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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Reporting Period
The results reported here are from work conducted from September 2007 to February 2008.

Introduction

It is widely accepted that blockage of xylem vessels by *Xf* is a major (if not the sole) factor leading to the development of PD. 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 wildtype *Xf* is able to move against the transpiration stream within grape to colonize vines, mutants without type IV pili were impaired in basepital movement (Meng et al. 2005). Mutants lacking shorter, type I pili, moved faster than the wildtype indicating that type I pili serves 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 wildtype and to mutant strains that have only type IV pili (Burr and Hoch 2006, Li et al. 2007). Biofilms formed by the wildtype have a denser-appearing phenotype (Figure 1) and therefore we hypothesize that type IV pili play an important role in secondary structure. Mutants that do not produce type I pili form biofilms that are sparse but

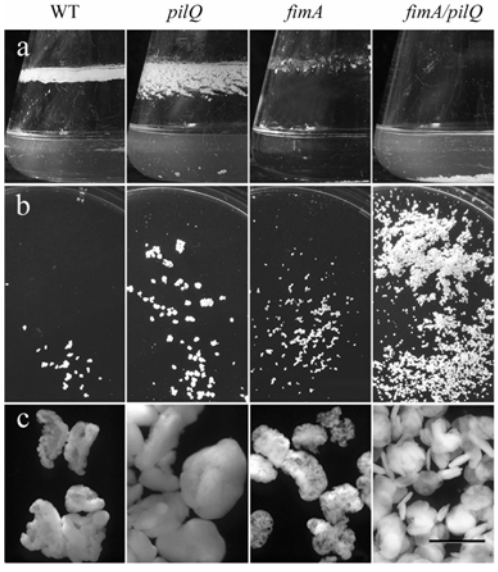


Fig. 1. Biofilm formation and cell-cell aggregation of *Xf* wild-type (Temecula) and various pilus-mutants following seven days growth in culture. a) biofilm on inner surface of flasks; b and c) free-floating cell aggregates from the bottom of the flasks corresponds inversely to the amount of biofilm on flask sides. *pilQ*, short type I pili only; *fimA*, longer type IV pili only and *fimA/pilQ* double mutant lacking both pili types (from Li et al. 2007).

appear to be made of dense clusters of cells again suggesting a role for type IV pili in cell-cell attachment and thus secondary structure of biofilms.

It has also been demonstrated that a number of bacterial species secrete DNA into the extracellular environment and that the DNA plays a major role in biofilm development and integrity. Determining whether DNA is secreted from *Xf* and is associated with biofilm structure and integrity will be important as it provides opportunities for developing strategies to reduce or eliminate biofilm formation.

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).

Activities conducted to accomplish each objective

1.a. Developmental stages and architecture of biofilm formation.

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). We refer to biofilm here as the multi-layered cellular aggregates observed on xylem vessels of PD symptomatic grapevines, on the walls of culture flasks at the air-media interface in late log or stationary phase or the large aggregates observed in microfluidic chambers. We cannot ascertain at this point whether these are structured communities with specialized cell functions or merely a consequence of cell to cell aggregation. However, we do observe that pili-defective mutants form biofilms with different adhesiveness, probably with altered secondary structure which is an indication that *Xylella* indeed forms a structured biofilm.

When analyzed in microfluidic chambers, freshly introduced *Xf* cells quickly become firmly attached to the glass substrate, either prostate or upright, attached by one pole (Fig. 2A). The next step of biofilm formation by Xf is characterized by the grouping of cells that still can break apart (Fig. 2B). These small cell masses retain mobility and over time merge to form larger clusters (Fig. 2C). Regions immediately around them consist of more sparse populations of "free" cells. This directed movement suggests a secreted chemical attractant since upstream movement is also observed. The functionality of a chemical attractant is further reinforced by observations in microfluidic chambers containing high cell-density (~10^9 cells/mL). Although preliminary, our observations indicate that under this condition the cell communities are formed much faster, suggesting a cell density-dependent aggregation process.

We are initiating experiments with a defined medium, XDM2 (Lemos et al. 2003), to verify the effects on biofilm formation and to have a chemically defined framework to conduct further experiments.

