BIOLOGY OF THE *XYLELLA FASTIDIOSA*-VECTOR INTERFACE

Principal Investigator:
Rodrigo Almeida
Dept. Environ. Sci., Policy, & Mgmt.
University of California
Berkeley, CA 94720
rodrigo@nature.berkeley.edu

Researcher:
Nabil Killiny
Dept. Environ. Sci., Policy, & Mgmt.
University of California
Berkeley, CA 94720
nabilkilliny@nature.berkeley.edu

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ABSTRACT
Xylella fastidiosa (*Xf*) has complex life histories because it must colonize both plant host and its vectors for successful dissemination. The switch from host to vector environments may require changes in gene expression prior to the *Xylella*'s departure from the plant. We found that structural polysaccharides of plant host origin are important in regulating *Xf* gene expression and mediating vector transmission of this pathogen. Through the addition of pectin and glucan to a simple defined medium we showed dramatic changes in *Xf*'s phenotype and gene expression profile. Cells grown in the presence of pectin become more adhesive than in other media tested. In addition, the presence of pectin and glucan in media result in significant changes in the expression of several genes previously identified as important for *Xf*'s pathogenicity in plants. Furthermore, vector transmission of *Xf* is induced in the presence of both polysaccharides. Our data show that host structural polysaccharides mediate gene regulation in *Xf* which results in phenotypic changes required for vector transmission. A better understanding of how vector-borne pathogens shift from host to vector, and vice-versa, may lead to novel disease control strategies.

INTRODUCTION
The interactions between *Xylella fastidiosa (Xf)* cells and the foregut of vectors probably are complex and specific, as other xylem-limited bacteria such as *Leifsonia xyli* are acquired but not transmitted by insects (Barbehenn and Purcell 1993). However, little is known about the specific interactions between *Xf* and the foregut of vectors. In previous work, we used different approaches to determine how *Xf* cells interact with the cuticular surface of the foreguts of vectors. We demonstrate that *Xf* binds to different polysaccharides with various affinities and that these interactions are mediated by cell surface carbohydrate-binding proteins. In addition, competition assays showed that *N*-acetylglucosamine inhibits bacterial adhesion to vector foregut extracts and intact wings, demonstrating that attachment to leafhopper surfaces is affected in the presence of specific polysaccharides. *In vitro* experiments with several *Xf* knockout mutants indicated that hemagglutinin-like proteins are associated with cell adhesion to polysaccharides (Killiny & Almeida 2009). These results were confirmed with biological experiments in which hemagglutinin-like protein mutants were transmitted to plants by vectors at lower rates than that of the wild type. Furthermore, although these mutants were defective in adhesion to the cuticle of vectors, their growth rate once attached to leafhoppers was similar to that of the wild type, suggesting that these proteins are important for initial adhesion of *Xf* to leafhoppers. It seems that *Xf* colonization of leafhopper vectors is a complex, stepwise process similar to the formation of biofilms on surfaces. In order to more characterize these complex interactions, setting up an artificial diet system with which *Xylella* cells can be acquired by the insect vector, is essential. The artificial diet system would allow the delivery of cells directly to sharpshooters without the requirement of source plants. Because cells interact with plants, are unevenly distributed in the xylem network, and have different gene expression profiles depending on individual vessel colonization stage, it has always been challenging to study how *Xf* colonizes its vectors. In addition, an artificial diet system would permit us to control the delivery of cells and compounds that may block transmission, to sharpshooters under various conditions. We and other groups have tried to develop this protocol, on and off, for 30 years. Here we report on our successful efforts to deliver transmissible *Xf* cells to vectors. This is an essential component of our project, as now we can easily compare cells that are transmissible if grown on certain conditions with others that are not transmissible. Here we describe for the first time a successful *Xf* transmission through sachet.

OBJECTIVES
1. Establishment an artificial diet system to deliver *Xf* to vectors.
2. Characterization of chitin-binding proteins in the *Xf* surface.
3. Identification of molecules that disrupt *Xf* adhesion to vector foregut surface.

We focus on Objective 1 in this report due to space limitations. We have preliminary data for objective 2 and have already set up experiments to test a large number of molecules to block *Xf* transmission (Objective 3, results pending).

RESULTS AND DISCUSSION

Host polysaccharides induce phenotypic changes necessary for the vector transmission of *Xf*.

