UNDERSTANDING *XYLELLA FASTIDIOSA* COLONIZATION AND COMMUNICATION IN XYLEM LUMINA

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ABSTRACT
Microfluidic chambers were fabricated using photolithographic and soft-molding methods. The chambers were made to mimic the physical parameters of grape xylem vessels in which *Xylella* cells were studied temporally and spatially for colonization and biofilm development. *Xylella* bacteria were observed to migrate by 'twitching' motility against a rapid flowing medium in microfludic chambers. Twitching motility is important in explaining how this pathogen is able to migrate against the flow of the plant's transpiration stream to colonize previously non-invaded xylem vessel regions. Mutant strains with insertions in *pilB, pilQ*, and *fimA* genes established the roles of short pili, and longer type IV pili in biofilm development and long distance migration of the bacterium.

INTRODUCTION
Pierce's disease of grape is generally recognized as being caused by restricted sap flow and resultant water stress due to plugging of xylem elements by live bacterial aggregates and associated mucilage. It is not clear whether the extracellular polymeric mucilage is of bacterial and/or plant origin. Based on the analysis of the complete genome sequence of *Xylella fastidiosa*, gums produced by the *X. fastidiosa* are similar to the 'xanthan gums' produced by *Xanthomonas campestris* pv *campestris*, although they may be less viscous (Simpson et al., 2000). In addition, tylose development in xylem vessels in response to the presence of the bacterium further restricts sap flow (Mollenhauer and Hopkins, 1976). These general concepts regarding *X. fastidiosa* pathogenicity are readily recognized; although, it is not understood how the bacteria become established in the turbulent habitat of a 'fluid conduit' i.e., xylem vessels and tracheae, to form colonies. In addition, how the bacteria are disseminated throughout the xylem vessels from insect-vector feeding sites has long been a particularly puzzling and important question. Long-distance intra-plant migration of the bacteria is even more perplexing since xylem sap flow is always down the pressure gradient, viz., with the transpiration stream that flows toward the leaf. Even under nocturnal conditions when leaf stomates are mostly closed, cuticular transpiration maintains sap flow toward the leaf, albeit at slower rates. Sap flow is seldom stagnant, and rarely, if ever, moves in a reverse direction away from the leaves. Since *X. fastidiosa* is a non-flagellated bacterium, one hypothesis for its ability to migrate against the normal flow of the plant's vascular system has been through the slow and incremental expansion of the bacterial colony through repeated cell division along xylem vessel walls. Another possibility is that occasional cavitation of the water column causes momentary reversal and short distance flow of the sap, thereby carrying the bacteria down the xylem elements. Neither of these scenarios satisfactorily explains colonization of upstream xylem regions.

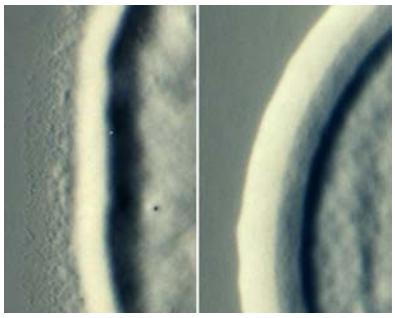
Investigations conducted during the last research period concentrated on understanding biofilm development and how *Xylella* bacteria are able to colonize regions 'upstream' from their initial site of introduction. Toward this, we generated mutant strains to help answer these queries, and we used microfluidic chambers in which we were able to examine the temporal and spatial aspects of bacterial colonization.

OBJECTIVES
To understand how the physical parameters of xylem tracheae and vessels influence *Xylella fastidiosa* colonization. Toward this, we evaluated bacterial movement, colony formation, and biofilm development. Our approach has been to use microfabricated 'artificial' vessels that mimic topologies and chemistries of xylem vessels.

