EVALUATING THE ROLES OF PILI IN TWITCHING AND LONG DISTANCE MOVEMENT OF *XYLELLA FASTIDIOSA* IN GRAPE XYLEM AND IN THE COLONIZATION OF SHARPSHOOTER FOREGUT

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ABSTRACT
Our investigations have focused on intra-plant movement and colonization by *Xylella fastidiosa* (*Xf*). This study is particularly directed toward elucidating how *Xf*, once introduced into xylem vessels, moves in these elements farther upstream (down shoots and canes). In addition, it reports on the influence of the physical and chemical environments of the xylem as they relate to *Xf* attachment, colony development, and biofilm formation in both plant tissues as well as microfludic chambers fabricated to mimic xylem elements. Toward these goals, we have made extensive use of mutants deficient for various traits of pili and fimbriae.

INTRODUCTION
How *Xylella fastidiosa* (*Xf*) moves in xylem elements upstream against the flow of the transpiration stream and into petioles from the leaf or down shoots and canes has long been a particularly puzzling and important question, the answer of which could provide clues about better disease control practices through knowledgeable timing of pruning or roguing. Certainly, it would provide significant advances in the biology of the pathogen. Our studies have been directed toward elucidating movement of *Xf in planta* and *in vitro*, as well as how these bacteria colonize and establish biofilms. Toward this, we have made use of a number of *Xf* mutants deficient in traits important for cell attachment, movement, and colonization. Previously, we reported that migration of individual *Xf* bacteria occurred both *in vitro* and *in planta*, and that such migration occurred against a strong current of flowing media (Meng et al., 2005; Hoch, 2005). Such movement is characteristic of twitching motility that occurs in some gram-negative bacterial species. It is mediated by type IV pili (Mattick, 2002). There are several important implications of this observation: this was not only the first observation of twitching movement by a non-flagellated plant pathogenic bacterium (albeit, *Ralstonia solanacearum*, that sometimes has flagella, has been shown to exhibit colony features characteristic of twitching (Liu et al., 2001)), it was also the first time that such movement by *Xf* was observed. Such motile behavior is important in explaining, in part, the query posed above about how the bacteria spread in the grapevine from an inoculation point to upstream locations.

Type IV pili are long (1-5.8 μm in length) filamentous appendages (a.k.a, fimbriae) located at either one or both poles, depending on the species (Bradley, 1980), are generally 5-7 nm in diameter, and may be up to several micrometers in length. They are assembled primarily from single structural protein subunits, pilin (PilA) (Mattick, 2002). Twitching movements are generated as the pili are retracted and dissembled. Because the pili tips are attached to the substratum, the cell moves toward that point of contact as the pili shorten (Mattick, 2002; Skerker and Berg 2001). Genomic analysis of *Xf* indicates that there are at least 26 genes related to pili synthesis and function (Simpson et al., 2000). In addition to the type IV pili, *Xf* has shorter (0.4-1.0 μm in length) type I pili, also positioned on the same cell pole. The unique dual-pili composition of the wild type *Xf* presents an opportunity to study the two types of pili comparatively in the same experimental setting. Biofilm deficient mutants (e.g., 6E11), the result of a disruption of the *fimA* gene (lacking type I pili), were previously shown to continue to migrate since they still possess the type IV pili; whereas, mutants deficient in genes that code for type IV pili (e.g., 1A2) are migration deficient and develop robust biofilms (Meng et al., 2005). Attachment of *Xylella* cells at their polar ends is well documented in the precibarium region of the sharpshooter foregut. At this point, however, little is known about how they attach in this orientation (other than the conjecture that the pili may be involved) to this preferred region, as opposed to other foregut regions. Additionally, little is known about how they detach from this region.

OBJECTIVES
Our goal is to understand how *Xf* colonizes plants and insects. One aim is to identify factors that contribute to attachment (and detachment) and migration of *Xf* cells on surfaces. Using wild-type and mutants of *X. fastidiosa*, we have proposed to

examine temporal and spatial interactions on both native and artificial surfaces using a microfabricated *in vitro* microfluidic system that mimics features of xylem vessels. It has thus far provided significant new insight into the dynamics of *Xf* cell-surface relationships.

