UNDERSTANDING CONTROL OF *XYLELLA FASTIDIOSA* CELL AGGREGATION: IMPORTANCE IN COLONIZATION AND BIOFILM DEVELOPMENT IN GRAPEVINE AND SHARPSHOOTER FOREGUT

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ABSTRACT
Our investigation focused on aggregation and 'autoaggregation' of *Xylella fastidiosa* (*Xf*) cells. This study is particularly directed toward elucidating how *Xf* cells aggregate to cause PD symptoms. We have documented that both type IV and type I pili are important in the process. Furthermore, it was discerned that surface properties of WT cells change from being more hydrophobic to more hydrophilic as the cells age, and that cell surface charge does not prevent aggregation. In addition, all cells with pili were effectively transmitted by BGSS while the double mutant, *fimX/pilO*, was apparently not transmitted which may not be surprising since no pili are present on these mutants to adhere the cells to the insect foregut regions.

INTRODUCTION
The ability of *Xylella fastidiosa* (*Xf*) to cause symptoms of Pierce's disease is generally assumed to be related to its colonization of grapevine xylem where the pathogen forms biofilms and cell aggregates. From a disease standpoint such aggregates and biofilms are important for several reasons, including possible direct blockage of sap flow through xylem vessels or indirect blockage through initiation of tylose formation. Cell aggregates may also facilitate pathogen spread from vessel element to vessel element via enzyme digested pit membranes (Newman et al., 2004) — individual cells likely lack sufficient 'enzymatic power' to breach pit membranes, but a compact aggregate of cells would be much more effective in this regard. Furthermore, enzyme production may not be expressed in individual cells, but be regulated in aggregates associated with quorum sensing. From the standpoint of the pathogen, cell aggregates and biofilms likely facilitate nutrient adsorption, protection from environmental stresses, and phytochemicals.

Determining how *Xylella fastidiosa* is able to inhabit the xylem environment and block the transpiration stream through the production of biofilms and bacterial cell masses would be informative toward facilitating development of novel control approaches. Furthermore, insight into the selective acquisition, retention, and transmission of *Xf* by leafhopper vectors represents a priority area of interest. Earlier, we demonstrated several unique and important features of *Xf* biology not previously recognized, including the observation that the bacteria posses functional type IV pili that allow the cells to migrate via twitching motility upstream in grape xylem elements (http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/; Meng et al., 2005), that they posses type I pili that function in adhering the cells to xylem (De La Fuente et al., 2007a; 2007b; Li et al., 2007), and more recently that at some as yet undefined time or condition individual bacteria that are separated by relatively great distances 'autoaggregate' into large masses. In our *in vitro* studies, this occurred after six or more days of growth (initiated from only a few cells) in PD2 media (http://www.nysaes.cornell.edu/pp/faculty/hoch/agg/). Aside from a slow population build up of cells in xylem vessels at or near sites of sap flow constrictions (pits, element end-wall openings) which we consider cell aggregates, it is possible that many individual cells normally distributed throughout xylem elements are able to quickly autoaggregate into large cell masses contributing to vessel blockage. This phenomenon may explain, in part, why PD symptom development (reddening and drying of leaf margins) often occurs within a short time span—from overnight to a few days.

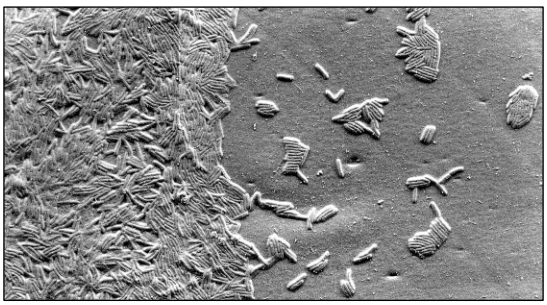

Figure 1. SEM of frozen-hydrated *Xf* colony edge exhibiting a peripheral fringe of twitch-migrating cells in 'rafts.'