RESULTS
Development of Artificial Xylem Vessels (Microfluidic Chambers)
Microfluidic chambers were fabricated from polydimethylsiloxane (PDMS), supported by a microscope slide with the channel side sealed with an air plasma treated cover glass. The mold for the PDMS device was prepared in silicon wafers using photolithographic procedures. 'In' and 'out' ports and tubing were sealed to the microfluidic chamber. Flow of media through the chambers was facilitated with a syringe pump. Chamber dimensions were as previously reported, but generally were 50-100 μm in width and depth, and up to 14 cm in length.

Mutagenesis of Xylella

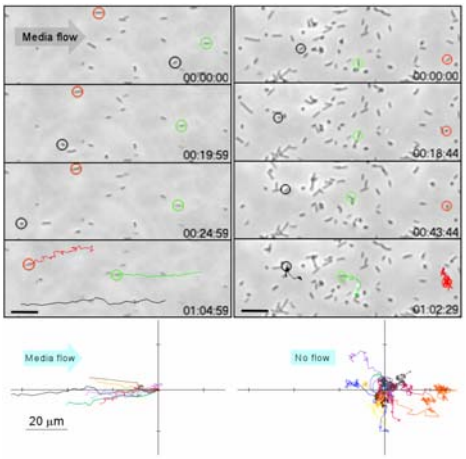
The EZ::TN Transposome system was used to generate *X. fastidiosa* mutants (Guilhabert et al., 2001). Two types of mutants were sought: biofilm modified mutants, and mutants deficient in 'twitching' (type-IV pili) movements. Ninety-six well polystyrene microtiter plates were used to screen for biofilm-modified mutants. The wild-type strain was used as a baseline control for biofilm development. Crystal violet, added to each well, served as an indicator for the presence of biofilm. Wells exhibiting either enhanced or decreased biofilm expression as compared to the wild-type strain were identified visually. Subsequently, biofilm development was assessed by dissolving similarly stained biofilms with DMSO and quantifying by absorbance (A620) in a microtiter plate reader. Screening for twitch minus mutants was performed on modified PW solid medium (Davis et al., 1981). Colonies with a peripheral fringe were designated as having a normal twitching phenotype characteristic of wild-type *X. fastidiosa*. Colonies lacking a peripheral fringe were designated as having a twitching defect.



Light micrographs of wild-type and twitch-minus mutant (1A2) colonies on agar medium with and without a peripheral "fringe."

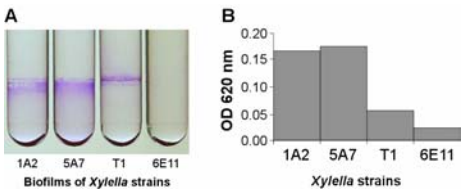
Movement and Biofilm Development of Xylella Bacteria

Wildtype *Xylella fastidiosa* (Temecula) exhibited a colony morphology, viz. fringed margin, consistent with twitching motility that is observed in other bacterial species. Time-lapse imaging of bacteria at the colony edge, revealed both individual bacteria and aggregates of cells that migrated between 0.01-0.32 μm min^{-1}, generally in a direction away from the colony periphery. When the bacteria were introduced into a microfluidic chamber, twitching movements propelled migration of individual cells in various directions depending on the rate and direction of medium flow. Under stagnant no-flow conditions, the cells exhibited no directional preference for migration. However, when the medium was passed through the chamber at approximately 20,000 μm min^{-1} (volumetric flow rate = 0.20 μL min^{-1}), a rate comparable to grapevine xylem sap flow under high transpiration conditions (Braun and Schmid, 1999a; Braun and Schmid, 1999b; Lascano et al., 1992; Peuke, 2000), the bacteria migrated predominately against the direction of flow. Under both flow and no-flow conditions the cells were either prostrate on the substratum or, often they were erect and attached at one pole. Maximum twitching speed for *X. fastidiosa* cells examined under flow conditions was 4.9 ±1.1 μm min^{-1} (n = 17), a speed comparable to the observed rate of bacterial spread within grapevines assessed through destructive sampling (Newman et al., 2004).
(Also see, http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/)

