**PROJECT TITLE: HIGH-THROUGHPUT LIVE CELL SCREEN FOR SMALL MOLECULES TARGETING TOLC EFFLUX PUMP OF *XYLELLA FASTIDIOSA*.**

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## ABSTRACT

Type I secretion (T1S) by *Xylella fastidiosa* (Xf) is required for multidrug efflux, a pump critical for survival of Xf in grapevines. In Xf, T1S depends on a very limited number of genes, possibly making this system more vulnerable to inhibition by small molecule treatments than T1S found in most bacterial pathogens, which typically carry redundant T1S systems. Xf single gene mutations in the T1S system are much more sensitive to the surfactant Silwet L-77 than wild type Xf. High throughput screening assays of Xf cell viability were developed using fluorescence and optical density measurements both with and without 200 ppm Silwet L-77. GFP-marked Xf strain Temecula1 was used to screen two Prestwick combinatorial small molecule libraries (phytochemical and FDA approved drugs; 1600 chemicals in total) for Xf cell growth inhibition. Significant (>50%) inhibition of Temecula1 growth was observed in presence of 50 µM of 215 different chemicals, 6 of which exhibited even higher (24% - 40%) stronger inhibition in the presence of Silwet L-77, indicating these 6 chemicals possibly target T1S efflux. One hundred and twenty one chemicals inhibited growth by >90% @ 50 µM; while forty six chemicals reduced growth >100%, indicating Xf cell lysis. Seven chemicals, including four phytochemicals, reproducibly lysed Xf at 25 µM levels. One chemical was identified that may be synergistic with native plant defense responses.

## LAYPERSON SUMMARY

*X. fastidiosa* (Xf) survival in grapevine and in many culture conditions depends on a Type I multidrug resistance (MDR) efflux pump system, which plays a critical function in pumping out environmental toxins and host antimicrobial compounds and antibiotics that leak into the bacterial cell and would otherwise kill Xf. Any method that could block or disrupt specific components of this system would likely result in both control of Pierce's Disease (PD) and elimination of Xf from infected plants. Portions of the outermost efflux pump protein, TolC, are embedded in the protective outer membrane (OM) of the bacterium and form the exit portal of the efflux pump. Both the OM and TolC are exposed at the Xf cell surface, making small molecule chemical treatments that target TolC or even the OM barrier attractive chemical targets. Many small molecule combinatorial libraries are commercially available for screening, some including synthetic and exotic chemicals that would likely require considerable testing to meet the high bar set for food safety and agricultural use. Also available are the highly diverse and complementary Prestwick natural phytochemical library and the Prestwick FDA approved drug combinatorial library, together representing 1,600 different small molecules in total. Forty six chemicals have been identified that appear to lyse Xf cells, including one that may be synergistic with native Vitis vinifera chemicals in xylem sap.

## INTRODUCTION

This is a new project that is based on two discoveries made during the course of two earlier CDFA funded projects. The first discovery is our demonstration that the Type I multidrug resistance (MDR) efflux system of *X. fastidiosa* (Xf) is absolutely required for both pathogenicity and even brief survival of the Pierce's Disease (PD) pathogen in grape (Reddy et al., 2007). Knockout mutations of either *tolC* or *acrF* (manuscript in preparation) render Xf nonpathogenic, and in addition, the *tolC* mutants were so highly sensitive to grape chemicals that the mutants are not recovered after inoculation. Inoculation of very high titers of Xf strain Temecula *tolC* mutants in grape results in rapid, 100% killing of inoculated bacteria. These results demonstrated a critical role for Type I efflux in general and TolC and AcrF in particular for defensive efflux by Xf of plant antimicrobial compounds, such as phytoalexins.

