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Title of project: Exploiting a chitinase to suppress Xylella fastidiosa colonization of plants and insects

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Abstract
Our research has shown that *Xylella fastidiosa* has a chitinase (ChiA), which is required for sharpshooter vector colonization, transmission to plants, as well as plant colonization. The goal of this project was to understand the function(s) of ChiA so that it can be exploited as a tool for control of Pierce's disease by disrupting *X. fastidiosa* interactions with both plant and insect hosts. A series of different approaches were used to better characterize the role of ChiA in *X. fastidiosa*. This report summarizes efforts aimed at experimentally addressing these goals.

Layperson Summary
The previously identified *X. fastidiosa* chitinase (ChiA) represents a unique opportunity to try to disrupt *X. fastidiosa* interactions with both insect and plant hosts, as well as sharpshooter transmission, because all of these processes are affected in the mutant strain that does not have this enzyme. The goal of this project was to better understand how ChiA impacts plant and insect colonization so that it can be exploited to limit Pierce's disease spread.

Introduction
Xylella fastidiosa has a chitinase (ChiA) that was identified a few years ago in studies of *X. fastidiosa*-sharpshooter interactions (Killiny et al. 2010). We have shown that *X. fastidiosa* is able to use chitin as its sole carbon source in vitro, and that ChiA is required for both plant and insect colonization. Lastly, we have demonstrated that ChiA itself does not bind to its substrate and that other proteins are necessary for its enzymatic activity. In other words, all data available indicate that ChiA plays an essential role in *X. fastidiosa* biology. It is clear that ChiA represents a unique target for control of both *X. fastidiosa* colonization of host and Pierce's disease spread. However, there are significant gaps on our knowledge of the role of ChiA in *X. fastidiosa* biology. This project aimed at further characterizing ChiA, as it represents a novel target to understand how this important pathogen colonizes insect and host plants.

Objectives
The original objectives of this project are listed below. In addition to these, to address technical challenges, we also performed other experiments that are described below to better understand how chitin impacts *X. fastidiosa* biology. The results are summarized below.

i) to identify *X. fastidiosa* proteins or protein complexes that bind to ChiA and are required for its chitinolytic activity.
ii) to screen potential substrates cleaved by ChiA.
iii) to functionally demonstrate the role of ChiA partners during insect and plant colonization.

Results and Discussion

To identify the ChiA partner *X. fastidiosa* (Temecula) was grown on solid XFM media supplemented with either galacturonic acid (GA) or colloidal chitin (0.1g of either) in order to simulate a plant or insect environment and induce phenotypic changes in the bacteria (Killiny and Almeida 2009, Killiny et al. 2010). Cells were scraped after 10 days and resuspended (PBS initially, then TE; both have worked well), with 1µM PMSF added to inhibit protease activity (Killiny et al. 2010). Cells were lysed via 5 cycles of freeze-thaw, and concentration was determined according to Bradford. An IMAC Ni resin column (BioRad) was used in an attempt to isolate ChiA binding partners. After purified ChiA (produced by our group following standard protocols, recombinantly expressed in *E. coli* cells) was bound to the column, whole cell lysate was applied and allowed to incubate for 30 minutes. Working on the assumption that the proteins of interest would remain bound to the ChiA and elute with it, the column was then washed with increasing concentrations of imidazole in PBS. The fractions were then run in SDS-PAGE, which denatures and should therefore cause protein complexes to disassociate, against a purified ChiA control, and stained with Coomassie blue. No proteins were found to elute with the ChiA fractions, the experiment was repeated several times and, for now, we have decided to not pursue this approach any longer.

Alternatively, more recently a solution of 4g colloidal chitin from shrimp shells (Sigma) was prepared. Five milliliters of the solution was allowed to settle in the column, which yielded a column volume of approximately 1ml. Ten volumes of 2M NaCl were washed through the column prior to loading, and then the column was equilibrated with the aforementioned solution. Whole-cell lysate from Temecula grown on XFM-GA was applied to the column and allowed to incubate for 30 minutes. Increasing concentrations (0.025M, 0.05M, 0.1M, 0.25M, 0.5M, and 1M) of methyl-alpha-D-glucopyranose were used to elute proteins bound to the column. Fractions were run on SDS-PAGE and stained with Coomassie blue. Many bands appeared in the early washes, indicating non-specificity for chitin. One prominent band appears in all subsequent fractions. There should be more bands in the later fractions, but we expect those to have lower concentrations and silver staining will now be used. This approach is simpler than others we hope to run with a postdoctoral researcher, but has now yielded satisfactory results and we expect to soon work on repeating this work so that samples can be sent out for identification of promising bands in late elutions.

