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
Xylella fastidiosa cells were shown to exhibit twitching motility 'upstream' in microfabricated 'artificial xylem vessels'. Such motility is due to extension and retraction of type IV pili present on the poles of the bacteria. Importantly, such upstream migration was subsequently demonstrated *in planta*. A survey of isolates from California, Texas and South Carolina revealed that all possessed motility characteristics. A number of mutants deficient in genes associated with type IV pilus functions were created, many of which may be useful in exploring targets for slowing development of the bacterial mass in xylem vessels. Type IV and type I pili were shown to have pronounced effects on colony and biofilm development.

INTRODUCTION
Once *Xylella fastidiosa* is introduced into xylem vessels in leaf, petiole, or other susceptible green tissues, how does it move in xylem elements farther upstream, e.g., into petioles from the leaf or down shoots and canes? This has long been a particularly puzzling and important question since xylem sap flow during the growing season is nearly always down the pressure gradient, viz., toward the leaf. It is seldom stagnant. Since *Xf* are non-flagellated bacteria, the consensus (albeit unproven) for their appearing in previously non-invaded regions upstream has been through the slow expansion of the colony through repeated cell division along xylem vessel walls. Lateral movement, from xylem element to element, has been proposed through dissolution of border pit membranes (Newman et al., 2004); but again, this does not explain long distance upstream movement.

Our investigations have focused on the effects of physical and chemical environments on attachment, colony development, and biofilm formation by *Xf* in microfludic chambers fabricated to mimic xylem elements. This has resulted in identifying unmistakable long distance migration of individual bacteria. Even more interesting was the observation that they were able to migrate against a strong current of flowing media (Meng et al., 2005; Hoch, 2005). The movement was characteristic of twitching motility that occurs in some gram-negative bacterial species (Mattick, 2002). There are several important implications of this observation: this is 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; Roine et al., 1996)), it is also the first time that such movement by *Xf* has been observed. Such motile behavior may be important in explaining how the bacteria spread in the grapevine from an inoculation point to upstream locations.

Type IV pili are filamentous appendages (fimbriae) located at either one or both poles, depending on the species (Bradley, 1980; Henrichsen, 1983), 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; Wall and Kaiser, 1999; Wolfgang et al., 2000). Type IV pili function and biogenesis in *Pseudomonas aeurginosa* involves more than 35 genes with conserved homologs existing in other bacteria that express twitching via type IV pili (Mattick, 2002). *Xf* likely produces type IV pili as its genome carries at least 26 genes that are related to pili synthesis and function (Simpson et al., 2000).

Xylella produces fimbriae that are thought to function in adhesion of the bacterium. Biofilm deficient mutants (e.g., 6E11), the result of a disruption of the fimA gene, continue to migrate since they still possess the type IV pili; whereas, mutants deficient in genes that code for type IV pili 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, nothing is known about how they detach from this region.

OBJECTIVE

Our goal is to understand how *Xf* colonizes plant and insect habitats. One aim is to identify factors that contribute to attachment (and detachment) and migration of *Xf* cells on habitat surfaces. Using wild-type and mutants of *Xf*, we will examine temporal and spatial interactions on both native and artificial surfaces using a microfabricated in vitro system that has thus far provided significant new insight into the dynamics of *Xf* cell-surface relationships.

RESULTS

Pili and fimbria mutants

The EZ::TN Transposome system was used to generate Kanamycin-resistant mutants from the Temecula isolate of *Xf* (Guilhabert et al., 2001). Mutants were sought with deficiencies in pilus and/or fimbrial gene products that would affect colony and biofilm development, and the ability to migrate via type IV pilus twitching motility. We previously reported that *Xf* mutants, designated as 1A2, 5A7, and 6E11 were deficient in the genes pilB (pilus biogenesis protein), pilQ (pilus assembly protein), and fimA (fimbrial subunit precursor), respectively (Meng et al., 2005). The first two mutants are deficient in twitching motility characteristics since they lack type IV pili, while the later mutant retains its motility feature, having type IV pili, but lacking the shorter-class of pili that we tentatively correlate with type I pili. Now, we have generated more than 30 single-site mutations representing deficiencies in more than 14 genes associated with pili and fimbria function. In addition, several others are yet to be sequenced. A second round of mutagenesis using trimethoprim (as the selection agent) of the 6E11 (fimA) *Xf* mutant has resulted in several 'double' mutants deficient for the genes fimA/pilC, fimA/pilO, fimA/pilX, and fimA and other second genes that have yet to be fully characterized. All are deficient for twitching motility as evidenced by colonies lacking a 'peripheral fringe.'

