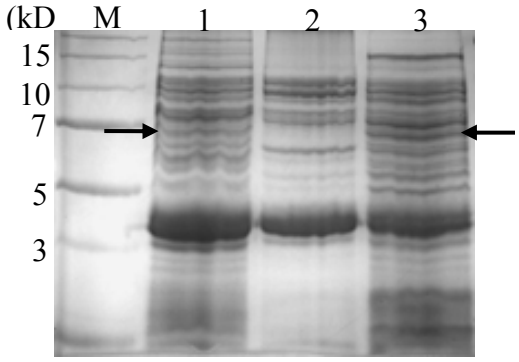

FIGURE 1. Membrane protein profiles.
Membrane proteins were isolated from the wild-type Temecula strain (Lane 1), the PD0528 deletion mutant (Lane 2), and from a strain containing both the PD0528 deletion mutant and pAM61, which carries a wild-type copy of the PD0528 gene (Lane 3). The membrane proteins were separated on an 8% 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 PD0528 protein on the gel is indicated by the arrows.

We next examined how reintroduction of PD0528 impacted the phenotypic changes observed in the PD0528::Km mutant. As reported in the Symposium Proceedings for 2005, the strain containing the PD0528::Km mutation has a number of distinctive phenotypic properties: it grows faster than wild-type strains, it is impaired in its ability to form cell-to-cell aggregates in liquid culture, and it is able to form a continuous lawn on solid medium. We expected that the reintroduction of PD0528 to the PD0528::Km mutant would restore the wild-type phenotype. However, this was not the case. As summarized in Table 1, the phenotypic properties of the PD0528::Km mutant strain are not altered by reintroduction of the wild-type PD0528 gene on plasmid pAM61. This would suggest that there is a second mutation(s) in the PD0528::Km mutant and that this second

mutation(s) is responsible for the distinctive phenotypic properties of the PD0528::Km mutant. Since the properties of the second mutation(s) could be masking the phenotypic defects caused by the PD0528::Km mutation, we cannot draw any conclusions concerning how the absence of PD0528 impacts *Xf* cell physiology.

Table 1. Summary of the phenotypic comparison

	Wild-type	PD0528::Km (a)	PD0528::Km + pAM61 (b)
Growth in liquid culture	~7-10 days	~4-5 days	~4-5 days
Biofilm	Yes	No	No
Confluent lawns on plate	No	Yes	Yes
Produce PD symptoms	Yes	No	No

(a) These characteristics are present in both a PD0528::Km Temecula strain and a PD0528::Km Travers strain.
(b) After obtaining these results, we extracted pAM61 from the PD0528::Km mutant and resequenced the PD0528 gene on pAM61 to confirm that a functional copy of PD0528 had in fact been introduced into the strain.

We considered two possible explanations, which are not mutually exclusive, for the presence of this second mutation(s). One possibility is that the second mutation(s) is a consequence of the way we generated the PD0528::Km mutation. However, it is also possible that the PD0528 gene is essential. According to this hypothesis, the presence of the second mutation(s) is necessary for *Xf* survival in the absence of PD0528. To determine if either of these explanations is correct, we decided to regenerate the PD0528 null mutation. In one experiment, we introduced the PD0528::Km mutation using the gene replacement plasmid pAM12, but selected for transformants on PD3 plates containing either 5 µg/ml or 10 µg/ml kanamycin. In a second experiment, we introduced a PD0528::Cm mutation using a gene replacement plasmid similar to pAM12, which carried a chloramphenicol resistance marker. As a control, we also generated similar gene replacement plasmids for another *Xf* gene, PD0939, which encodes a phage-related protein. Transposon insertions into this gene have no effect on *Xf* physiology or pathogenicity (Guilhabert and Kirkpatrick, unpublished).

