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

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

ABSTRACT
Investigations reported herein focused on aggregation and 'raft' development by *Xylella fastidiosa* (*Xf*) cells. This study was particularly directed toward elucidating how *Xf* cells aggregate, to cause Pierce's disease symptoms. It was shown that 100% grape xylem sap, for the first time, could be routinely used to culture *Xf*. Furthermore, the biofilm formed in sap was significantly different than biofilms formed in the more commonly used nutrient rich PD2 media. Additionally, we were able to visualize using immunocytochemical approaches, type I and type IV pili by light microscopy.

INTRODUCTION
Colonization of grapevine xylem by *Xylella fastidiosa* (*Xf*) develops over an extended period of time before symptoms of Pierce's disease are recognized. Such colonization, initially as individual cells, then as aggregates of a few cells, and finally as very large multicellular aggregates, coalesce to from a biofilm. 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.

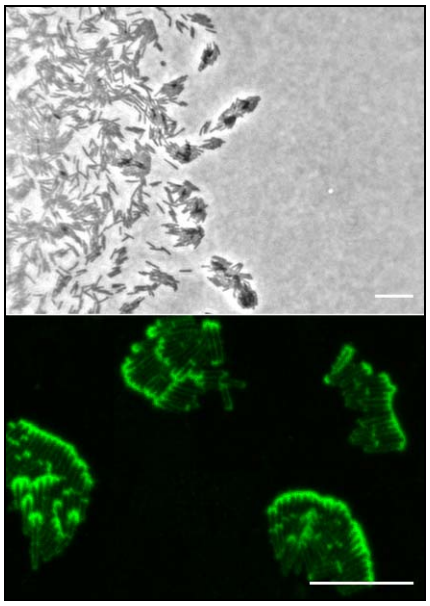

Figure 1. 'Rafts' of *Xf* cells actively migrate outward from colony margins (upper). Migration is through extension and retraction of type IV pili at one pole of the cell. Immunostaining of similar rafts with an antibody (Agdia) reveals the common polar site of both type I and type IV pili and the cell alignment that allows raft migration. Bars, 10μm.

Ascertaining how *Xf* inhabits the xylem environment and how it blocks the transpiration stream through the production of biofilms and bacterial cell masses is deemed 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 against the transpirational flow in grape xylem elements (http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/; Meng et al., 2005; **Figure 1**), that they possess 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 (De La Fuente et al., 2008). In our *in vitro* studies, this occurred after six or more days of growth (initiated from only a few cells) in PD2 media. 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 Pierce's disease symptom development (reddening and drying of leaf margins) occasionally occurs within a short time span.

OBJECTIVE

Understanding the relationship of *Xf* cells within the confines of the xylem environment is our long term goal. To that end, this project centers on the development and importance of bacterial cell aggregates and biofilms, and their involvement in expression of Pierce's disease. We previously reported that *Xf* cells 'autoaggregate' as the cell population matures in PD2 media within microfluidic devices (De La Fuente et al., 2008). That observation has led us to examine the biological and genetic mechanism associated with this phenomenon by generating aggregation and autoaggregation-defective mutants. Such mutants were 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. More recently we have directed our attention toward similar aggregation phenomena and biofilm development in these artificial xylem vessels using grape xylem sap in addition to the nutrient rich PD2 medium.

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 the first objective, and related attachment mutants already in hand, for acquisition, retention, and transmission by sharpshooter vectors.

We reported previously many aspects of the results of our studies toward these objectives (in 2005, 2006, 2007 Pierce's Disease Research Symposium Proceedings, as well as in several publications—De La Fuente et al., 2007a; 2007b; 2008). This report summarizes our most recent observations pertaining to aspects of the objectives which remained to be investigated, as well as reporting coincidental observations made during the course of our studies.

RESULTS

Mutants, aggregation, rafts. Numerous *Xf* mutants, including *pilB*, *pilO*, *fimA*, *pilY1*, *hecA*, *xadA*, double mutants *fimA-pilO*, etc, were generated and screened for deficiencies in the ability to form cell aggregates when suspended in a fluid environment. Thus far, only the *xadA* mutant from Lindow's group exhibited such a deficiency; all other mutants formed aggregates. Furthermore, mutants deficient in either type I or type IV pili, or both (e.g., *fimA-pilO*), aggregated from cell suspensions. This indicates that cell aggregation is not entirely dependent upon pilus-related adhesins, but more likely on cell surface adhesins. In addition, raft formation *in vitro* on firm surfaces (agar, cellophane, etc.), a phenomenon we relate to aggregation in liquid environments (xylem sap, PD2, etc.) occurred in *fimA* mutants, indicating that type I pili were not necessary for this phenotype and that the sole presence of type IV pili was sufficient for rafts to form. Rafts do not form in mutants deficient for type IV pili, e.g., *pilB*, because they cannot twitch-migrate (Meng et al., 2005). The side-to-side arrangement of *Xf* cells in rafts is likely reliant upon cell surface adhesins, in addition to the presence of type IV pili (for movement and alignment). We were not able to relate the surface adhesin of *xadA* to raft development since this mutant was either developed from a twitch-deficient parent or this characteristic was lost in to course of the mutation. Our next goal will be to site direct a *xadA* mutation into an existing *fimA* mutant to test this possibility.