In the process of investigating the increased sensitivity of the MDR efflux mutants to plant-derived antimicrobial chemicals, we also discovered that even wild type Xf, with its lone MDR efflux system, is much more sensitive to plant-derived antimicrobial chemicals than most other plant pathogens, which carry multiple efflux systems. Both *tolC* (encoding the outer membrane and periplasmic tunnel component of Type I secretion) and *acrF* (encoding the inner membrane pump component of Type I secretion) are essential for MDR efflux in Xf, which has only one copy of each gene and only one such MDR efflux system. By contrast, most plant pathogens have redundant MDR efflux systems and multiple *tolC* genes. These results suggest that Xf should be much more vulnerable to treatments affecting Type I efflux than other bacterial plant pathogens.

MDR efflux mutants in other systems have provided proven, highly sensitive and quantitative screening methods for antimicrobial chemicals (Tegos et al., 2002). The goal of this new project is to exploit the increased vulnerability of Xf and our knowledge of particular chemicals that require efflux in a high throughput assay that screens small molecule combinatorial libraries and Xf-resistant grapevines for chemicals that may disable Type I secretion directly or indirectly. Since: 1) there is only a single *tolC* gene (and TolC is the sole Type I secretion system outer membrane component) in Xf strains, and 2) PD strains are clonal and there is little variation in TolC among PD strains, this makes TolC a particularly attractive molecular target. This should make PD strains highly vulnerable to any blocking agent, including small molecules, that specifically targets TolC or AcrF, disrupts the TolC / AcrF interaction or generally affecting Type I efflux. A highly sensitive live cell assay that is well suited for high throughput screening was developed and is proposed for use for such a screen.

## OBJECTIVES

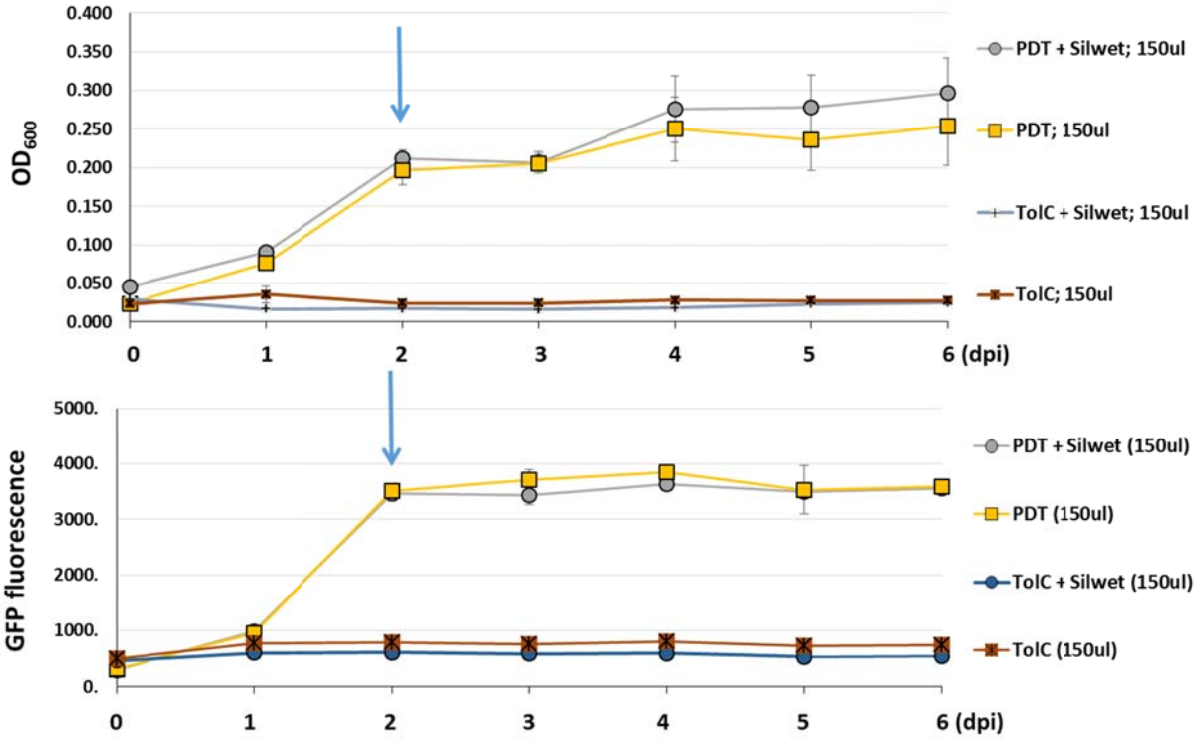
The specific objectives of this one year proposal are:
1. Screen two Prestwick combinatorial libraries for chemicals affecting Type I efflux from Xf.
2. Screen sap and crude extracts from *V. vinifera* grape plants subjected to freezing treatments (sufficient to cure PD) for potential effects on Type I efflux from Xf.
3. Determine if sap and crude extracts from PD resistant *Muscadinia rotundifolia* contain more and/or more effective chemicals affecting Type I efflux from Xf than susceptible *V. vinifera* plants.