RESULTS

Mutants. As previously reported (Hoch and Burr, 2005), the EZ::TN Transposome system was used to generate Kanamycin-resistant mutants from the Temecula isolate of *Xf* (Guilhabert et al., 2001). Mutants with deficiencies in pilus and/or fimbrial gene products were sought that would affect colony and biofilm development, and the ability to migrate via type IV pilus twitching motility. We previously reported that *Xf* mutants (1A2, 5A7, and 6E11) were deficient in the genes *pilB*, *pilQ*, and *fimA*, respectively (Meng et al., 2005). The first two mutants are deficient in twitching motility characteristics since they lack type IV pili, while the latter mutant retains its motility phenotype, having type IV pili, but lacking the shorter type I pili. We have now generated more than 30 single-site mutations representing deficiencies in more than 14 genes associated with pili and fimbria function. 'Double mutants' were generated through a second round of mutagenesis using trimethoprim (as the selection agent) of the 6E11 (*fimA*) *Xf* mutant and has resulted in mutants deficient for the genes *fimA/pilC*, *fimA/pilO*, *fimA/pilX* (Table 1).

Table 1. Partial listing of *Xf* Temecula mutants.

Gene	Single Mutation	ORF	Gene Product	Observed Characteristics *in vitro*
pilB	1A2	PD1927	Pilus biogenesis protein	No twitching motility.
pilQ	5A7	PD1691	Fimbrial assembly protein	No twitching motility.
fimA	6E11	PD0062	Fimbrial subunit precursor	Twitching motility. Colony 'fringe' wider than wild type.
pilX	20D10	PD0022	PilX protein	Twitching motility in chamber not examined. Colony 'fringe' deficient.
pilO	TM1	PD1693	Fimbrial assembly membrane protein	No twitching motility
pilO	TM5	PD1693	Fimbrial assembly membrane protein	Twitching motility in chamber not examined. Colony 'fringe' deficient.
pilC	TM6	PD1923	Fimbrial assembly protein	Twitching motility in chamber not examined. Colony 'fringe' reduced.
pilR	TM7	PD1928	Two component system regulator	Twitching motility in chamber not examined. Colony 'fringe' deficient.
pilY1	TM14	PD0023	PilY1 protein homolog, pilus tip protein	Twitching motility. Colony margin smooth to crenulate.
pilR	TM17	PD1928	Two component system regulator	Twitching motility in chamber not examined. Colony 'fringe' deficient.
	Double Mutation			
fimA, pilO	DM12	PD0062 PD1693	Fimbrial subunit precursor Fimbrial assembly membrane protein	No twitching motility. Colony 'fringe' deficient.
fimA, pilX	DM16	PD0062 PD0022	Fimbrial subunit precursor PilX protein	No twitching motility. Colony 'fringe' deficient.
fimA, pilC	DM15 DM11	PD0062 PD1923	Fimbrial subunit precursor Fimbrial assembly protein	No twitching motility. Colony 'fringe' deficient.

Transmission electron microscopy (TEM), atomic force microscopy (AFM), and/or confocal microscopy (LSCM) using Agdia's antibody to *Xylella* (Carbajal et al., 2004) were used to assess for the presence of pili. In addition, light microscopy in conjunction with time-lapse imaging and image analysis was used extensively to assess motility, motility rates, adhesiveness of *Xf* wild-type and mutants to substrata, colony development, and cell 'autoaggregation'. Microfluidic devices fabricated to mimic xylem vessel characteristics were prepared similar to the protocols previously described (Meng et al., 2005).

Disease development in grapevines infected by pili-defective mutants. To date, a number of pilus-defective mutants have been introduced into greenhouse grown Cabernet Sauvignon vines and evaluated for PD symptom development. Notable is that the wild-type Xf Temecula isolate and the mutant 6E11 (type IV pili only) expressed symptom development at about the same time (9 weeks following needle inoculation), where as the double mutant DM12 (Table 1) remained asymptomatic until the 18th week. The single mutant TM7 remains asymptomatic to date. Similar results with these mutants are being observed by our cooperator Rodrigo Almeida in an ongoing project..