Cells were grown in vitro, suspended in buffer, confined in parafilm sachets and given to sharpshooters in small cages. Insects are then transferred to grape seedlings for an inoculation access period. Our group and others have successfully shown acquisition of cells from such a system before. However, those cells were never transmitted to plants. Our work started with two assumptions. First, the most commonly used medium to grow *Xf*, PWG, is too rich, does not mimic conditions cells encounter in nature, and likely results in pathogen gene expression profiles that are not correlated with those in plants or insects. Therefore, we modified a previous published medium (Almeida et al. 2004) to obtain XFM, a simple defined medium to grow *Xf*. Our second assumption was that a plant component induced pathogen transmission, as cells colonizing plants are obviously transmitted by insects. We highlight that, although our work focuses on transmission, these results should be useful for the *Xf* community in general, especially those interested in how this bacterium colonizes plants. Our approach to understand the biology of our system was to compare 4 different media for different phenotypic, biochemical and molecular characteristics (**Figures 1** and **2**). These media are: PWG (rich medium, most commonly used for *Xf* research), XFM, XFM-glucan (XFM with glucan), and XFM-pectin (XFM with pectin). We choose glucan and pectin as plant polysaccharides because they occur on pit membranes and xylem cell walls, surfaces with which *Xf* interacts and is capable to degrade. The *pglA* mutant was also used to confirm results (**Figure 3**)

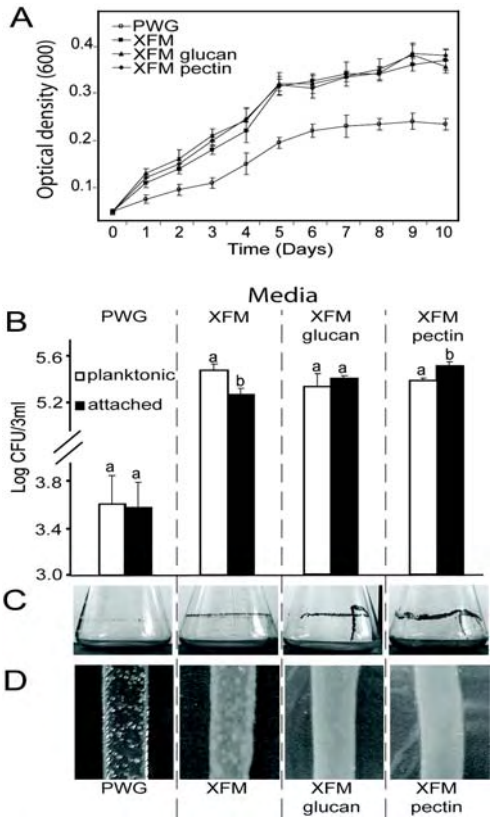

Figure 1. Growth characterization of *Xf* on XFM media. *Xf* (Temecula isolate) cells reached higher populations on all XFM-based media compared to PWG (A). These results are interesting, suggesting that although PWG is the most robust medium for growth of different *Xf* strains, it either suppresses growth or does not provide adequate growth conditions as the much nutrient poorer XFM. Similar results can be observed in (B), where populations were higher on XFM-media than PWG.

Of interest to our work, and of many other groups, are *Xf*'s adhesion phenotypes. In (B) we compared the number of planktonic versus attached cells grown in tissue culture tubes in these media. Both glucan and pectin induced cell adhesion, compared to the basal XFM medium. *Xf* adhesion is mediated by a large number of surface proteins, such as pili and hemagglutinins.

Figure (C) shows adhesion 'rings' on culture flasks when cells were grown on these different media, illustrating the data presented in (B) In addition, colony phenotype on these media were different on solid surfaces. On solid media (D), cells formed a uniform, glossy lawn on XFM-glucan and –pectin, when compared to individual colonies on PWG, which were more typical of *Xf*. Colonies on XFM medium were intermediate among these treatments. These results suggest, among other things, that gum is up-regulated in XFM media.