The results from this analysis, which are presented in Table 2, allowed us to draw a number of conclusions. First, when we select for either the PD0528::Km mutation or the PD0939::Km mutation on PD3 medium containing 5µg/ml kanamycin, we obtain fast growing transformants that are unable to form biofilms. We have performed this analysis multiple times and monitored the plates for the appearance of slower growing transformants over time. Nonetheless, in spite of repeated attempts, slow growing transformants were not obtained using PD3 medium containing 5µg/ml kanamycin as the selective medium. Second, we were able to obtain slow growing PD0939::Km transformants when we used PD3 medium containing 10 µg/ml kanamycin and PD0939::Cm transformants when we used PD3 medium containing 5 µg/ml chloramphenicol. Since the phenotypic properties of these transformants are the same as those of the PD0939::EZ-TN KAN-2 mutant, we have concluded that the fast-growing transformants we obtained by selecting on PD3 medium containing 5µg/ml kanamycin arose as a consequence of our selection procedure and that these transformants have probably acquired a second mutation(s). Third, although we made repeated attempts, we have been unable to generate PD0528::Cm transformants or PD0528::Km transformants when selecting on PD3 medium containing 10 µg/ml kanamycin. This would suggest that PD0528 is an essential gene. Experiments are currently underway to test this hypothesis.

Table 2. Summary of the transformation results

	Selection	*Xf* transformants	Growth in liquid	Biofilm	Confluent lawn
PD0939::EZ-TN KAN-2	Km-5µg/ml	Yes	7-10 days	Yes	No
PD0939::Km	Km-5µg/ml	Yes	4-5 days	No	Yes
	Km-10µg/ml	Yes	7-10 days	Yes	No
PD0939::Cm	Cm-5µg/ml	Yes	7-10 days	Yes	No
PD0528::Km	Km-5µg/ml	Yes	4-5 days	No	Yes
	Km-10µg/ml	No	-	-	-
PD0528::Cm	Cm-5µg/ml	No	-	-	-

During the last year, we have also examined the feasibility of using the PD0528 promoter for expressing genes in *Xf*. For this analysis, we generated fusion constructs between the PD0528 promoter and the firefly luciferase gene on plasmid pBBR1MIC-3, which confers tetracycline resistance (Kovach *et al.* 1995). The resulting plasmid was then introduced into the *Escherichia coli* strain DH5α and into the *Xanthomonas campestris pv. campestris* (*Xcc*) strain Xcc8004. Our initial results are very promising. Although the PD0528-*luc* fusion is expressed at a very low level in *E. coli*, it is highly expressed in *Xcc*. Because there is not a homolog to PD0528 in *Xcc*, it might be possible to gain insights into the factors involved in the regulation of PD0528 in *Xf* by examining its regulation in *Xcc*.

REFERENCES

Bateman, A., et al. 2004. The Pfam protein families database. Nucleic Acids Res 32:D138-41.

Feil, H., W. Feil, J. Detter, A. Purcell and S. Lindow. 2003. Site-directed disruption of the fimA and fimF fimbrial genes of *Xylella fastidiosa*. Phytopathol 93:675-682.

Gabriel, D. W. 2005. Role of type I secretion in Pierce's disease, p. 158-161. In M. Athar Tariq et al. (eds.), Proceedings of the 2005 Pierce's Disease Research Symposium, 5-7 December 2005, San Diego, CA.

Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez, and D. Ala'Aldeen. 2004. Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev 68:692-744.

Hopkins, D., and A. Purcell. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. Plant Disease 86:1056-1066.

Igo, M. 2003. The *Xylella fastidiosa* Cell Surface, p. 56-58. In M. Athar Tariq et al. (eds.), Proceedings of the 2003 Pierce's Disease Research Symposium. 8-11 December 2003, Coronado, CA.

Kovach, M. E. et al. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 166:175-176.

Meidanis, J., M. D. Braga, and S. Verjovski-Almeida. 2002. Whole-genome analysis of transporters in the plant pathogen Xylella fastidiosa. Microbiol Mol Biol Rev 66:272-99.

Preston, G., D. Studholme, and I. Caldelari. 2005. Profiling the secretomes of plant pathogenic Proteobacteria. FEMS Microbiol Rev 29:331-360.

Smolka, M. B. et al. 2003. Proteome analysis of the plant pathogen *Xylella fastidiosa* reveals major cellular and extracellular proteins and a peculiar codon bias distribution. Proteomics 3:224-37.