OBJECTIVES

Our overall goal is to understand the relationship of *Xf* cells within the confines of the xylem environment. This project centers on the development and importance of bacterial cell aggregates and biofilms, and their involvement in expression of Pierce's disease. Recent observations in our lab revealed that *Xf* cells 'autoaggregate' as the cell population matures. That observation has led us to examine the biological and genetic mechanism associated with this phenomenon by generating aggregation and autoaggregation-defective mutants. Mutants thus obtained will be examined for their activities within microfabricated 'artificial' xylem vessels (which provide superior observation opportunities) as well as in *bona fide* xylem vessels, for disease development, and for vector transmission.

Specific objectives are to:

1. Identify genes associated with aggregation and autoaggregation of *Xf* cells.
2. Assess spatially and temporally aggregation and autoaggregation activities as they occur *in planta* and in microfluidic 'artificial' xylem vessels.
3. Assess selected aggregation and autoaggregation-defective mutants *in planta* for disease development and movement within the plant.
4. Assess aggregation mutants generated in Objective 1, and related attachment mutants already in hand, for acquisition, retention, and transmission by sharpshooter vectors.

RESULTS

Mutant generation. In this period we screened for mutants with deficiencies in the ability to form tight 'rafts at colony peripheries (Figure 1). A number of interesting and potentially useful mutants have been identified as a result of our mutational screenings; however, to date we have not identified colonies with 'raftless' peripheries, viz., individual cells not closely associated 'side-by-side.' The mutation and screening process is continuing. We are encouraged about the prospects of identifying such mutants, in part because the screening procedure is straight forward and relatively quick. In addition to screening for raftless mutants, we have identified and isolated a number of cell lines that are deficient in biofilm formation and in the formation of large aggregates—all of which are being further characterized.

Table 1. *Xylella fastidiosa* Temecula mutants referenced in this report. Gene, ORF, and Gene Product designations are according to the recently revised annotation for *Xylella* (http://www.xylella.lncc.br/)

Gene	Single Mutation	ORF	Gene Product	Observed Characteristics *in vitro*
pilB	1A2	XP2038	Type IV fimbrial assembly protein PilB	No twitching motility.
cheY2	3E10	XP1759	two-component system, regulatory protein	Very poor twitching motility.
fimX	6E11	XP0065	Fimbrial adhesin protein	Twitching motility. Colony 'fringe' wider than wild type.
pilY1	TM14	XP0026	Type IV pilus assembly protein, tip-associated adhesin PilY	Twitching motility. Colony margin smooth to crenulate.
pilL	TM25	XP0876	PilL/ChpA fusion protein: pili sensor histidine kinase-response regulator / chemotaxis-related protein kinase	Reduced twitching motility
	Double Mutation			
fimX, pilO	DM12	XP0065 XP1782	Fimbrial adhesin protein Fimbrial assembly protein PilO	No twitching motility. Colony 'fringe' deficient.

Influence of pilus type on autoaggregation. Using microfluidic chambers (Meng et al., 2005) coupled with time-lapse microscopy, we observed a process previously referred by our group as "autoaggregation." During this process, dispersed WT *Xf* cells formed compact, aggregates over 3-10 hours (Figure 2a). This autoaggregation process occurs after cells were grown in PD2 broth medium within microfluidic chambers for 7-11 days. We investigated the influence of both type I and type IV pili in this process by using a series of mutants defective in biosynthetic and structural genes (Meng et al., 2005, Li et al., 2007) (Table 1). *pilY1* mutants (deficient in encoding for a putative tip adhesin protein of type IV pili) exhibited similar aggregation processes as the WT cells. Mutant cells lacking type I pili [*fimX* (previously designated *fimA*)] also formed cell aggregates, but the process differed from the WT in that the cells repeatedly aggregated and dispersed, but eventually formed relatively stable, albeit smaller aggregates (Figure 2b). Adherence among *fimX* cells appeared reduced, probably due to the lack of strong attachment conferred by type I pili (De La Fuente et al., 2007a; 2007b). Mutants deficient in type IV pili (*pilB*) did not form spherical aggregates; instead, loose lace-like cell-cell associations were formed (Figure 2c). These looser cell

aggregates were generally non-adherent to the chamber walls. Mutants deficient for both type I and IV pili (*fimX*, *pilO*) did not form cell aggregates, and as a result generally remained separated from each other (Figure 2d).