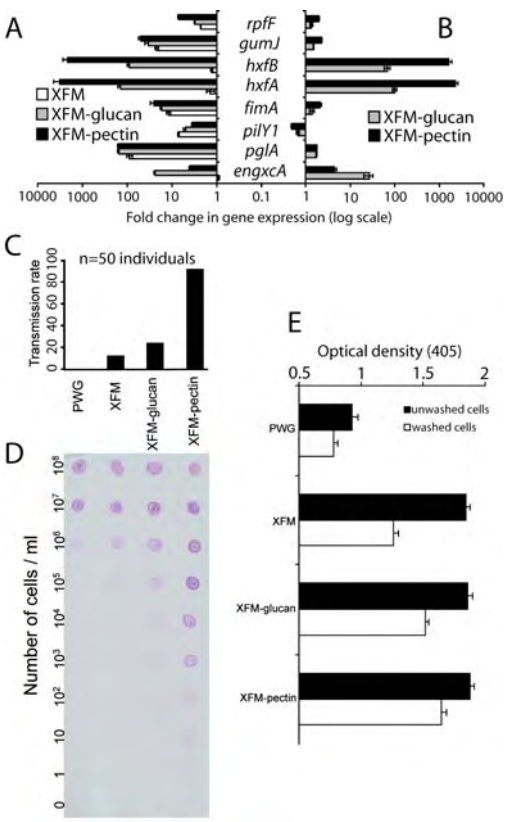

Figure 2. In (A) we compared gene expression of multiple *Xf* genes when cells were grown in different XFM-based media compared to PWG. Equal gene expression was represented by the value of '1.' In (B) we compared the role of glucan and pectin on transcription, in this case '1' represents expression equal to XFM. We focused on adhesion and pathogenicity genes. It is clear that all these genes are repressed in PWG. In relation to XFM, however, hemagglutinins were up-regulated many fold, while other genes were up-regulated but not as much. The type IV pilus gene tested (*pilY1*) was down-regulated. These data suggesting that these carbohydrates induce a cell 'adhesion' phenotype and limit movement within plants; similarly to *Xf* occurring in high cell density. In (D) we quantified the amount of hemagglutinin-like proteins on cells grown in the four media. We have previously shown that these proteins were associated with vector transmission of *Xf* to plants. The results show that the proteins were expressed at higher rates in cells grown on pectin and glucan compared to PWG and XFM. Our in vitro transmission tests (C), using the artificial diet system described above, show that transmission was above 90% efficient if cells were grown on XFM-pectin, while no transmission was observed on PWG. It is interesting to note a good correlation between the quantity of hemagglutinin on cells and vector transmission of *Xf* using the in vitro system. Lastly, in (E), we compared the amount of gum produced by cells on these different conditions. Gum production was similar in all XFM media, but much lower on PWG, which may explain why colonies have different phenotypes on solid media (previous Figure)

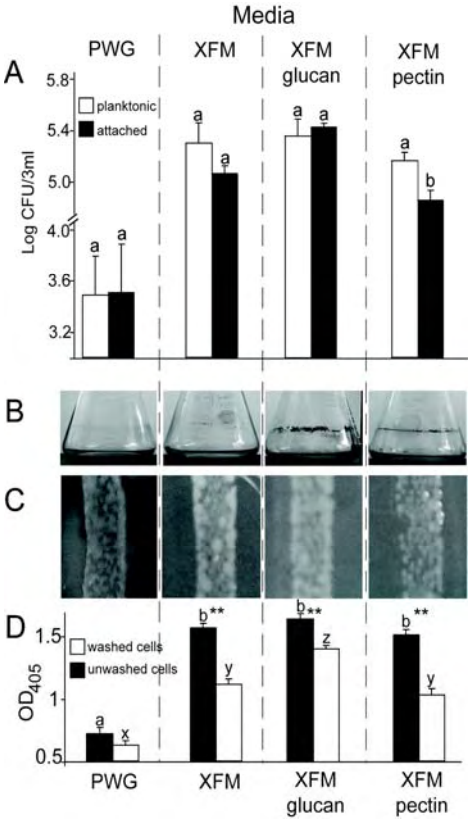

Figure 3. Growth characterization of *Xf pglA* mutant grown in four different media. (A) Populations of planktonic versus glass-attached cells grown *in vitro*, bars with the same letter are not different from each other within media treatments (t-test, $P < 0.05$); and B) biofilm formation at air/broth interface in different media. (C) visual aspect of bacterial lawns on solid media, 'glossy' phenotype likely associated with exopolysaccharide production. D) EPS production quantified immunologically in four media (unwashed cells – filled bars, washed cells – empty bars), asterisks ($P < 0.05$ for one, $P < 0.001$ for two) indicate within media differences, bars with the same letter are not different from each other within wash treatments.