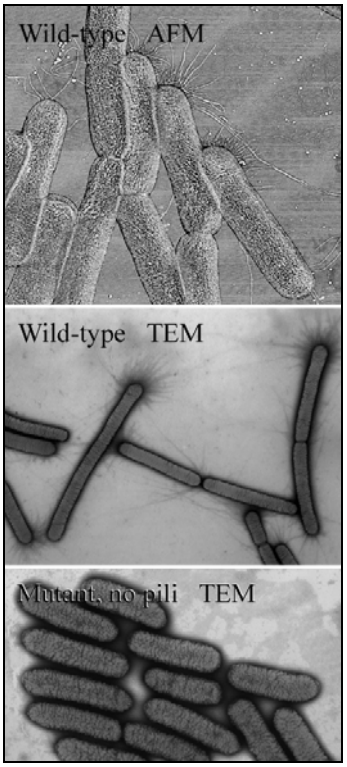

Figure 1. Pili of wild-type and mutant *Xf*. AFM and TEM images. Wild-type cells have an abundance of short pili, and fewer long type IV pili.

Presence or absence of pili were assessed by transmission electron microscopy (TEM), atomic force microscopy (AFM), and/or by confocal microscopy (LSCM) using Agdia's antibody to *Xylella* (Carbajal et al., 2004). *Xf* type IV pili are 1-6 μm in length as seen in wild-type *Xf* and mutants such as 6E11. In addition to the longer type IV pili, 0.4-1.0 μm long pili are also present on wild-type strains and on mutants such as 5A7 (Figure 1). *Xylella* strains with the abundant 'tuft' of type I pili (wild-type, and mutants, e.g., 1A2, 5A7) revealed a brightly staining spot at one pole of the cells when exposed to the Adgia antibody; whereas, those mutants, e.g., 6E11, with only the more sparse type IV pili or no pili at all did not have a polar staining spot (Figure 2).

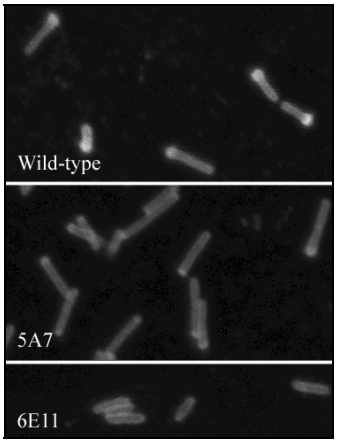

Figure 2. Antibody staining of cell surface and polar regions (wild-type, 5A7) bearing type I pili; polar staining is absent in 6E11 which lacks type I pili.

Upstream movement *in planta*

Vitis vinifera cv. Chardonnay plants were needle-inoculated at the seventh internode from the shoot apex with cell suspensions of wild-type and mutant (1A2, 5A7, and 6E11) *Xf*. After 11 weeks, vines were cut from the main trunk, surface sterilized, and 1-cm sections aseptically excised at measured distances basipetally from the original point of inoculation. The sections were crushed and the triturate was spread onto PW agar and subsequently examined for the presence of *Xf*. The wild-type bacteria and the 6E11 mutant were recovered from grapevine sections considerably more basipetal from their respective sites of inoculation than were the non-twitching mutants 1A2 or 5A7 (Figure 3). Fluorescent latex beads similarly introduced into grapevines were observed in xylem vessels 10-20 cm basipetal from the introduction sites after 2 hours, indicative of 'initial' passive transport following cavitation of the xylem water column. The bacteria were also likely transported this distance as well; thus, the meaningful distance that the bacteria moved over the 11-week period is beyond this range.

