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. Over a dozen natural antibiotics and phytochemicals were identified as strongly inhibitory (>80%) at 50 µM, including the phytoalexin gossypol and the alkaloids remerine and olivicine. A dose-dependent effect of selected chemicals and sap and crude extracts from PD resistant grapevines on Xf cell viability and T1S efflux is underway. This study is expected to result in the identification of small molecules that are synergistic with natural plant resistant compounds that could be developed into an effective chemical treatment of PD.

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. This proposal is to adapt an existing and tested live bacterial cell count assay originally tested using mutants affecting Type I efflux in another bacterium to enable high throughput screening of the two Prestwick libraries and also fractionated extracts of Xf-resistant. The result could be an immediately applicable phytochemical spray to control PD or one requiring a minimum of regulatory approval for use on *Vitis vinifera* grapevines.

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.

For the primary screen, plates were preloaded with Temecula1 cells with or without 200 ppm Silwet L-77 and with each tested chemical loaded at a concentration of 50 μM. Each chemical in the Prestwick Phytochemical and Chemical libraries was screened in two separate experiments. The statistical parameter (Z′) was used to evaluate the quality of the assays exactly as described (Zhang et al., 1999). The overall Z′ value for the Prestwick Phytochemical library was 0.76 and the overall Z′ for the Prestwick Chemical library was 0.78; these values are within the statistically "excellent" reproducibility range (Z′ > 0.75; Zhang et al., 1999).

Significant growth inhibition (>50%) of Temecula1 was observed with 22 phytochemicals (Fig. 1), 8 of which exhibited strongly significant growth inhibition (>90%). Greater than 100% inhibition occurred when the optical density (data not shown) and the fluorescence emitted (shown in Fig. 1) was reduced to below that of the starting cell values, and indicated lysis. None of the 320 phytochemical library compounds was found to enhance growth. None of the 320 phytochemical library compounds exhibited enhanced inhibition in the presence of 200 ppm Silwet L-77, indicating that none of these compounds directly affected T1S. Eleven phytochemicals, including some natural antibiotics, were identified as strongly inhibitory (> 80%) at 50 µM, including the phytoalexin gossypol and the alkaloids remerine and olivicine.