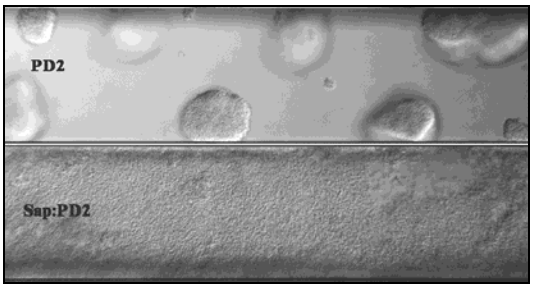

Figure 2. Aggregation of *Xf* WT cells on PD2 medium and in a 50:50 mixture of grape (Chardonnay) sap and PD2 after 7-8 days of growth.

***Xf* aggregation and biofilm development in PD2 and xylem sap.**

Until now all our studies with cell motility, aggregation and biofilm development were in PD2 broth—in both microfluidic devices and in culture flasks (Meng et al., 2005; De La Fuente et al., 2008). This was because we were not able to culture *Xf* in grape xylem sap, even though that is where the organism lives *in planta*. Either collected sap was oxidized and became inhospitable to *Xf*, or possibly *Xf* lost its ability to grow in sap after being continually cultured in a nutrient rich media such as PD2. We were able to grow *Xf* in summer grape xylem sap (Chardonnay, provided by Hong Lin, USDA, Parlier, CA) following initial growth in PD2, and gradually increasing the percentage of sap over several days. Subsequently, we used spring sap obtained from Chardonnay as well as from *Vitis riparia* and *Vitis labrusca* grapes grown in Geneva, NY and noted no difference in *Xf* growth. *Xf* grown in side-by-side microfluidic channels, one with PD2 and one with a mixture of 50:50 (PD2:Chardonnay sap) exhibited different growth

habits; grown in PD2, *Xf* developed cell aggregates by seven days as observed previously (De La Fuente et al., 2008), while in the PD2:sap mixture *Xf* growth occurred as a 'lawn' of cells (biofilm) (**Figure 2**). Best growth occurred in mixtures of 20:80 (PD2:sap), although continual growth and biofilm formation also occurred in 90 and 100% sap in microfluidic devices (**Figure 3**). Also, while not quantitatively assessed, it appeared that *Xf* cell type IV pili motility was significantly greater in sap and sap mixtures than in PD2 alone. Being able to grow *Xf* in grape sap provides a more natural environment in the microfluidic devices to assess other aspects of *Xf* biology. Since culturing of *Xf* in sap in microfluidic devices, we have now been able to grow it in tubes and flasks containing sap, again by increasing sap concentrations gradually to 100%. Also, we are now able to store *Xf* at -80 C in 100% sap, and retrieve viable cells, shortening the time interval for sap media culture. Notable is the observation that not only is biofilm formation in 80-90% sap significantly more robust and greater than in PD2, but cell growth is also greater in higher percentage mixtures of sap and PD2 (**Figure 4**).

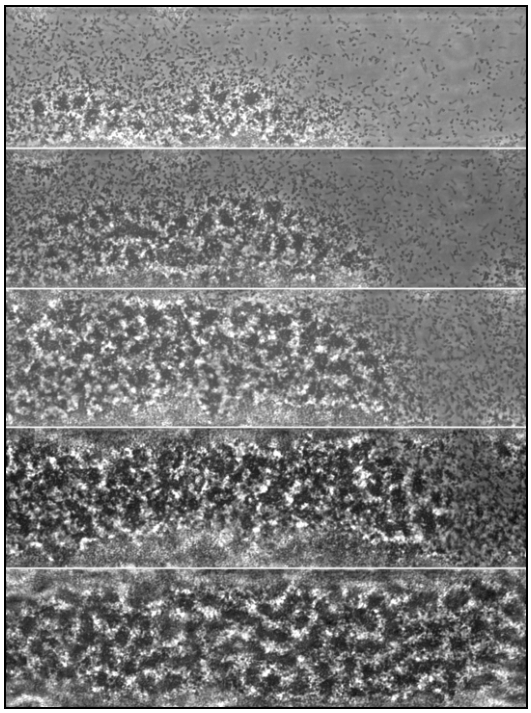

Figure 3. Sequential development of WT *Xf* biofilm over 6 days in a mixture of 10:90 PD2:Chardonnay sap in microfluidic channels.