One last approach has been tested. This approach to determine ChiA binding partners involved overlaying an agarose gel containing 4-MU(GlcNAc)3 (Sigma) on a native PAGE gel containing whole cell lysate from both chitin- and GA-grown cells incubated with purified ChiA. As 4-MU(GlcNAc)3 only fluoresces when the MU subunit has been cleaved, fluorescence indicates the presence of a functional chitinase (Killiny et al. 2010). Initial assays suggest that optimization of pH in native gels must be performed. No detectable ChiA migrated in the native PAGE, but aliquots from the same sample displayed a positive band in the SDS-PAGE, the pH of the gel may be incompatible with the isoelectric point of the protein. An effort is being made to optimize these experimental conditions. The detectable band in native gel will be cut out and prepared for sequencing following standard protocols.

In summary, three approaches were tested. The first one aimed at identifying proteins binding to ChiA failed and will not be continued, at least for now. Another had promising results and will lead to the identification of chitin-binding proteins in the near future. The last approach should identify a protein that is required by ChiA for its activity in gel, but the protocol still requires some optimization. Therefore, the project is successfully moving forward, albeit slowly than originally proposed.

One method proposed for determining binding partners for ChiA was to incubate purified ChiA with cell lysate of Temecula grown on XFM supplemented with either colloidal chitin or galacturonic acid. This mixture would then be run on a native PAGE gel, which would preserve protein folding and therefore enzymatic activity ; make an overlay gel containing 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotrioside, which fluoresces when cleaved would then be poured onto the native PAGE and imaged under UV light. Areas of fluorescence would indicate

enzymatic activity. We found, however, that the isoelectric point of our chitinase was too close to the pH of the gel, and we were not able to overcome that technical limitation.

The current method we are investigating is to reversibly bind the purified ChiA to its partners using DTSSP. We plan to incubate purified ChiA with cell lysate of Temecula grown on XFM supplemented with either colloidal chitin or galacturonic acid. This mixture will then be bound to a Ni++ ion exchange column, and eluted with imidazole. The fractions will be run on SDS PAGE, with 5% 2-mercaptoethanol acting to cleave the DTSSP and release the ChiA and its binding partners, resulting in multiple bands, which will then be excised and processed via mass spectroscopy. Initial tests provided multiple bands, but we are now working on reproducing the results prior to protein identification.

Alternatively, work was performed to determine *X. fastidiosa* substrates that may require ChiA activity. Two different biolog plates (PM1 and PM2) containing 190 different carbon sources were tested to determine what carbon sources can be degraded and used by *X. fastidiosa*. By comparing the activity of WT cells with the *chiA* mutant cells, we might be able to (i) determine what are the carbon sources which could be used by *X. fastidiosa* and (ii) which ones are directly degraded by the chitinase. We used cells scraped from XFM plates and resuspended into a 25 μM glutamine solution to obtain a final 0D600 ranging from 0.1 to 0.2. 100 μL of this solution as well as Dye G were added to each well of PM1 and PM2a MicroPlate Carbon Sources, each plate containing 96 different carbon sources (Biolog, USA). The plates were then incubated at 28°C for a week. The results were read at 0D590 on a VersaMax microplate reader (Molecular Devices). We further analyzed the carbon sources which were found to be positive on Biolog plates by comparing growth on XFM medium depleted for carbon sources initially present in the medium (ΔXFM) and supplemented or not with the carbon source of interest as previously performed (Killiny and Almeida 2009; 2010). This experiment was done with both *X. fastidiosa* wild-type and *chiA* mutant.

Among the 192 carbon sources tested, positive results were obtained: Tween 20, Tween 40, Pyruvic acid, L-Malic acid, D,L-Malic acid, Fumaric acid, D-galactonic acid gamma lactone, L-ornithine, L-phenylalanine, L-pyroglutamic acid, L-arginine, Inulin, Mannan, Pectin. We note that these represent our own clear positives however we cannot completely exclude other carbon sources for *X. fastidiosa*. So far we have been able to demonstrate with the confirmation assays on plates that both the WT and chiA mutant can use pectin and galacturonic acid as sole carbon sources. Interestingly, we were also able to confirm that Tween20 and Tween40 as sole carbon sources, additional experiments are being performed to follow up on these observations.

