BLOCKING *XYLELLA FASTIDIOSA* TRANSMISSION

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ABSTRACT
The insect-transmitted bacterium *Xylella fastidiosa (Xf)* colonizes the foregut of its insect vectors. We provide evidence suggesting that chitin in the cuticle of leafhopper vectors of *Xf* may serve as a carbon source for this bacterium. Chitin-enhanced media resulted in *Xf* growth to larger populations. In addition, chitin induced phenotypic changes such as increased adhesiveness. Furthermore, transcriptional changes in the presence of chitin were observed. We demonstrated chitinolytic activity by *Xf* and identified an ortholog of chitinase A (*chiA*) in the *Xf* genome. *chiA* encodes a protein of 351 amino acids with an expected molecular mass of 40 kDa. *chiA* is in a locus that consists of genes implicated in polysaccharide degradation; ChiA was determined to be secreted by cells. We cloned *chiA* into *Escherichia coli* and endochitinase activity was detected in transformants. These findings show that *Xf* has chitinolytic activity and that chitin utilization may be important for vector colonization. Moreover, chitin induces *Xf's* gene regulation and biofilm formation. The data presented here were recently published (Killiny *et al* 2010)

INTRODUCTION
Xylella fastidiosa (Xf) has a complex life history since it has to interact with host plants and insect vectors. In our previous work we characterized the molecular interactions of *Xf* with the surface of its vector's foregut. We showed that the initial attachment occurs through carbohydrate binding proteins including hemagglutinins (Killiny & Almeida 2009b). Moreover, we found that pectin from the host plant induced the transmissibility of *Xf* by regulating gene expression and making cells stickier to surfaces. This adhesive state is essential for the initial attachment to foregut and eventually transmission to plants (Killiny & Almeida 2009a). In this report we focus on the characterization of a chitinase we found in *Xf*. Similarly to pectin, chitin induces strong phenotypic changes in this bacterium. Chitin can also be used as the sole carbon source by *Xf*. The utilization of chitin as a signal resulting in phenotypic changes to cells, including adhesiveness, may be essential for sharpshooter colonization.

OBJECTIVES
1. Molecular characterization of the *Xf*-vector interface.
2. Identification of new transmission-blocking chitin-binding proteins.

RESULTS
I- Chitin enhances *Xf* growth and induces the adhesiveness: *Xf* has chitinase activity.
Our previous work revealed the importance of plant polysaccharides in inducing *Xf* cells into a transmissible (adhesive) state (Killiny & Almeida 2009a). Additionally, we demonstrated that *Xf* cells have carbohydrate binding proteins, including hemagglutinin-like proteins and fimbrial adhesins, which are required for the efficient transmission (Killiny et al 2009b). These proteins bind to the cuticle surface of the vector foregut in the initial step of mouthpart colonization. We showed that these proteins are up-regulated in a defined medium (XFM) supplemented with pectin. Once the cells are acquired by the insect vector they attach to the surface of foregut, multiply, and form a biofilm. Adult vectors then can transmit *Xf* throughout its life. Here we provide evidence that chitin affects gene expression and maintains *Xf* cells, possibly in a 'permanent' adhesive state. **Figure 1** describes the effect of chitin in *Xf* growth, phenotype, and the gene expression of genes implicated in the adhesions to surfaces. We also provide the first evidence of the chitinase activity presence in *Xf* cells.

II- *Xylella fastidiosa* uses chitin as a sole carbon source.
The *in silico* analyses revealed the presence of an ortholog of chitinase A (PD1826) in the genome of *Xf* (**Figure 2**). We confirmed the chitinase activity of PD1826 by the expression of gene in *E. coli* and then detected the activity using ([4-MU(GlcNAc)$_3$] (**Figure 3D**). The growth of *Xf* in XFM, which contains colloidal chitin as a sole carbon source (**Figure 3A**), and on Chitin-yeast plates (**Figure 3C**) suggest that *Xf* uses chitin as a carbon source. Using quantitative PCR, we showed that the expression of *chiA* (PD1826) is correlated by bacterial population growth through time (**Figure 3A**). By using [4-MU(GlcNAc)$_3$**],** we also detected an increase in the chitinase activity (**Figure 3B**)**.** In **Figure 4** we used the hind wings of the glassy-winged sharpshooters to mimic the foregut surface. GFP-*Xf* cells were initially suspended on XFM medium with no carbon source and then this suspension was placed on the wing tissue. The increase of fluorescence over time reflected the growth of cells and confirms that *Xf* can utilize chitin from the wing surface as a carbon source. GFP-*rpfF*-*Xf* cells (Newman *et al* 2003) did not grow as much as the wildtype strain. It is known that *rpfF* mutant is not transmissible due to lack of the diffusible signaling factor (DSF) (Newman *et al* 2004). DSF regulates the expression of important genes

such as the fimbrial and afimbrial adhesions and also ChiA. We predicted genes implicated in the chitin utilization machinery similar to those in other chitinolytic bacteria. Here, we present a Hypothetical model for this machinery in *Xf* (**Figure 5A**). **Figure 5B** provides evidence for degradation of blue-green sharpshooter foregut surface by *Xf*.