FUNDING AGENCIES

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

ISOLATION, CHARACTERIZATION AND GENETIC MANIPULATION OF *XYLELLA FASTIDIOSA* HEMAGGLUTININ GENES

Project Leader:
Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA
bckirkpatrick@ucdavis.edu

Cooperators:
Tanja Voegel
Department of Plant Pathology
University of California
Davis, CA
tmvoegel@ucdavis.edu

Jeremy Warren
Department of Plant Pathology
University of California
Davis, CA
jgwarren@ucdavis.edu

Michele Igo
Section of Microbiology
University of California
Davis, CA
mmigo@ucdavis.edu

George Bruening
Department of Plant Pathology
University of California
Davis, CA
gebruening@ucdavis.edu

Paul Feldstein
Department of Plant Pathology
University of California
Davis, CA
pafeldstein@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2006 to September 2006.

ABSTRACT
Xylella fastidiosa (*Xf*) possesses genes for hemagglutinins (HAs), large adhesion proteins involved in cell-cell aggregation and biofilm formation. Mutations in either one of the functional HAs, HxfA (PD2118) or HxfB (PD1792), result in hypervirulent strains that move faster and cause more severe disease in grapevines. Computer analyses of the HA proteins identified several regions that might be possible adhesion domains (ADs) responsible for cell-cell and/or cell-surface binding. We cloned 6 *Xf* HA fragments that may contain potential ADs into protein expression vectors and to date have prepared antibodies against 1 AD protein fragment that is conserved in both HxfA and B. Recombinant proteins from the other 5 ADs are being purified and prepared for injection. Western blot analyses of using *Xf* proteins extracted from *Xf* cells grown in liquid culture showed a very faint reaction with an *Xf* protein of approximately 220kd but this result needs to be confirmed using a higher quality antibody. Recent discoveries in the Bruening lab using phage technology indicate that HA are more abundant in cells grown on solid medium compared to liquid medium and we are now repeating our Western analyses using cells grown on solid medium. Once *Xf* HA cell-cell binding domains are identified they will be expressed in transgenic tobacco and grapevines where we hope the proteins will act as a "molecular glue" to aggregate insect-inoculated *Xf* cells, retard their ability to systemically colonize plants and potentially provide a unique form of resistance against PD.

INTRODUCTION
Xylella fastidiosa (*Xf*) hemagglutinins (HAs) are large secreted proteins (200-300kD) that play important roles in mediating cell-cell contact and plant pathogenicity. Mutations were made in both *Xf* HA genes, HxfA (PD2118) and HxfB (PD1792), by transposon mutagenesis and the resulting mutants did not form aggregates in liquid culture and they had reduced biofilm formation *in vitro* and *in planta* (Guilhabert and Kirkpatrick 2005). When inoculated into grapevines the mutant cells showed hypervirulence and more rapid colonization of xylem vessels (Guilhabert and Kirkpatrick 2005). The premise of this research is to determine whether over-expressing *Xf* HA adhesion domains in the xylem, either by transformation of grapevines or inoculation of grapevines with HA expressing endophytes, the HA will act as a "molecular glue" which clumps the *Xf* cells and retards their ability to systemically colonize grapevine and cause Pierce's disease (PD).

Because of the large size of the HA genes (10kb), we cannot transform grapevines with the whole HA gene. Therefore we are trying to identify the active adhesion domains (ADs) responsible for cell-cell aggregation by dividing the HA genes into several smaller fragments that we believe will contain the cell-cell AD. Recombinant proteins derived from these fragments are being expressed in *E. coli*, purified and injected into rabbits to produce AD specific antisera. The resulting antisera will be used in ELISA, Western blot analysis, immunolocalization studies and cell-cell clumping experiments to determine which of the HA fragment(s) contain functional ADs that can later be transformed into plants.

OBJECTIVES
1a. Use antibodies we have prepared against a conserved, putative binding domain (AD2) that is present in both *Xf* hemagglutinins (HA), which we have named HxfA and HxfB, to determine the native size and location of *Xf* HA in cultured *Xf* cells and PD-affected grapevines.
b. Determine if these antibodies (Fab fragments) can prevent cell-cell clumping in liquid *Xf* cultures.