Table 2. Aggregation assay.

Gene	Single Mutation	Observation
---	WT	Aggregation observed immediately
pilB	1A2	Aggregation observed immediately
pilO	TM1	Aggregation observed immediately
fimX	6E11	Aggregation observed immediately
pilY1	TM14	Aggregates did not form, or formed very slowly; aggregation was not as pronounced
pilY1	TM23	Aggregation observed immediately; larger clumps
hecA	Lindow	Aggregation observed immediately; larger clumps
xadA	Lindow	No aggregation observed
	Double Mutation	
fimX, pilO	DM12	Aggregation observed immediately

Toward the pursuit of assessing aggregation, we noted that WT cells removed from PW agar plates and suspended in water or buffer aggregated shortly thereafter, within 1-2 minutes. We thus explored this approach as a means of screening mutant cells for deficiencies in aggregation (Table 2). Most notable were the observation that *pilY1* and *xadA* mutants remained in a homogenous suspension and that after 1 hour the *pilY1* mutant suspension appeared as if it may have been starting to clump. The *hecA* mutant exhibited clumps that were noticeably larger than in the wild-type. All other mutants and the WT clumped within seconds.

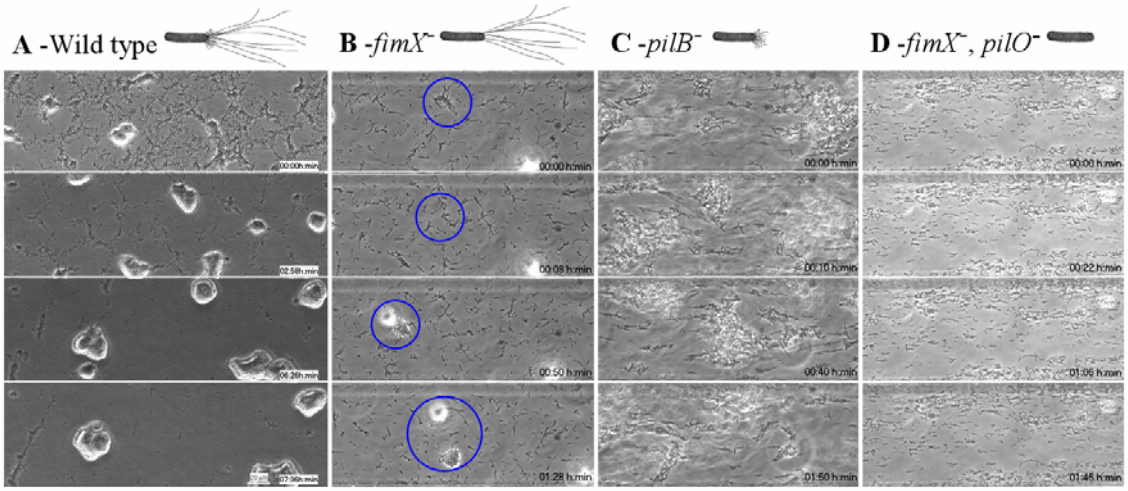

Figure 2. Autoaggregation of *Xf*. A) wild-type *Xf* densely dispersed cells merged into small aggregates within hours. B-D: mutants defective in type I and/or type IV pili genes.

Cell surface physical properties related to autoaggregation: To ascertain that autoaggregation occurred as a result of pilus or other cell surface adhesin characteristics, we investigated other factors that may be involved—namely, cell surface hydrophobicity and surface charge.

- *Surface hydrophobicity*. The BATH (Bacterial Adhesion to Hydrocarbons) technique (Rosenberg et al., 1980) was used to measure the adhesion of *Xf* cells to three hydrocarbons (n-hexadecane, n-octane and p-xylene). WT cells were grown in flasks on PD2, harvested, washed, and subjected to phase separation with each of the hydrocarbons. The affinity for the organic phase (measured as a reduction in the optical density of the aqueous phase) is an indication of the hydrophobicity of the cells. Three-day old cell aggregates were more hydrophobic than 8-days old cells (Figure 3). The hydrophobic properties of 'young' cells may help on the process of cells aggregation, since they are immersed in a hydrophilic environment (PD2 medium/xylem sap). The increase in hydrophilic properties

of older cells may be a consequence of exopolysaccharide, since these molecules are generally hydrophilic (Sutherland, 2001; Schär-Zammaretti and Ubbnik, 2003).