The main subunit of pectin is responsible for gene induction in *Xf*.
We showed that pectin mediates the transmission of *Xf*, but pectin is a very large and complex molecule. Thus, we also studied the effect of different subunits of pectin in the changes in phenotype and gene expression we observed. Pectin is primarily composed of galacturonic acid with rhamnose side chains; the ratio of these sugars is host plant species dependent (Cho et al 2001, Coutinho & Henrissant 1999). We tested the effect of rhamnose and galacturonic acid separately. Results are described in **Figures 4 and 5**.

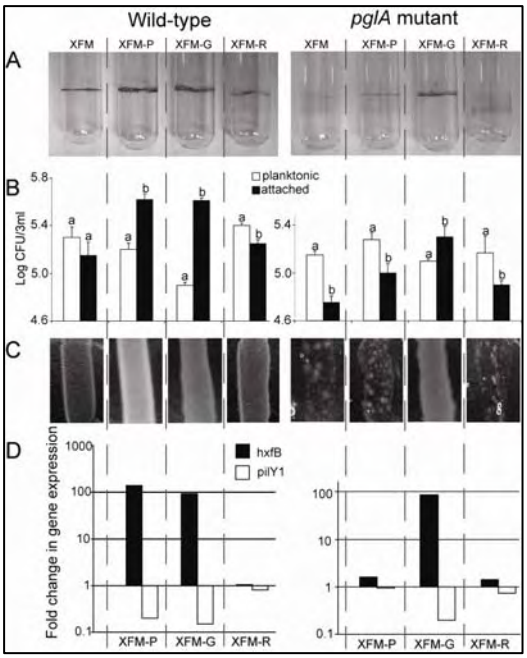

Figure 4 . The main subunit of pectin is responsible for gene induction in *Xf*. A) Biofilm formation at air/broth interface for the *Xf* wild-type and polygalacturonase (*pglA*) mutant in four media; XFM, and XFM supplemented with -P(ectin), -G(alacturonate-Na) and -R(hamnose). B) Populations of planktonic versus glass-attached cells grown in four media, bars with the same letter are not different from each other within media treatments (t-test, $P < 0.05$); and C) visual aspect of bacterial lawns on solid media. D) Quantification of *hxfB* and *pilY1* transcription under the same conditions for the wild-type and mutant; value of 1 indicates transcription level equal to cells grown in the basal medium XFM.

Taking together, it seems that galacturonic acid is the selective sugar for *Xf* and it is the responsible of the change in phenotype and gene expression profiles. In order to confirm these results we used the *pglA* mutant, the mutant grow in the presence of galacturonic acid just like the wild type in the presence of pectin, which corroborates other observations that this sugar is responsible for the phenotypic changes we found.

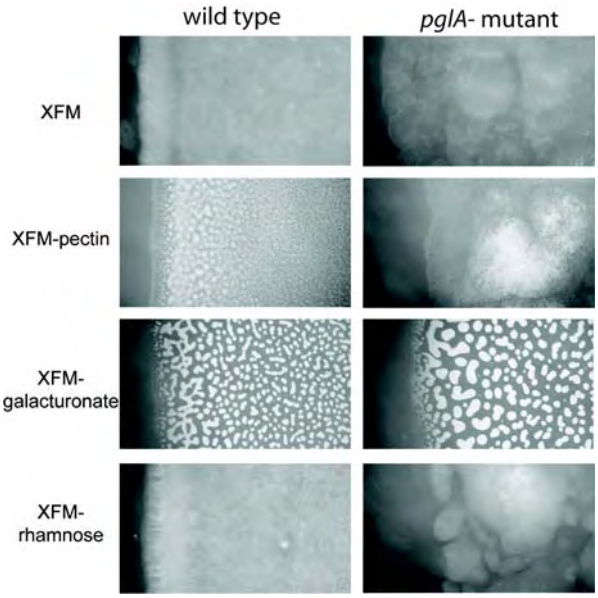

Figure 5. Phenotype of wild type and *pglA* mutant colonies on XFM media supplied with pectin or one of its major component. Pictures focus on the edge of plated cells for comparative purposes Images were taken using LEILKA M125 steromicroscope with 100X total magnification. Inoculums were adjusted to OD600 of 0.2 before plating on the media.

Host polysaccharides induce changes in gene regulation profiles.
In order to better understand the changes to cells when grown on XFM-pectin, we carried out a gene expression microarray analysis for *Xf*'s whole genome. Not surprisingly given the phenotypic changes observed, we found the presence of pectin induced many changes on the transcriptome of *Xf* (**Figure 6**).