Production of antibodies against *Xf*. Characterization of *Xf* pili and other cell surface characteristics, e.g., adhesins, is important to understanding the biology of the pathogen. One means of approaching this is to use antibodies and specifically monoclonal antibodies (MAb's) to visualize localization of these properties. In addition, such antibodies may be useful in inhibition of migration and colonization of *Xf in vitro*, and possibly *in planta*. We have thus produced MAb's in mice toward various surface proteins of *Xf*. A number of cell surface localizations are visualized using these antibodies (**Figure 5**). In addition, blood serum (polyclonal antibodies) from immunized mice exhibited excellent recognition of *Xf* cell surface antigens including both type I and type IV pili (**Figure 5**).

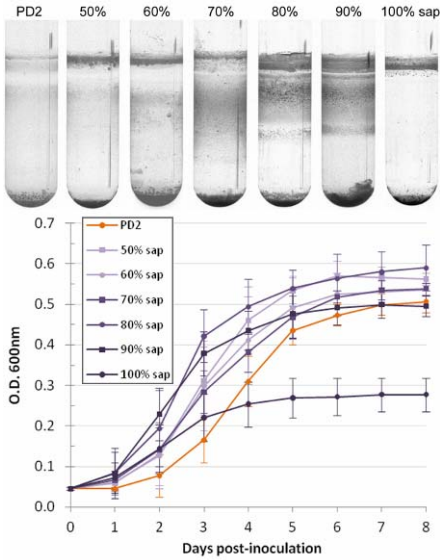

Figure 4. Biofilm formation and growth of *Xf* in mixtures of PD2 and grape xylem sap. *Xf* biofilms were most robust and greatest in 80-90% sap following 7 days growth (upper). Similarly, growth of *Xf* was greatest in sap:PD2 mixtures (lower).

Presentation of antibodies, ligands, dispersion chemicals, etc. to *Xf* cells. One of our goals is to examine temporally, activities of *Xf* cells when presented with various chemicals that may affect cell motility, aggregation, and biofilm development. We wish to do this in a way that excess non-bound chemical can be removed from the cell environment, and at the same time observe cell behavior. To accomplish this, we developed microfluidic devices with valves that can be activated open or closed have been devised in which the environment around treated cells can be flushed, and the treated cells moved to an adjacent chamber for observation (**Figure 6**). We will next expose cells to fluorescent protein stains such as CY3 to observe activities of the type IV pili under different environmental conditions, as well as to treatments that may influence cell activities.

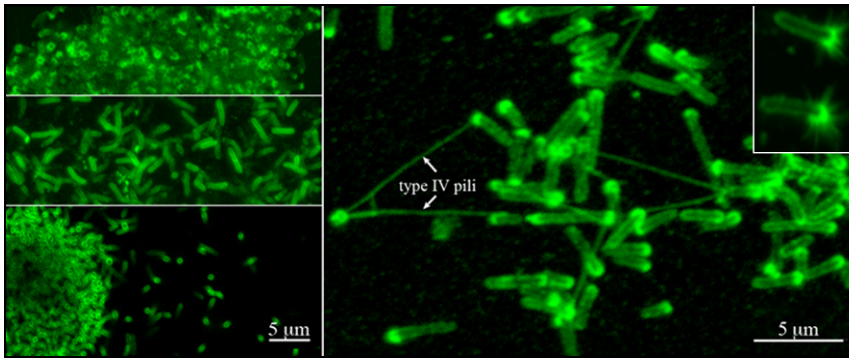

Figure 5. Immunocytochemical localization of cell surface components of WT *Xf* with MAb's reveals predominately one cell pole (left top), the site of type I pili, and outer surface proteins (left middle, left bottom). Polyclonal mouse serum localizes several cell surface features, including cell surface, type IV pili, and type I pili as a bright pole (right), and individual type I pili (right inset).

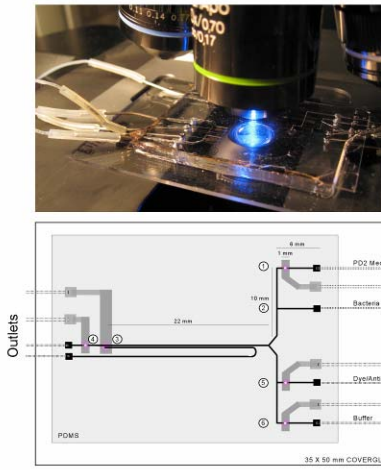

Figure 6. 'Valved' microfluidic device (upper) and schematic of components (lower). Five pneumatic independently operated valves (gray components) control flow and exposure of *Xf* cells to various treatments.

CONCLUSIONS

Observations from this period demonstrate the pronounced role that pili 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*. To our knowledge, this is the first time that *Xf* has been reported to be cultured in 100% grape xylem sap, and advancement that should provide better insight into *Xf* cell colonization and biofilm development.

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FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program.