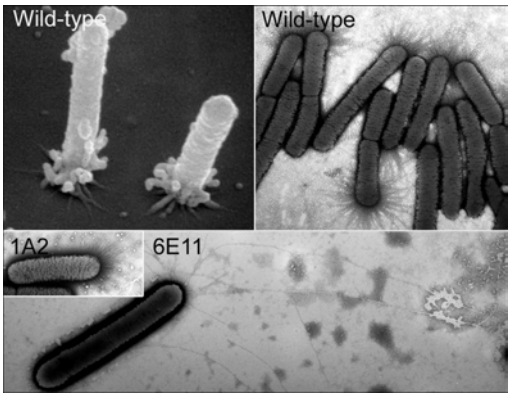


Light micrographs of time-lapse series depicting paths of three (circled red, green, black) wild-type twitching bacteria in microfluidic channels under flow (left) and no flow (right) conditions. Scale bar, 10 μm. Time (h:min:sec). Lower figure, cumulative twitching motility paths for 17 cells under corresponding conditions for 60 min, respectively.

A number of mutant strains were identified as twitching-minus mutants; two (1A2, 5A7) are reported here. Colony peripheries of 1A2 and 5A7 were well demarcated and without bacteria distinctly separated from the main colony mass (lack of peripheral fringe). Colony expansion for these two mutants occurred through repeated cell division and gradual spread as the cell mass increased. When examined in the microfluidic chambers, neither mutant strain exhibited migration, with or without medium flow. Both of these strains were biofilm enhanced. Another mutant, 6E11, was found to be biofilm deficient but still produced colonies with a peripheral fringe and exhibited active twitching, similar to that observed for the wild-type strain. Growth rates of all mutants were not significantly different from the wild-type strain. Sequence analysis of mutants 1A2, 5A7, and 6E11 indicated that transposon insertion occurred in ORFs PD1927, PD1691 and PD0062 of the Temecula genome corresponding to putative genes *pilB*, *pilQ*, and *fimA*, respectively. PilB is known to function as a nucleotide binding protein supplying energy for pilin subunit translocation and assembly, whereas PilQ is a multimeric outer membrane protein that forms gated pores, through which the pilus is extruded (Wall and Kaiser, 1999; Alm and Mattick, 1997; Strom and Lory, 1993). Mutants deficient in these proteins have smooth colony edge phenotypes, do not twitch, and are generally devoid of type IV pili (Kang et al., 2002; Huang and Whitchurch, 2003; Alm and Mattick, 1997; Strom and Lory, 1993). Disruption of *fimA* in *X. fastidiosa* (Feil et al., 2003) as well as in *E. coli* (Orndorff et al., 2004) indicates that the gene encodes for an essential protein of type-I pili that functions in surface attachment and biofilm formation.



Biofilm formation by *X. fastidiosa* wild-type (T1) and mutant strains 1A2, 5A7, and 6E11 following 7 days growth.



SEM and TEM of wild-type cells attached to the substratum at the pili-bearing polar ends. Mutant strains 1A2 and 6E11 depicting only short pili and only longer type-IV pili, respectively.

Electron microscopy substantiated the presence of polar pili on the wild-type and many of the mutant strains.

Negative staining of TEM preparations of the wild-type strain revealed an abundance of pili, the majority of which were 0.4-1.0 μm in length with many additional filaments 1.0-5.8 μm in length. Mutant strains 1A2 and 5A7 had only the shorter class of pili, whereas strain 6E11 had predominantly long pili. The correlation between the presence of long and short pili on the wild-type *X. fastidiosa* strain, the occurrence of essentially only long pili on the twitching, biofilm-deficient strain (6E11), and the absence of long pili on the twitching-minus, biofilm-enhanced mutants (1A2 and 5A7), clearly relates to distinct functional roles for two length classes of pili.

CONCLUSIONS

Microfabricated fluidic chambers were created to mimic plant xylem vessels, in which we studied the non-flagellated *Xylella fastidiosa* bacterium. We discovered that the bacteria migrate 'upstream' by twitching motility, which explains, in part, how they are able to travel against the flow direction of xylem sap to invade non-colonized plant regions.

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