(*A*) Hierarchical clustering analysis of microarray expression data for 187 genes found to be differentially regulated during growth in PWG, XFM, XFM-pectin, and XFM-glucan, respectively. (*B*) Up-regulated genes in the presence of pectin. (*C*) Down-regulated genes in the presence of pectin when compared to XFM. Note the gradient increasing or decreasing gradient of expression data are the average of 4 independent replicates of each treatment. The most intense red and blue colors correspond to increased or decreased expression values respectively. Genes having ratios of ≥ 1.6 or ≤ 0.6 fold for expression were selected as up-regulated or down-regulated respectively. PWG was used as baseline for other media. (*D*) Scatter plots of XFM media against PWG showing difference on gene expression.

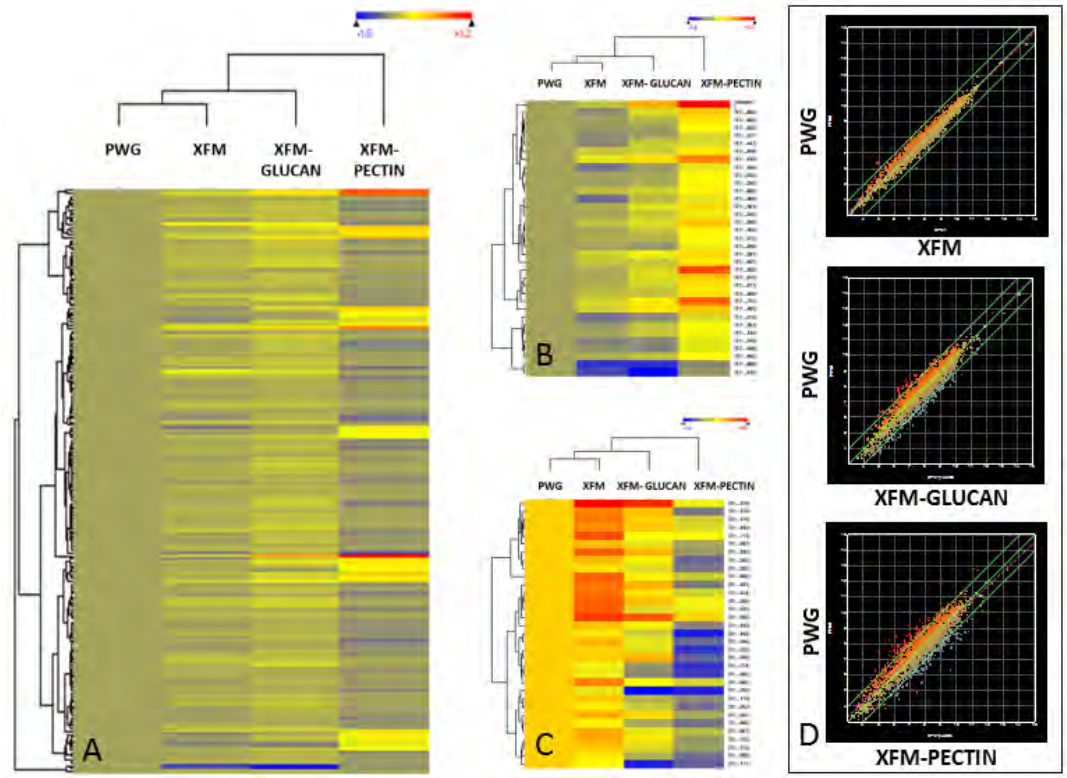

Figure 6. Microarray analysis for gene expression of cells grown in different media.

CONCLUSIONS

In this report we described the first successful transmission through artificial diet systems for *Xf*. We found that *Xf* cells grown in a minimal media XFM supplied with polysaccharides from host, especially pectin were capable to be acquired and transmitted by the vector to grape plants. Pectin induced gene regulation and subsequently, a different growth phenotype than cells grown in the complex medium PWG. Hemagglutinin-like proteins and exopolysaccharides were found to be significantly over expressed and thus associated with the transmission. These results support our previous work (Killiny & Almeida 2009). This artificial diet system (transmission through sachet) will be a powerful tool to study and identify molecules may disrupt the transmission using competition assays. Furthermore, we can study the transmissibility of certain mutants that may grow in lower populations in plants than required for the transmission from plant to plant by insects. Lastly, our results should be useful to other *Xf* research groups interested in plant-pathogen interactions.

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