- *Surface charge*. The net charge at the surface of a particle affects the distribution of ions that surrounds it. Zeta potential is one means of assessing surface charge and is defined as the potential of the most outer stable ion layer surrounding a particle. When a particle moves, ions within this boundary layer move with it, but ions beyond this boundary layer do not. WT *Xf* cells from PD2 broth culture were washed with PBS (pH 7.4), and their zeta potential was determined using a Zetasizer (Malvern Instruments Ltd, Worcestershire, UK). No differences were noted for the zeta potential between two or seven day old cells (Figure 4). The average zeta potential for two-day old cells was -16.0 mV, and -9.60 mV for seven-day old cells. In general, particles with zeta potentials more negative than -30 mV are considered stable. Zeta potential values for WT *Xf* cells indicate that these bacterial cells are 'non-stable' and, thus the force is not strong enough to prevent cell aggregation. These results indicate that *Xf* cells from both time points have adequate zeta potential to form aggregates.

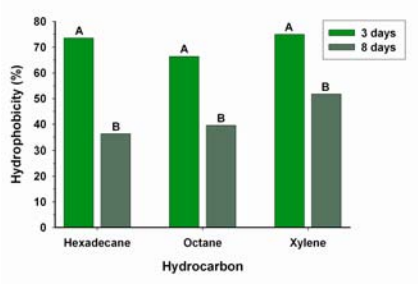

Figure 3. Surface hydrophobicity of *Xf* WT cells was measured by adhesion to hydrocarbons (hexadecane, octane, and xylene).

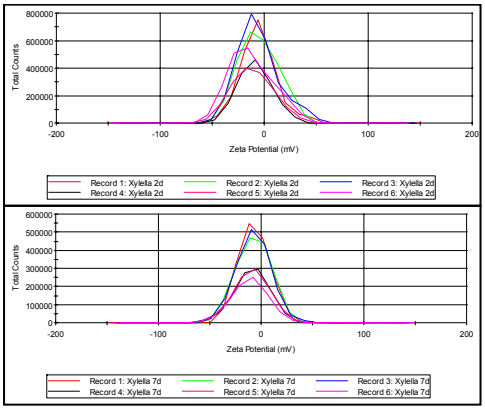

Figure 4. Zeta potential of WT *Xf* cells grown for 2 days (upper) and 7 days (lower).

Assessment of acquisition, retention, and transmission by sharpshooter vectors

For this objective we supplied our collaborator, Rodrigo Almeida *fimX*, *pilB*, and *fimX/pilO* (double mutant) mutants with which he inoculated greenhouse confined plants, and subsequently assessed transmission efficiencies by sharpshooters. In brief, both WT and the *fimX* mutant expressed severe symptoms in needle inoculated plants, *pilB* less severe symptoms, and *fimX/pilO* very mild symptoms, all consistent with our previous observations. However, both WT and the *fimX* mutant were efficiently transmitted by BGSS followed by severe symptom development. *pilB* mutant cells were also efficiently transmitted (which we would expect due the presence of type I pili), but it did not move in the infected plants. *fimX/pilO* were apparently not transmitted and this may not be surprising since no pili are present on these mutants.

CONCLUSIONS

Observations from this period demonstrate the pronounced role that pili and fimbriae have in *Xf* attachment, aggregation, and biofilm formation. We have demonstrated that microfluidic devices can effectively serve as 'artificial xylem vessels' to gain valuable information about the biology of *Xf*, and to infer roles for these phenomena *in planta*. In this report we show that autoaggregation in *Xf*, a phenomenon that could explain the rapid development of symptoms in grapevines affected by PD, is reliant upon the presence of pili.

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