THE SIGNIFICANCE OF *XYLELLA FASTIDIOSA* TYPE I AND TYPE IV PILI IN BIOFILM STRUCTURE, BACTERIAL SURVIVAL IN BIOFILMS, AND DNA SECRETION AND UPTAKE

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ABSTRACT
We have determined that type I and type IV pili of *Xylella fastidiosa* (*Xf*) play essential roles in biofilm development, based on their individual contributions to cell adhesion and motility. Our recent studies with grape xylem sap indicate that it triggers the development of a more robust and structured biofilm than caused when the bacterium is grown in PD2 medium. Cell viability in the *Xf* cell matrix showed the interior of biofilms to contain a high level of dead cells whereas the outer periphery consists of mainly live cells. We also found that the signal molecule DSF inhibits twitching motility as does the chemical stressor EDTA.

INTRODUCTION
Biofilm formation is recognized as a major virulence factor of *Xf*, being essential for bacterial survival *in planta* and disease development (Newman et al., 2004; Koide et al., 2004; Li et al., 2007). Maintaining *Xf* in axenic culture over several passages gradually changes the expression of pathogenicity factors and leads to loss of virulence (de Souza et al., 2003). It has been shown previously that when *Xf* is grown in media that are intended to mimic xylem fluid chemistry growth, biofilm formation and aggregation are affected. (Andersen et al., 2007). Here we show that culturing *Xf* in xylem sap is more suitable for biofilm studies than standard culture media broadly used, such as PD2 and PW, given it enhances the adhesive characteristic of the bacterium and induces a more realistic *in planta* phenotype.

We have demonstrated that two distinct classes of *Xf* pili are associated with the cell's ability to move in grapevine xylem (via twitching motility) and to form biofilms and cellular aggregates (Meng, et al., 2005; Burr and Hoch 2006, Li et al., 2007). Whereas wild-type *Xf* is able to move against the transpiration stream within grape to colonize vines, mutants without type IV pili were unable to move (Meng et al., 2005). Mutants lacking shorter, type I pili, moved faster than the wild-type indicating that type I pili serve to anchor and slow movement (De La Fuente et al., 2007b). This scenario is supported by the fact that mutants with only type I pili form biofilms that have a more spreading phenotype on surfaces as compared to the wild-type and to mutant strains that have only type IV pili (Burr and Hoch 2006, Li et al., 2007). Biofilms formed by the wild-type have a denser-appearing phenotype and therefore we hypothesize that type IV pili function in secondary structure. Mutants that do not produce type I pili form biofilms that are sparse but appear to be made of dense clusters of cells again suggesting a role for type IV pili in cell-cell attachment and secondary structure of biofilms.

The signal molecule DSF (Diffusible Signal Factor) produced by *Xf* has been recently identified and shown to be required for insect colonization and to reduce its virulence to grape (Simionato et al., 2007; Chatterjee et al., 2008).

OBJECTIVES
1. Assess and understand the biology and role of *Xf* biofilms in Pierce's disease. For this objective, we will be particularly interested in:
   a. Understanding the developmental stages and architecture of biofilm formation.
   b. Determine how the presence of type I and type IV pili affect biofilm morphology and integrity.
   c. Assess the viability of *Xf* cells temporally and spatially in biofilms.
   d. Determine whether *Xf* secretes DNA into the extracellular environment and how it affects biofilm morphology and integrity.
   e. Evaluate effect of DSF on motility and biofilm regulation.
2. Determine how the stage of biofilm development and structure (dependent on pili) influence *Xf* sensitivity to chemical and environmental stresses.

3. Determine the role of type IV pili in *Xf* uptake of extracellular DNA (natural transformation).

RESULTS

Developmental stages and architecture of biofilm formation.

Whether or not *Xf* forms a structured community with specialized cell functions or if it is merely a consequence of cell to cell aggregation has been a point of interest of our group. We recently started growing *Xf* in pure grape sap, allowing a more "natural" environment for growth. This was done by initially transferring the cells growing in PD2 medium to a mixture containing 50% sap and then gradually increasing the sap concentration in each passage. The supplementation with sap not only induced faster growth (Hoch and Burr et al., 2008), but also increased the attachment of cells to glass surfaces, as assessed in test tubes and on microscope slides (Hoch and Burr et al., 2008). Besides the greater biomass visible in both assays, the biofilm formed in tubes containing 100% sap or sap:PD2 mixes were denser and difficult to disrupt by vortexing (Hoch and Burr et al., 2008). We are quantifying this by crystal violet staining and spectrophotometry.