As the chitinase mutant is not able to move within grapes - no cell was found at 15 cm above the inoculation point in the 20 plants inoculated with the chitinase mutant whereas they were found in 15 out of the 19 plants inoculated with the WT cells - we tested whether this lack of movement is due to the inability of the mutant to degrade components of the pit membrane. *X. fastidiosa* has indeed been shown to move from one xylem vessel to another by degrading pit membranes. The ability of the WT strain, the chitinase mutant and the complemented strain to degrade cellulose, xylan and pectin was tested. Three different media containing 1.5g/L K2HPO4, 1g/L KH2PO4, 1g/L MgSO4-7H2O, 10 mL/L of a 0.2% phenol red solution, 10 mL/L of a Hemin chloride (0.1% in 0.05% NaOH) solution, 10g/L of gelrite, 3g/L of BSA and 0.2% of methylcellulose or xylan from oat spelts or 0.1% of pectin from apple were prepared. Eight 10μL droplets of the WT, chitinase mutant or the chitinase complemented strain (OD600 = 1.4-1.5) were spotted on each medium. After 6 days of incubation at 28°C, the cellulose and xylan plates were flooded with 1 mg/mL of congo red for 15 min. The congo red was then poured off and a solution containing 1M NaCl was added for an additional 15min. 1 M HCl was finally poured on the plates for longer visualization of the hydrolysis zone (Teather et al. 1982). The pectin plates were flooded with 1% cetrimide solution for 3 days after a 6 day incubation at 28°C (Beg et al. 2000). The experiment was repeated three times per medium and per strain. Halos were observed for the wild type, the chitinase mutant and the chitinase complemented strains for the three different plant polysaccharides (cellulose, xylan and pectin) tested.

Another potential function of the chitinase -not previously evoked in the project- could be to degrade a bacterial defense elicitor, like peptidoglycan, to evade the plant immune system. If this is the case, there should be some differences between the expression of certain plant defense genes in plants infected by the WT cells and the chiA mutant cells. This hypothesis will be tested by measuring by qPCR the expression of some defense-associated genes -such as the genes encoding for PR1 and PR5- on plants infecting by the WT strain, the chiA mutant strain, the chiA complemented strain or mock-inoculated. Besides, an exogenous application of the elicitor (the example of peptidoglycan is here used) either pre-treated or not by a functional chitinase (a complex of the chitinase with its partners) to the plant should led to a colonization of the plant and a virulence respectively similar to the ones observed in plants infected by WT or chiA mutant cells.

To determine if ChiA is involved in interactions with the immune system of plants, one month *Nicotiana tabacum* cv. Xanthii 'Glurk' were needle inoculated at the base of the fourth (or fifth) petiole with 20 µL of an inoculum containing log 9 CFU/ml of either wild-type, *chiA* mutant or *chiA* mutant that was complemented with *chiA*. Mock inoculation with succinate-citrate phosphate buffer was performed on the same number of plants as a control (12-14 plants). Two days post inoculation, the inoculated leaves were harvested from half of the plant for each treatment. The other half was collected 4 days post inoculation. The leaves were immediately frozen and kept at -80°C until further analysis. In parallel, 14 grapevines (var. Cabernet Sauvignon) per treatment were inoculated with 5 µL of an inoculum containing log 9 CFU/ml on the stem. Half of the leaves situated above the inoculation point was collected at 2 dpi whereas the other half was collected at 4 dpi. RNA was extracted from these plants to determine if there are differences in key plant response genes among treatments. Results suggested no differences among treatments, although results were inconclusive.

Conclusions

The work confirmed that ChiA has multiple roles in *X. fastidiosa*, both in plant and insect colonization. Although it was demonstrated that s ChiA cleaves a range of substrates, results were not conclusive in demonstrating a specific role in plant colonization. Efforts to identify host specific protein partners were not successful.

Publications and Presentations

. Exploiting a chitinase to suppress *Xylella fastidiosa* colonization of plants and insects. Pierce's Disease Research Symposium, Sacramento, CA, Dec 2014.

. Pierce's disease in winegrapes and olive. 3rd Annual Vineyards & Wineries Continuing Education Class Series, Nov 4, 2014, Napa, CA. Sponsored by UCCE, Farm Bureau and the Napa Ag Commissioner's Office.

. Emerging Vector-borne Diseases; Forum on Microbial Threats, Institute of Medicine, National Academy of Sciences, Washington DC.

. Talk at UCCE VitTech Extension Meeting at Napa, Jan 13, 2016. 'Pierce's disease in the North Coast - What is known and what is going on?'

. 'Blocking the transmission of a vector-borne bacterial pathogen' At: 2015 American Phytopathological Society Annual Meeting - Symposium: Blocking the Transmission of Vector-Borne Plant Pathogens, Dream or Reality?. Pasadena, CA, July 30-Aug 3.

. Seminar series - Department of Entomology, University of Illinois at Urbana-Champaign, May 2017.

. Invited speaker, Gordon Research Conference - Animal-microbe symbiosis. Mount Snow, VT. June 2017.

. Almeida, R.P.P. 2016. *Xylella fastidiosa* vector transmission biology. In: Vector-mediated transmission of plant pathogens, p. 165-173, Ed. J.K. Brown. APS Press Book, St. Paul, MN.

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