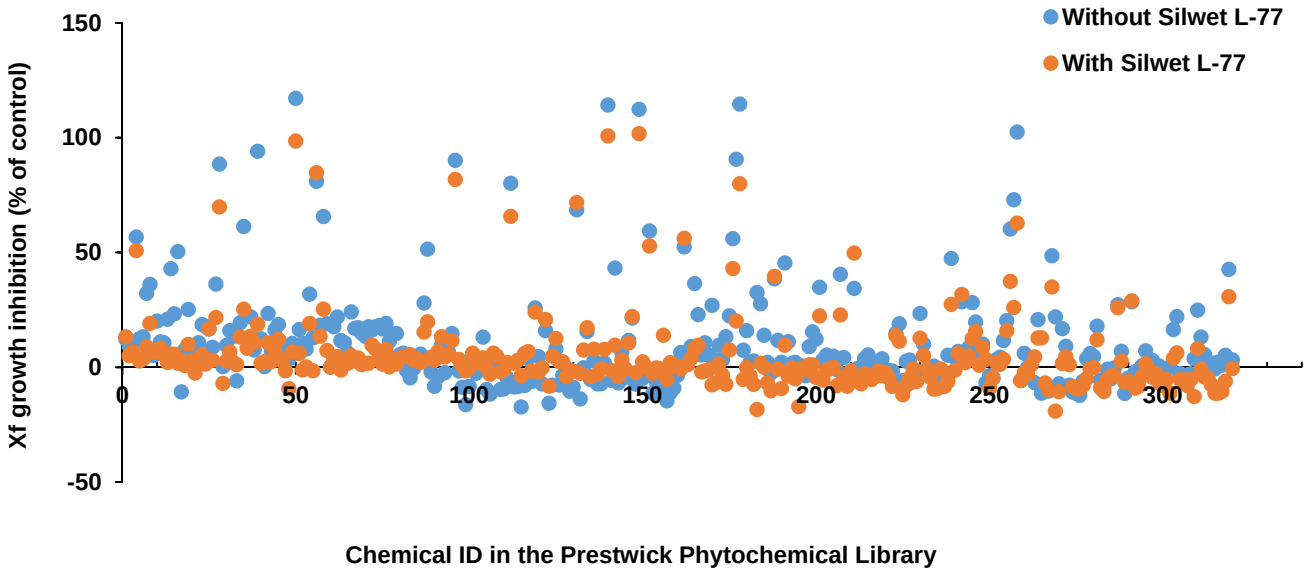

Fig 1. Screening of the Prestwick Phytochemical Library of 320 compounds for growth inhibition of Xf, both with and without Silwet L-77. Two day old cultures of GFP-marked Temecula1 cells (OD600= 0.25) were diluted to starting OD=0.05 and used for seeding 96-well microtiter plates for high throughput screening of the Library both with (orange dots) and without (blue dots) 200 ppm Silwet L-77. Both OD and GFP fluorescence were measured initially. The plates were incubated at 28° C for two days, and both OD and GFP fluorescence again measured. Growth inhibition was calculated as the difference between the change in OD (not shown) or GFP fluorescence between treatments and the respective untreated control. Chemicals exhibiting at least 50% of growth inhibition relative to the respective untreated control were selected for additional screening at different concentrations (dose effect).

Significant growth inhibition (>50%) of Temecula1 was observed with 193 chemicals from the Prestwick Chemical library (Fig. 2), 121 of which exhibited strongly significant growth inhibition (>90%). Greater than 100% inhibition occurred when the optical density (data not shown) and the fluorescence emitted (shown in Fig. 2) was reduced to below that of the starting cell values, and indicated lysis. Notably, 6 chemicals exhibited not only direct growth inhibition (ranging from 53% - 90%), but this inhibition was enhanced (>24% more) by Silwet L-77, indicating that these chemicals possibly target T1S efflux. These chemicals include a thiazolide antiparasitic agent, several antibiotics and a calcium antagonist.

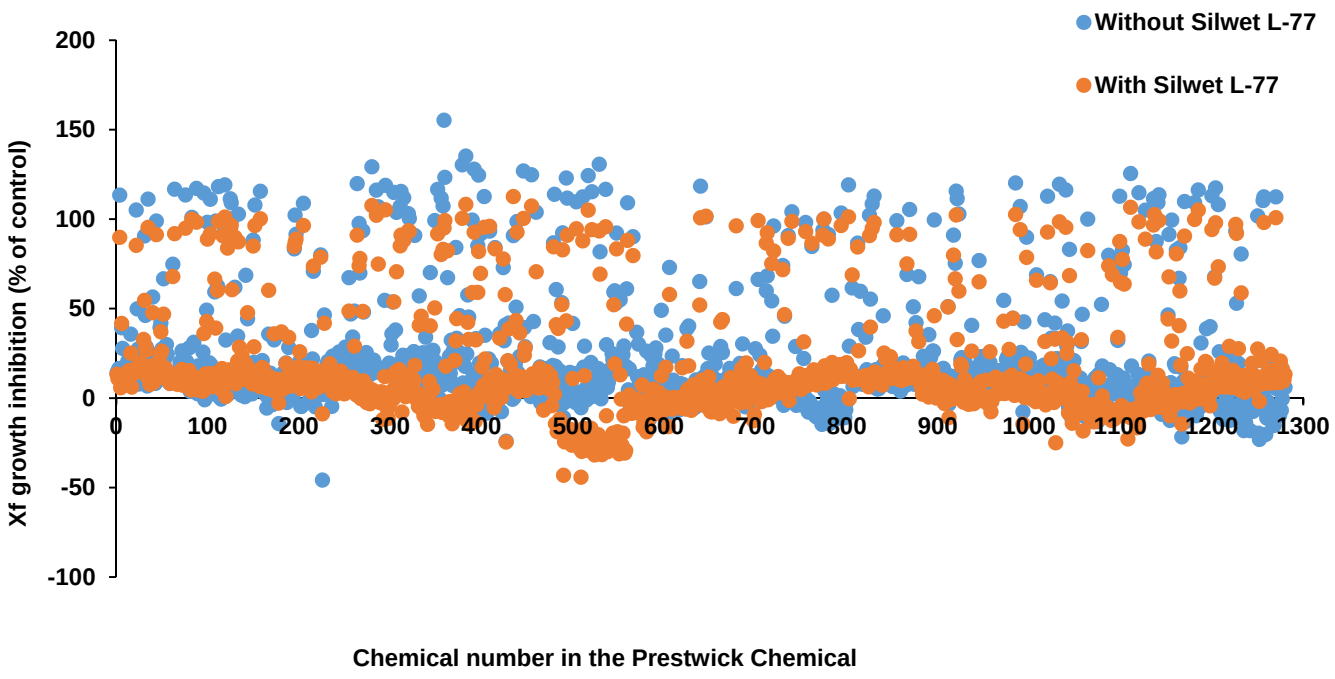

Fig 2. Screening of the Prestwick Chemical Library of 1,280compounds for growth inhibition of Xf, both with and without Silwet L-77. Legend as in Fig. 1.

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 will be ready for cold treatment within 3-4 weeks.

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 are being locally procured.

LITERATURE CITED

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