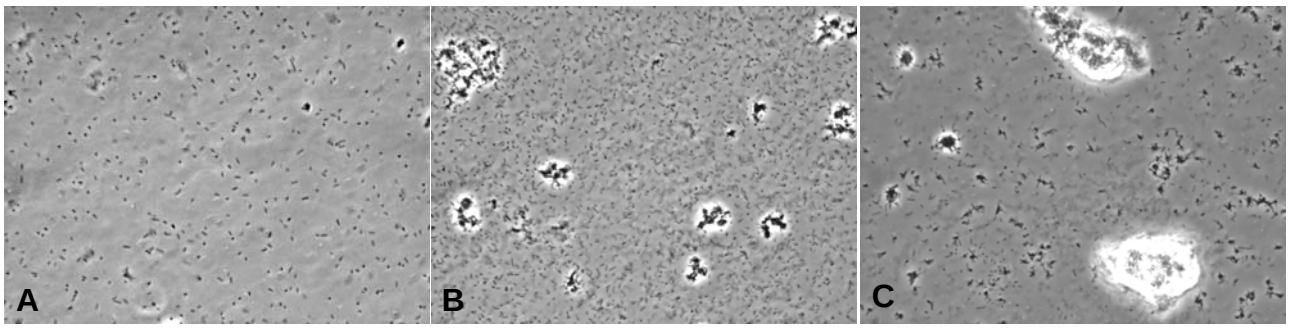


Figure 2. Stages of biofilm formation by *X* in microfluidic chambers. Cells initially attach to surface and begin clustering (A). After 24 hours a denser population is observed and larger cell clusters that retain mobility are seen (B). After 6 days of growth inside the microfluidic chamber most cells are tightly grouped into larger groups characteristic of biofilms (C).

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

We have been conducting studies to investigate if the type of pili (I and IV) effect biofilm development and structure. Using microfluidic chambers (Meng et al., 2005) coupled with time-lapse microscopy, we can compare biofilm formation by the different pilus-deficient mutants. Mutant cells lacking either type I (*fimA)* or type IV pili (*pilB*) showed reduced biofilm formation, consistent with reduced adherence 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 (Fig. 1D), and as a result generally remained in a planktonic state. Our results suggest that the type of pili affects cell clustering and biofilm morphology. Further studies will use fluorescence-tagged strains to study the biofilm architecture in different types of devices and *in planta*.

1.c. Viability of *Xf* cells temporally and spatially in biofilms.

Work in progress.

1.d. Secretion of DNA into the extracellular environment and how it affects biofilm morphology and integrity.

Several studies have reported the importance of extracellular DNA (ecDNA) to the maintenance of biofilm integrity, and secretion of ecDNA may involve type IV pili (Whitchurch 2002, Hamilton et al. 2005, Allesen-Holm et al. 2006).

The importance of ecDNA to biofilm morphology and integrity will be assessed by exposing cells in the chambers to DNase I. In vitro tests in our lab show that DNase I retains activity when added to culture media (without its original buffer), still being capable of digesting a PCR product. However, in a preliminary experiment in which DNase I was pumped into the microfluidic chamber with cells in advanced aggregation stage (after 6 days in chamber) biofilm integrity was not affected (not shown). Other tests will examine the effect of DNase I in earlier stages of biofilm development. The results will be compared to cells exposed with either proteinase K or β 1-4 glucosidase.

1.e. Effect of DSF on motility and biofilm regulation.

The effect of the signal molecule 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 Lindow's laboratory and re-suspended in 60% methanol. The DSF suspension (approximately 1 unit/µl) was used to supplement culture media. The effect of this molecule on *Xf* movement has been observed using three different approaches:

i) Agar plates diffusion assays

Petri dishes were prepared with PW media modified by omitting BSA (Galvani et al., 2007). A 5mm-diammeter well was made in the center of each plate and filled with the DSF suspension referred above (10, 20, 30, 40, 50 and 60µl) (Fig. 3). The solvent was let to dry allowing DSF to form a diffusion gradient on the agar. A control plate containing only the solvent (60% methanol in H_2O) was included. The diffusion of the solvent on the agar plates was tested by using a solution of a dye (propidium iodide) suspended in 60% methanol. Bacterial colonies of *Xf* WT and a *fimA* mutant (lacking type I pili) (Meng et al., 2007) were spotted at two different distances (8 and 15mm) from the center of the plate (Fig. 4). The experiment was repeated three times and two replicates were used for each strain.