## RESULTS AND DISCUSSION

### Objective 1: Screen two Prestwick combinatorial libraries for chemicals affecting Type I efflux from Xf.

This objective has now been fulfilled, and 121 chemicals have been identified that inhibited growth by >90% @ 50 μM, including 46 chemicals that appeared to lyse Xf cells. One chemical has been identified that may be synergistic with native plant defense responses. Seven chemicals proved to lyse Xf cells at 25 μM, including four phytochemicals. An earlier report details how the screen was performed.

Following the primary screen at 50 µM, the effect of different dose levels (25 µM, µM, and 100 µM) were evaluated using 3 replications of each level, in each case with and without Silwet L-77. This evaluation was performed both for confirmation purposes and to determine if a threshold level effect was present for some chemicals. No threshold effects were observed; initial results were confirmed at all dose levels. Silwet L-77 had no effect on any of the phytochemicals. However, Silwet enhanced the inhibition of six compounds from the Prestwick chemical library in primary screen. At different dose levels, only one compound consistently inhibited Xf growth more strongly in the presence of Silwet, and at all three treatment levels, indicating an effect of the chemical on multidrug efflux (Type I secretion).




Both optical density (OD) and GFP fluorescence were measured in a 96 well format, and each measurement sample was repeated in triplicate. Maximum growth was and fluorescence was observed after 2 days.

**Objective 2. Screen sap and crude extracts from *V. vinifera* grape plants subjected to freezing treatments (sufficient to cure PD) for potential effect on Type I efflux from Xf.**

*V. vinifera* grape plants are being cold treated.

**Objective 3. Determine if sap and crude extracts from PD resistant *Muscadinia rotundifolia* contain more and/or more effective chemicals affecting Type I efflux from Xf than susceptible *V. vinifera* plants.**

*Muscadinia rotundifolia* grapevines have been procured and propagated.

## LITERATURE CITED

Reddy, JD, Reddy, SL, Hopkins, DL, and Gabriel, DW. 2007. TolC is required for pathogenicity of *Xylella fastidiosa* in *Vitis vinifera* grapevines. Molecular Plant-Microbe Interactions, 20*:*403-410.

Tegos, G, Stermitz, FR, Lomovskaya, O, and Lewis, K. 2002. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrobial Agents and Chemotherapy 46:3133-3141.

Zhang, S, Chakrabarty, PK, Fleites, LA, Rayside, PA, Hopkins, DL, and Gabriel, DW. 2015. Three New Pierce's Disease Pathogenicity Effectors Identified Using *Xylella fastidiosa* Biocontrol Strain EB92-1. PloS one, 10: e0133796.

Zhang, JH, Chung, TD, and Oldenburg, KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomolecular Screening, 4:67-73.

Zhang, Y, Callaway, EM, Jones JB, and Wilson, M. 2009) Visualisation of hrp gene expression in *Xanthomonas euvesicatoria* in the tomato phyllosphere. European J. Plant Pathology, 124:379-390.

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