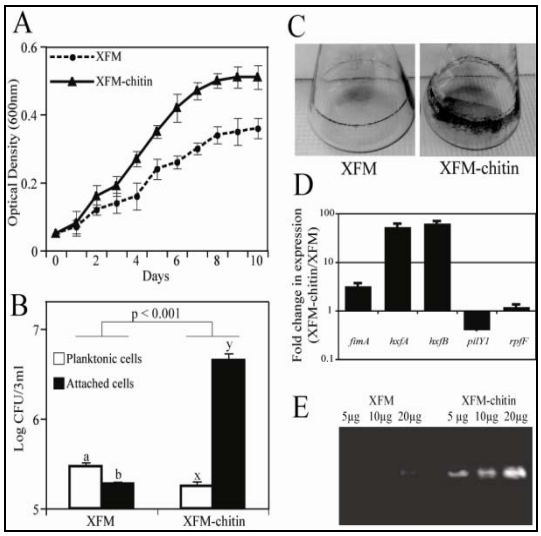

Figure 1. Effect of chitin on *Xf*. A) Growth curve of *Xf* in XFM in presence or absence of colloidal chitin. B) Log-transformed cell numbers present as planktonic or attached as a biofilm on glass in liquid XFM. Different letters on bars indicate statistically different treatments. C) Biofilm formation of *Xf* in a shaking culture; the biofilm is stained at the broth-air interface. D) Chitin induced transcriptional changes in *Xf*. E) In-gel chitinase activity ([4-MU(GlcNAc)$_3$] cleavage) of *Xf* whole-cell culture extracts.

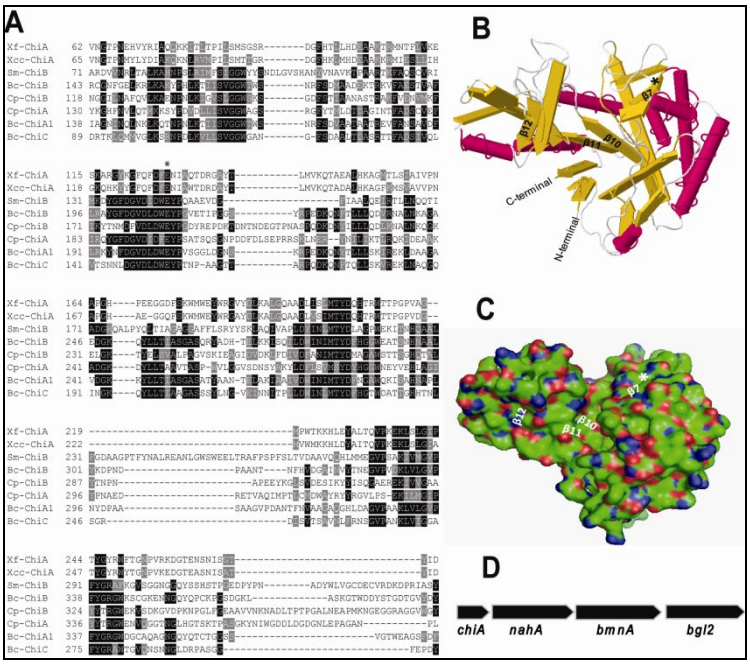

Figure 2. *In silico* analyses of *chiA* and its translated protein. A) Alignment of putative catalytic region in bacterial chitinases family 18 of *Xylella fastidiosa* (*Xf*), *Xanthomonas campestris campestris* (Xcc), *Serratia marcescens* (Sm), *Bacillus cereus* (Bc), *Clostridium paraputrificum* (Cp), and *Bacillus circulans* (Bc). Conserved amino acids are indicated with black shading and those with high similarity score are in gray. The glutamic acid residue identified as a proton donor necessary for activity is marked with an asterisk. B) Predicted three-dimensional structure and C) molecular surface of *Xf* ChiA; regions with similarity to the glycoside.