To study spatial-temporal biofilm development we are using microscope slides fixed inside 500mL jars that receive 20mL of culture media. The jars are shaken at 100 rpm and the biofilm is formed on the slide surfaces at the air-media interface. Biofilm development and architecture were found to be greatly influenced by medium i.e. PD2 or grape sap. Preliminary results using light and laser scanning microscopy indicate that aggregates up to 20µm in height were observed in PD2 whereas in a mixture of sap and PD2 (90% sap, 10% PD2) aggregates of 100µm in height were commonly seen. This might be related to a quicker aggregation of cells that appears to occur in sap. Whether in PD2 or sap, *Xf* characteristically forms small aggregates that retain mobility and eventually merge together. We observed that such aggregates are retained even when the extracellular matrix accumulates in later developmental stages. These denser cell aggregates consist mainly of live cells and are surrounded mostly by dead cells within the large aggregates **(Figure 1)**. At the substrate surface level, there is a higher proportion of live cells at the periphery of the biofilm.

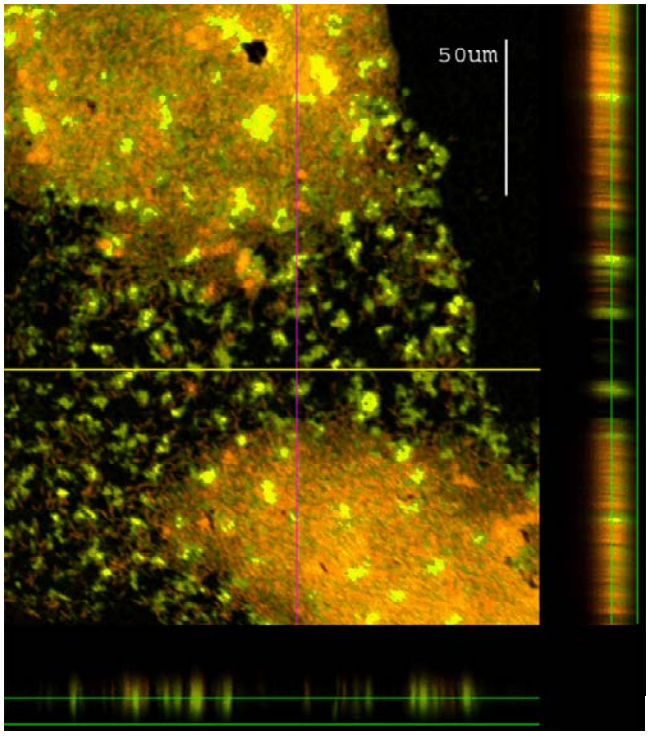

Figure 1. Cell viability within biofilm formed by *Xylella fastidiosa*. Cells were stained with SYTO-9 (green) and propidium iodide (red), equivalent to a "live and dead assay" (Boulos et al., 1999). The figure shows a portion of a mature biofilm formed after 7 days in PD2 medium. Small compact cellular aggregates can be seen embedded in and outside the biofilm matrix. The yellow and magenta lines indicate cross-sections in the Z-plane, shown on the bottom and right side of center image. The green lines on border images indicate the position of the plane shown on the center image. Obtained with confocal imaging.

Another distinctive feature of biofilm formation in sap is what appears as "fluid channels" throughout the matrix. They might function in increasing exchange rates of nutrients and toxic by-products of metabolism, enabling biofilms to develop as a functional unit. Initial investigations reveal what appears to channel formation in biofilms formed in microfluidic chambers and on surfaces of microscope slides (Hoch and Burr et al., 2008).

Further studies will use the fluorescence-tagged strain KLN59.2 (Newman et al., 2003) to study biofilm architecture in different types of devices and *in planta*. This strain displays attachment and motility on surfaces similar to wild-type Temecula.

Influence of type I and type IV pili on biofilm morphology and integrity.

We have been conducting studies to investigate how the type of pili (I and IV) affect biofilm development and structure. Mutant cells lacking either type I (*fimA)* or type IV pili (*pilB*) showed reduced biofilm formation, consistent with reduced adherence to surfaces among *fimA*$^-$ cells; probably due to the lack of the strong anchoring character conferred by type I pili (De La Fuente et al., 2007a; 2007b). Mutant cells deficient for both type I and IV pili (*fimA*, *pilQ*) did not form a biofilm on the glass surface (Li et al., 2007), and as a result generally remained in a planktonic state. Our results suggest that the type of pili affects cell clustering and biofilm morphology. We are also studying a chemosensory system of *Xf* involved in motility and biofilm formation (Burr and Hoch, 2008).