Figure 1. PD symptom expression of WT *Xf* vine 10 weeks after inoculation. Asymptomatic vine (17 weeks) inoculated with mutant DM12

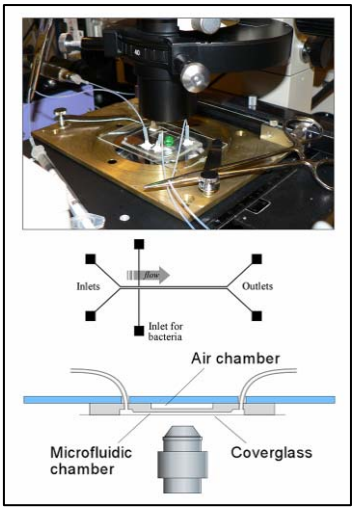

Figure 2. Microscope and dual channel (80 µm wide, 50 µm deep) microfluidic chamber configuration.

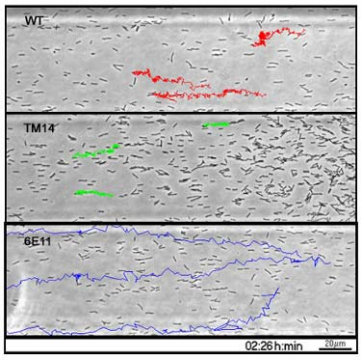

Figure 3. Relative rate of twitching motility of *Xf* wild type and pili-defective mutants. Color tracks correspond to twitching distance (right to left) over 2h 26min. Media flow, left to right.

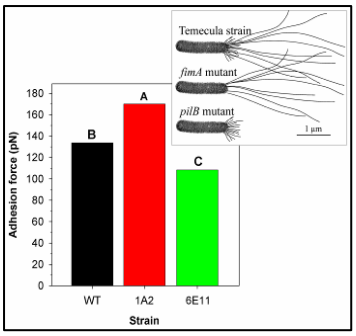

Figure 4. Adhesion forces required to remove *Xf*. Letters A, B, and C indicate differences in significance at 0.05.

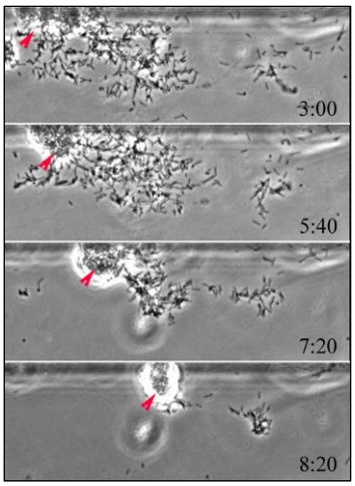

Figure 5. Autoaggregation in 9-day old culture, first noted as dispersed cells aggregated into developing cell mass (upper frame). Cells continued to be attracted to the developing aggregate (arrowheads). Times, h:min.

Motility of pili-defective mutants. We reported that *Xf* moves via type IV pili-dependent twitching-motility in 'artificial xylem' vessels against a flow (Meng et al., 2005). We now report that mutations in the pili genes influence not only the ability of the cell to exhibit twitching motility, but also the rate at which they move. Clearly, mutants without type IV pili, e.g., 1A2 (*pilB*), 5A7 (*pilQ*) are incapable of moving as has been previously demonstrated (Meng et al., 2005); however, there are a number of type IV pili mutations that do influence motility. To examine rates of movement, a microfluidic chambers was designed with side-by-side channels separated by a 50 µm-wide partition so that two mutants or isolates could be compared at the same time, under the same conditions (Figure 2). We compared the rate of twitching-motility of the wild type isolate with mutants TM14 (*pilY1*$^{-}$) and 6E11 (*fimA*$^{-}$) against a constant stream flow. The wild type isolate moved at 0.7 µm min^{-1}, whereas mutant TM14, which lacks a *tip protein* of the type IV pili, exhibited a significantly slower motility rate of 0.2 µm min^{-1} (Figure 3). Mutant 6E11 (lacking the short type I pili) moves 7 times faster than the wild type (5 µm min^{-1}). The short type I pili likely function as a "brake" reducing the speed of movement of wild type compared to the mutant 6E11.