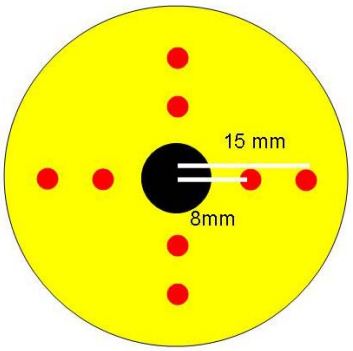


Figure 3. Schematic of the agar diffusion assays. The circle in the center represents the well where DSF or the control solvent were. The smaller circles indicate where bacterial colonies were spotted.

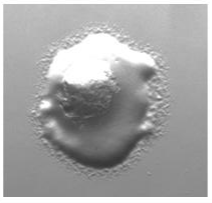
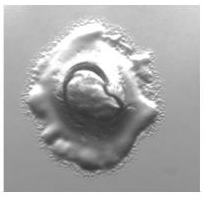
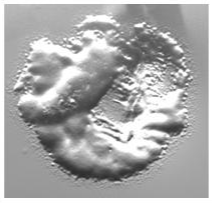
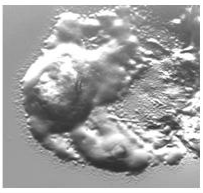
The presence of a fringe around the colonies, indicating twitching movement, was observed after 3-5 days. We observed an absence of fringe in WT colonies spotted closest to the DSF (8mm distance) and specifically the colony edge facing the DSF-containing well. Normal colony fringe was observed on the edge of the colony opposite to the center (15mm) (Fig. 4). The DSF had an effect whenever 40µl or higher volumes of the suspension were used (Fig. 4B). The effect of DSF on twitching of the *fimA* mutant was more subtle. A very small reduction of the fringe was periodically observed, but more studies are necessary to define the effect of DSF on *fimA* mutant. The control plates containing only the solvent in the center well (Fig. 4A) did not show any effect on the colony fringe. Colonies showing an impaired fringe together with control colonies were transferred to fresh plates and induction of fringe was observed in the absence of DSF (Fig. 4C).

ii) Microfluidic chambers: addition of DSF to culture media

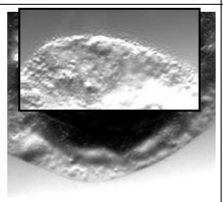
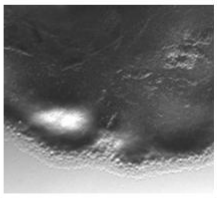
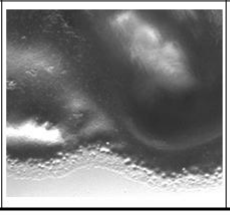
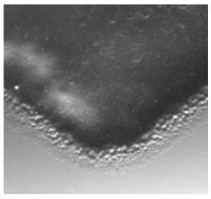
Dual channeled microfluidic chambers (De La Fuente et al, 2007a) were used to microscopically observe the direct effect of DSF on twitching movement. The channels were filled with PD2 medium, and bacterial *Xf* WT cells were grown and twitching movement was observed in both channels. After 5-7 days of incubation, the syringes supplementing media were exchanged. One of the channels was supplemented with PD2 containing 3.3 % of methanol, and it was used as a control of the solvent effect on movement. The other channel was supplemented with the DSF suspension at a final concentration approximately 33 units/ml. The feeding syringes were interchanged every 1-2 days, thus exchanging between fresh and supplemented media. The process was repeated at least three times. 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. Cells used as control, which received PD2 supplemented with methanol, never reduced their twitching movement.