Effect of DSF on motility and biofilm regulation.
The effect of Diffusable Signal Factor (DSF) (Newman et al., 2004) on *Xf* twitching motility is being studied by our group in collaboration with Steve Lindow (UC, Berkeley). Purified DSF produced by *Xf* was obtained from the Lindow laboratory and re-suspended in 60% methanol. The DSF suspension (approximately 1 unit/µl) was used to supplement culture media. The effect of DSF on *Xf* movement has been observed using three different approaches:

(i) Agar plates diffusion assays: a 5mm-diameter well was made in the center of each plate and filled with the DSF solution referred above (10, 20, 30, 40, 50 and 60µl) **(Figure 2)**. Bacterial colonies of *Xf* WT and a *fimA* mutant (Meng et al., 2007) were spotted at two different distances (8 and 15mm) from the center of the plate **(Figure 2)**.

We observed an absence of peripheral fringe in WT colonies spotted closest to the DSF (8mm distance) and specifically the colony edge facing the DSF-containing well. Normal colony fringe was observed on the edge of the colony opposite to the center (15mm).

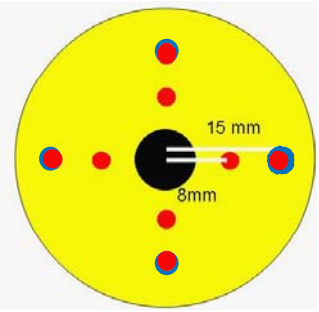

Figure 2. Schematic of the agar diffusion assays. The circle in the center represents the well where DSF or the control solvent were added. The smaller red circles indicate where bacterial colonies were spotted. The blue zones represent the fringes observed

(ii) Microfluidic chambers: addition of DSF to culture media: Dual channeled microfluidic chambers (De La Fuente et al., 2007a) were used to microscopically observe the direct effect of DSF on twitching movement. The feeding syringes were interchanged every 1-2 days, thus exchanging between fresh and supplemented media. Whenever DSF was introduced in the chambers, the twitching movement was greatly reduced after 8-12h. We observe only a few cells moving short distances in the presence of DSF. Control cells in PD2 supplemented with methanol, showed normal twitching activity.

iii) Twitching movement of DSF non-producing mutant: A mutant deficient in the production of DSF (*rpfF*-DIF2) was obtained from the Lindow lab. Preliminary observations on solid media **(Figure 3)** and in microfludic chambers showed that the speed of twitching movement of the *rpfF* mutant is slightly higher than the WT. The speed was calculated as 0.98µm/min (as compared to the reported 0.86µm/min for the WT strain) (De La Fuente et al., 2007b).

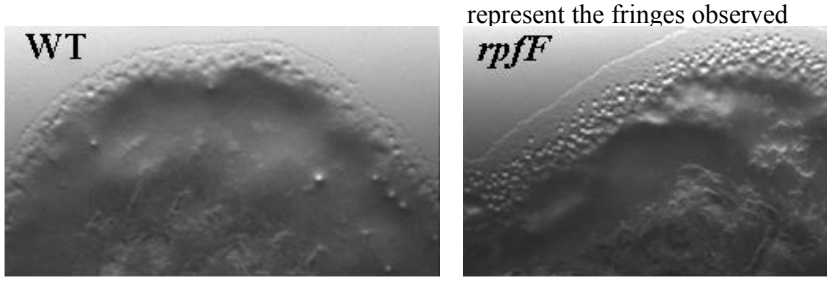

Figure 3. Colony fringe of WT and mutant *rpfF*-DIF2 deficient in DSF production.

The characterization of this mutant is still ongoing in our laboratory. Other observations indicate that aggregation of the *rpfF*-DIF2 mutant in chambers resembles the phenotype of mutants reduced in biofilm formation, such as *fimA*⁻ (see above). These results suggest that the presence of DSF reduces movement in *Xf*. We are continuing to investigate the effect of DSF on biofilm formation.

Effect of chemical and environmental stresses on movement and biofilms.
Previous studies with other microorganisms have shown the importance of biofilm in increasing resistance to detergents, toxins and environmental stresses like salinity, acidity and low humidity (Xavier et al., 2005). The protectiveness conferred by the biofilm can be studied by comparing cell viability between planktonic cells to those within biofilms after exposure to the stresses.

We have found that a chelating agent, such as EDTA has an effect on WT *Xf* cell movement. Whenever EDTA was added to the cells (8, 6, 4, and 2 mm) they slowly reduced movement and eventually stopped their displacement. Nevertheless, the presence of the chelating agent did not affect cell division or growth. We are now investigating the effect of EDTA on *Xf* cell aggregates. Based on the fact that movement affects morphology of cell clusters, we expect to see an effect of EDTA on biofilm formation.

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