_Xf_ cell aggregation, colony and biofilm development

Cell movement and colonization characteristics were evaluated in microfabricated 'artificial' xylem chambers (Meng et al., 2005). As previously reported, isolates and mutants with functional type IV pili exhibit twitching motilities, whereas mutants with only type I pili, or no pili, do not migrate. Morphological characteristics of the developing colonies is also dependent upon the type of pili present. Wild-type isolates of *Xf* and mutants with only type IV pili, e.g., 6E11, initially develop 'star-shaped' aggregates of cells (Figure 4). These aggregates retain functionality of the type IV pili and frequently move as a cell mass on the surface of the observation chamber. Individual cells or aggregates of cells frequently move and become associated with other aggregates, adding to the cell mass. Subsequently, as the cells divide and the mass enlarges, the colony become compact and fixed in situ. *Xylella fastidiosa* mutants with only type I pili, e.g., 5A7, do not form star-shaped aggregates, but instead develop looser aggregates of cells attached end-to-end and side-by-side (Figure 4).

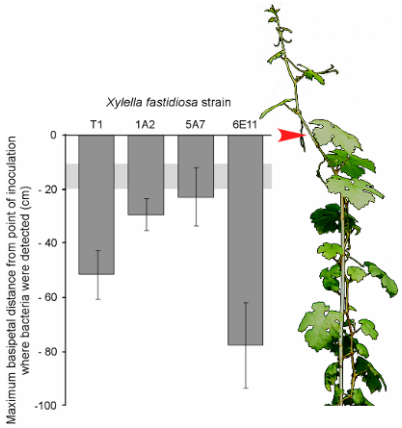

Figure 3. Basipetal translocation of *Xf in planta*. Maximum distance wild-type and mutant *Xf* cells were recovered from grapevine regions basipetal to the inoculation sites (represented by 0 of y-axis; arrow of illustrated vine) after 11 weeks. Light gray horizontal band represents max distance that 0.2 µm fluorescent latex beads traveled passively.

Colonization of xylem

We have developed hybrid microfabricated chambers in which we are able to insert bona fide grape xylem (as well as insect parts) and observe *Xf* cell movement and association. This work is preliminary and ongoing; however, already we have made some interesting observations. *Xf* cells appear to have a preference for xylem walls as opposed to other cell walls of grape, and they have a preference for attaching to xylem vessel walls over that of the glass and polydimethylsiloxane (PDMS) of our microfluidic chambers, although they do attach to the latter. *Xf* cells adhere to the xylem walls predominately by their polar ends (Figure 5), much as seen in the precibarium of the sharpshooter (Newman et al., 2004).