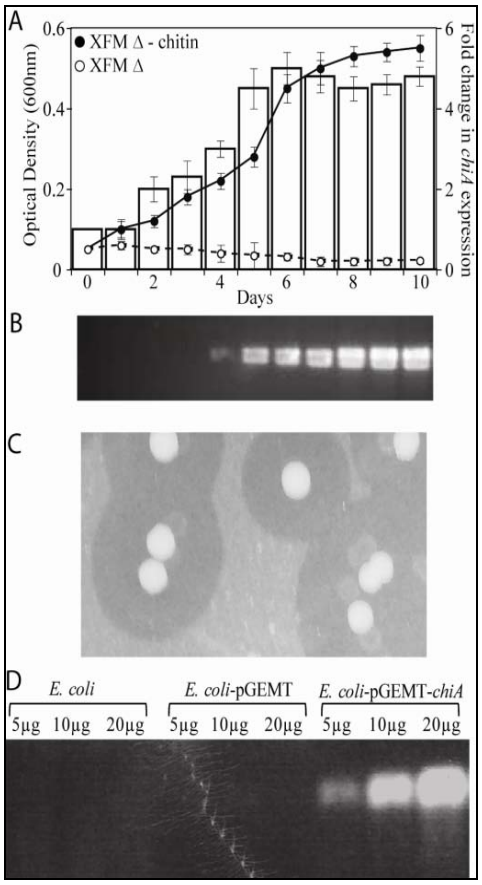

Figure 3. Chitin utilization by *Xf*. A) Bacterial growth curve in XFM medium without carbon sources (XFM Δ) or with chitin added as the only carbon source (XFM Δ-chitin); bars (second Y-axis) show the progression of *chiA* expression over time. B) In-gel chitinase activity ([4-MU$(GlcNAc)_3$] cleavage) over time for *Xf* culture filtrates in liquid XFM Δ-chitin, demonstrating ChiA secretion. C) *Xf* colonies grown on chitin-agar medium; the clear zones around colonies indicate chitin degradation. D) In-gel chitinase activity ([4-MU$(GlcNAc)_3$] cleavage) for *Escherichia coli* strains grown in LB medium.

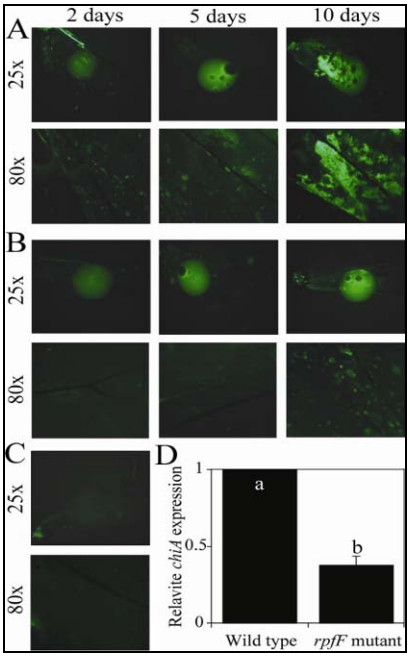

Figure 4. *Xf* growth and biofilm formation on hindwings of leafhopper vectors. Cells were suspended in XFM Δ and drops were placed on wings. A) Wild-type and B) cell-cell signaling *rpfF* mutant cells were incubated up to 10 days on wings. C) Control, medium XFM Δ without cells. The upper pictures were taken at 25x magnification and are suspension droplets; the lower pictures were taken at 80X magnification after removing the drop of medium and rinsing the wings with water. D) *chiA* expression in the cell-cell signaling mutant *rpfF* compared to the wild-type; different letters on bars represent statistically different treatments.

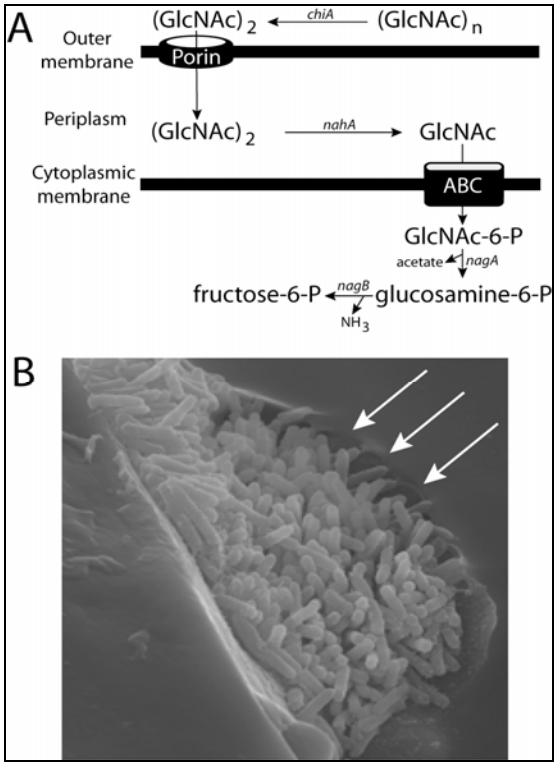

Figure 5. A) Hypothetical model for chitin utilization in *Xf*. Chitin is hydrolyzed to chitobiose outside the cell by *chiA* and passively transported into the periplasmic space as a dimer. *nahA* converts the substrate into *N*-acetylglucosamine, which is phosphorylated and transported into the cytoplasm via an ABC transporter. B) Scanning electron microscopy micrograph of *Xf* cells colonizing the mouthparts of a leafhopper vectors. The arrows indicate potential degradation of the chitinous surface at the fringe of microcolony.

CONCLUSIONS

The aim of this project is to learn more about molecular interactions of *Xf* with its insect vector and to identify moleculess that can be used to disrupt such interactions, and subsequently block the transmission. We found that *Xf* uses chitin as a carbon source. We also showed that fimbrial and afimbrial adhesions are up regulated in response to chitin utilization. These findings suggest that the chitin is second to pectin in its importance as an environmental factor regulating gene expression in *Xf*. While pectin is essential in introducing the adhesive state of the bacterial cells, chitin functions to maintain this state. Both components are required for the successful transmission of *Xf* by its leafhopper vectors to other host plants.

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