A- Methanol 60 µl

Strain	8mm	15mm
WT		
fimA⁻		

B- DSF 40 µl

Strain	8mm	15mm
WT		
fimA⁻		

C- Transferred from assay plates

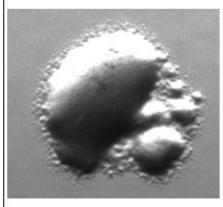
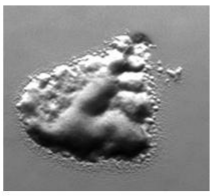
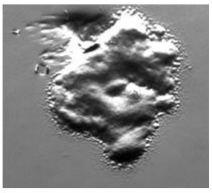
Strain	MeOH 40µl	DSF 40µl
WT		
fimA⁻		

Figure 4. Effect of DSF on development of peripheral fringe around *Xf* colonies. All bacterial colonies are oriented showing the lower surface facing the center well containing DSF or methanol solvent alone. The inset in (B) [WT at 8mm], shows the opposite edge of the colony. See text for more details.

iii) Twitching movement of DSF non-producing mutant

A mutant deficient in the production of DSF (*rpfF*-DIF2) was obtained from Lindow's lab. Preliminary data suggests that this mutant has a slightly increased twitching movement compared to WT. The phenotype of the mutant is been studied by observing fringe development in PW (without BSA) agar plates and in microfluidic chambers. The fringe formed by *rpfF*-DIF2 on agar plates is wider than the WT (Figure 5). Preliminary observations 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). The characterization of this mutant is still ongoing in our laboratory. Other preliminary observations indicate that *rpfF*-DIF2 mutant autoaggregation in chambers resemble the phenotype of mutants reduced in biofilm formation, such as *fimA* (see above).

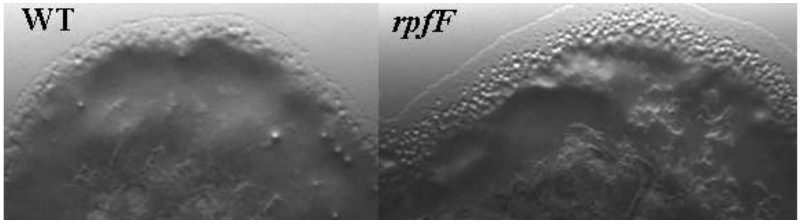


Figure 5. Colony fringe of *Xf* WT and mutant *rpfF*-DIF2 deficient in DSF production.

These results suggest that the presence of DSF reduces movement in *X. fastidiosa*. We have not yet deciphered the effect of DSF on biofilm formation. The reduction in motility in the presence of DSF may help in cells staying together in an area where later they will form aggregates and biofilm.

2. Biofilm influence on sensitivity to chemical and environmental stresses.

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. Preliminary studies towards this objective have shown that a chelating agent, such as EDTA has an effect on WT *Xf* cells movement. In a study using microfluidic chambers bacterial cells were grown in PD2 medium ("fresh") and PD2 supplemented with decreasing concentrations of EDTA (8 to 2 mM). After each addition of EDTA-supplemented media, the cells were immersed in "fresh" medium for a few hours to wash away the chelating agent. We observed twitching movement of WT cells anytime "fresh" medium was used. 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.

3. Role of type IV pili in uptake of extracellular DNA.

Work in progress.

Publications or reports resulting from the project

None (the project has been underway for 6 months)

Presentations on research

Burr, T. J., H. C. Hoch, L. Cursino, Y. Li, L. De La Fuente and C. Galvani. 2007. The roles that different pili classes in *Xylella fastidiosa* play in colonization of grapevines and Pierce's disease pathogenesis: chemosensory cluster controlling twitching motility. Proceedings of the Pierce's Disease Research Symposium 2007, p. 123-126.

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Research relevance statement

We aim to provide an improved understanding of how different pili types on *X. fastidiosa* affect biofilm development and Pierce's Disease development. This information will provide insight on how the bacterium colonizes grapevines and will be important for the development of PD controls.

Lay summary of current year's results

- This project started in September 2007 and in October a post-doctoral researcher joined our program. Since then we have learned more about the developmental stages of biofilm by *Xf* and we are now testing treatments to interfere with this process by using enzymes and the chemical messenger DSF. We have discovered that DSF inhibits motility of Xf cells.
- We have demonstrated the importance of both type I and type IV pili in formation of a mature biofilm.
- We have successfully implemented the methods for time-lapse imaging of biofilm formation inside microfluidic chambers and are planning methods to study different aspects of the biofilm in 96-well microtiter plates.
- We have learned that EDTA reduces cell movement without affecting cell viability.

Status of funds

Salary has been committed for the post-doctoral associate for the next 18 months. The post-doctoral associate (Paulo A. Zaini) has been working in the laboratory for 5 months. The current funding balance is $114,157.

Summary and status of intellectual property produced during this research project

None to date

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