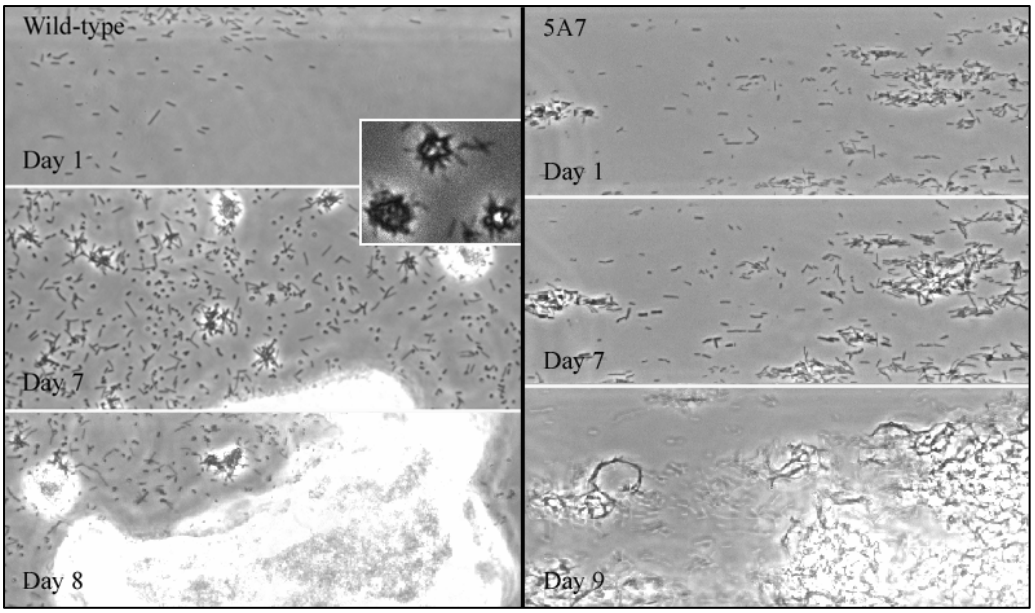

Figure 4. Colony development of wild-type *Xf* and mutant 5A7 over a period of several days

Pilus-mediated twitching among wild-type _Xf_ isolates

To ascertain that the twitching motility behavior of the Temecula isolate of *Xf* that we have been investigating is characteristic of all or most other *Xf* wild-type strains, we surveyed a range of isolates for this feature. Recently isolated bacteria from infected grapevines were obtained from California, Texas and South Carolina courtesy of A. Purcell, D. Appel, and C.J. Chang, respectively).

All isolates exhibited a colony 'fringe' which we have associated with twitching motility behavior and the presenceof type IV pili (Meng et al., 2005 Hoch, 2005) (Figure 6). There was a marked difference in the width of the fringe as well as colony vigor (diameter) between the various isolates; nevertheless, they all twitched, thus having implications for movement *in planta*.

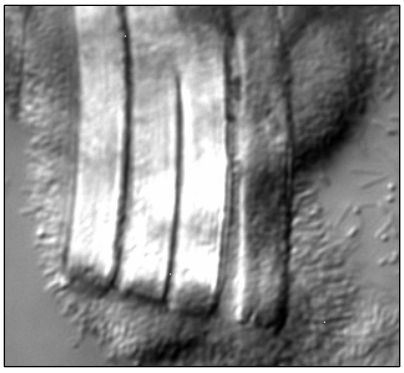

Figure 5. *Xylella* cells grown and attached to secondary xylem in a microfabricated chamber after 8 days.

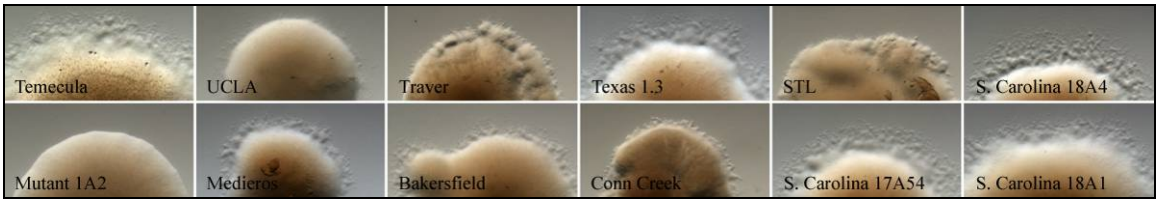

Figure 6. Representative *Xf* grapevine isolates from California, Texas, and South Carolina. All except mutant 1A2, which lacks type IV pili, exhibit twitching motility as evidenced by a 'fringe' at the colony periphery.

CONCLUSIONS

We have demonstrated that 'artificial xylem vessels' can be used to gain valuable information about the biology of *Xf*. Temporal and spatial data are not possible to obtain in the same plant, but with these devices we have been able to show that *Xf* moves via twitching motility, that small aggregates of cell can also migrate which likely occurs *in planta* and possibly promotes vessel plugging, and we have been able to extend and confirm these observations to 'upstream' movement of the bacteria *in planta*. Importantly, pili and fimbria have been shown to play important roles in *Xf* cell aggregation, cell movement, and in colony development. It may be possible to take advantage of these cell appendages to develop approaches that decrease or possibly control *Xf* expansion in the grapevine.

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