These results agree with our observations (see **Adhesiveness of type I and type IV pili**) that type I pili support the strongest adhesion force between the bacterial cells and a surface, as well as developing greater amounts of biofilm (Li et al., 2006).

Adhesiveness of type I and type IV pili. Adherence to a surface is a crucial early event in the process of bacterial biofilm formation. Adherence is especially important for *Xf* since it inhabits xylem vessels where sap is flowing. Using microfluidic devices we measured the adhesion force of *Xf* wild-type and pili-defective mutants. *Xf* cells were introduced into the chambers where they attached to the substratum. Adhesiveness of the cells was assessed by gradually increasing the flow of media through the chamber with a syringe pump. The number of cells remaining attached to the glass surface after each flow rate increase was captured digitally and scored. By means of a computational model we determined the adhesion force exerted by each bacterial cell. Using selected pili mutants, the role of pili-type in adhesion was determined. Mutant 1A2 (short type I pili-only) had a significantly greater adhesion force (170 pN) than the wild type isolate (both type I and type IV pili) (134 pN). Mutant 6E11 which posses only long type IV pili had the weakest adhesion (108 pN) (Figure 4). These results further confirm that type I pili play a dominant role in *Xf* attachment to a substratum. The presence of type IV pili reduces the strong attachment exerted by type I pili, as was observed in the wild type isolate.

Cell aggregation and autoaggregation. When, where, and how individual *Xf* cells come together to form aggregates and biofilms to block xylem vessels remains poorly understood. It has been assumed that such cell masses develop as sessile cells divide, remain in place, and slowly accumulate mass. Such 'slow' aggregation does not explain how symptom development (reddening and drying of leaf margins) often occurs within a short time span—from overnight to a few days. We discovered using time-lapse imaging that 'autoaggregation' of many dispersed *Xf* cells into large cell masses occurs over relatively short time periods following 7-11 days of growth. Such large aggregates developed over periods ranging from 3-10 hours (Figure 5).

Influence of BSA on twitching motility. It was previously shown that the wild-type *Xf* Temecula isolate developed a 'peripheral fringe' around colonies (Meng *et al*., 2005) grown on PW agar, a trait associated with type IV pilus-mediated twitching motility. During the course of a survey of various wild-type pathogenic *Xf* isolates to ascertain that twitching motility is a characteristic of other wild-type isolates, we discovered that the concentration of bovine serum albumin (BSA) in the medium dramatically influenced whether or not a peripheral fringe was associated with the colonies. For some isolates, as well as the *fimA* mutant 6E11, a barely perceivable fringe was frequently observed at the highest BSA concentration (3.5-6.0 g L^{-1}), while medium with no or low BSA (0-1.8 g L^{-1}) produced the widest and most pronounced fringe. At the same time, the higher BSA concentrations nearly always produced larger colonies which, in part, substantiated the original report describing the need for BSA in PW medium for *Xf* (Davis *et al*., 1980).

CONCLUSIONS

Observations from this period demonstrate the pronounced role that pili and fimbriae have in *Xf* attachment, movement, colonization, and biofilm formation, as well as being involved in the timing of disease expression. We have again demonstrated that 'artificial xylem vessels' can be used to gain valuable information about the biology of *Xf*, and to infer roles for these phenomena *in planta*. Temporal and spatial data are not easily obtainable *in planta* without destructive sampling. In this report we describe autoaggregation in *Xf*, a phenomenon that could explain the rapid development of symptoms in grapevines affected by PD. The data on the speed of movement and the adhesiveness to a surface by different pili-defective mutants provide information on the relevance of type I and type IV pili regarding cell attachment and motility. Type I pili have a decisive role in "slowing" down cell motility and strongly attaching the cells to the surface. We demonstrated that the appearance of a peripheral fringe around *Xf* colonies is suppressed by higher BSA concentrations, thus explaining why such a colony morphology (and twitching motility) has not been readily observed.

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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 (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