Project Report

Project Title: Use of peptide aptamers to explore blocking of Type I secretion of *Xylella fastidiosa*

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Objectives of the proposed Research:

As a result of previous work on this project, all Type I secretion of PD strain Temecula was theoretically eliminated by mutation of a common *tolC* gene, (PD1964). The resulting mutant was nonpathogenic on grape, and pathogenicity was restored by complementation using *tolC* (Reddy et al., 2007). Therefore, Type I secretion is critical for pathogenicity of PD strain Temecula. Not only was the resulting *tolC* mutant nonpathogenic; it also died so rapidly from exposure to plant sap that it could not be recovered, even following brief exposure (a few minutes) to sap. There are two main purposes for Type I secretion: multi-drug resistance (MDR) efflux (in this case, defense against anti-microbial chemicals in the xylem sap of grape), and toxin secretion (offensive, to promote pathogenicity). Clearly, *tolC* is needed for MDR efflux, regardless of its potential offensive use to promote pathogenicity. We proposed to synthesize peptides that correspond to the known chemical ligand binding target or choke point in the TolC protein. Specifically, we chose regions of the TolC protein that were 1) known from published literature to correspond to the externalized region of TolC, and 2) known from the published literature to correspond to bacteriophage binding sites. Similtaneously, we proposed to determine the minimum-inhibitory concentration (MIC) of various chemicals and to determine if any of the aptamers lowered the MIC values of an array of challenge antibiotics and plant-derived compounds.

Specifically, the 1 year objectives (extended to two years by a no cost extension) were:

1. Synthesize peptides of sequence “LLDVVQNQ”, using both keyhole lympet hemocyanin (KLH) conjugation and multiple antigen peptide (MAP) synthesis (no conjugation needed).

2. Use the synthetic peptides, in separate experimental series, to enrich two aptamer libraries. Following sequencing, a series of three short (eight amino acids in length) polypeptide aptamers will be identified following four cycles of enrichment using a random aptamer library displayed on M13 phage (New England Biolabs). Both the unconjugated (MAP) and conjugated

KLH-peptides will be separately used. Only aptamer sequences that are common to both enrichment pools will be chosen for further use.

3. Synthesize the eight amino acid aptamers, incubate strain Temecula wild type cells with the aptamers, and plate on PD-3 agar carrying various levels of antibiotic, plant-derived chemical or plant sap. At a minimum, the following will be tested: a) the detergent SDS, b) the hydrophobic chemical DOC, c) the antimicrobial agent from Rhubarb, Rhein, d) the isoflavonoid genistein, e) the alkaloid berberine and f) the grape phytoalexin resveratrol.

4. Determine the MICs of the different antibiotic, plant derived chemical or plant sap in comparison with wild type Temecula.

Research Results:

Objective 1: The following synthetic peptides were synthesized, both as MAP peptides and as KLH conjugates (6 total):

PD1964N: KSKAWGSSS
PD1964M: AMSALIPD
PD1964C: QPISDTN

In addition, the following was synthesized and conjugated to KLH:

DG1: LLDVVQNQ

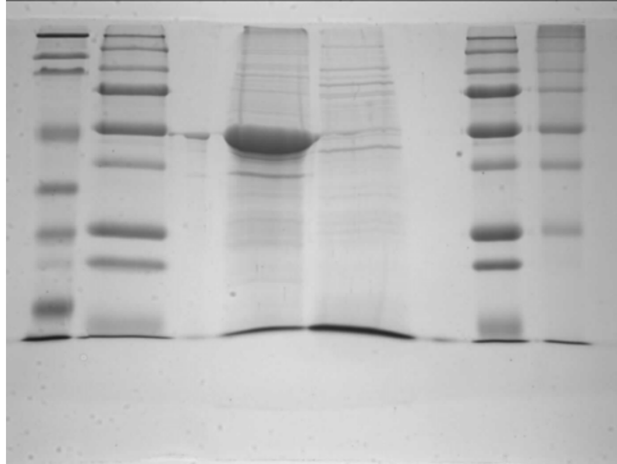
These 7 synthetic peptide versions were used in biopanning experiments as outlined in Objective 2.

In addition, mice were injected with each of the MAP peptides, and boosted twice after the primary immunization. Test bleeds were taken twice after each immunization. The adjuvant used was TiterMax Gold.

Thus, Objective 1 was more than fully realized, because it was becoming apparent very early from the results of Objective 2 that at least some of synthetic peptides failed to enrich for any particular protein sequence.

Objective 2: Four aptamer libraries were screened using six rounds of biopanning. PD1964N and DG1 enriched no particular consensus sequence after four rounds of biopanning using either MAP or KLH conjugated peptides. PD1964M revealed a partial consensus of PHT and TPP, but no single consensus. PD1964C revealed a consensus KHTHHRF aptamer binding sequence.

Surprisingly, none of the MAP peptides gave any indication of specific binding using ELISA assays using: 1) whole cells of Temecula (TolC+); 2) whole cells of M1 (TolC-), protein extracts from Temecula (TolC+); 3) protein extracts of M1 (TolC-); 4) induced protein extracts of *E. coli* BL21DE3/pET27B empty vector, and 5) induced protein extracts of *E. coli* BL21DE3/pET27B expressing cloned TolC (pictured in Lane 4 in the Coomassie blue stained SDS PAGE gel shown below).



In the Coomassie blue stained SDS PAGE gel photo to the left, the large band in Lane 4 corresponds to the expected size of expressed TolC. Surprisingly, not one of the three polyclonal antisera raised against synthetic peptide fragments corresponding to expected exposed areas of TolC bound to this protein in ELISA assays more extensively than negative controls. The controls were grown, induced and extracted under identical circumstances.

Objective 3: Based on our results with objective 4 (below), berberine was selected for use in these experiments. The KHTHHRF aptamer binding sequence was synthesized and incubated with Temecula wild type cells and plated on PD-3 agar with 100 ug/ml of berberine chloride. No effect was observed.

In order to determine if the aptamer was actually binding the outside of the cells, DNA primers were synthesized so as to create a gene fusion encoding KHTHHRF at the N terminus, a hinge region, and beta-glucuronidase (GUS) at the C terminus. This gene was expressed in pET27B, with nickel column purification. No binding was observed.

Objective 4: Antimicrobial plant chemicals berberine (5,6-dihydro-9,10-dimethoxybenzo-1,3-benzodioxoloquino-lizimium), genistein (4',5,7-Trihydroxyisoflavone), resveratrol (3,4',5-Trihydroxy-trans-stilbene) and rhein (9,10-Dihydro-4,5-dihydroxy-9,10-dioxo-2-anthracenecarboxylic acid) were purchased from Sigma-Aldrich Company (St. Louis). Stock solutions were: berberine, 50 mg/ml, dissolved in 100% methanol; genistein, 5 mg/ml, dissolved in 80% ethanol; resveratrol, 50 mg/ml, dissolved in 95% ethanol; and rhein, 10mg/ml, dissolved in 0.05 M sodium hydroxide and filter sterilized. Autoclaved PD3 agar medium was cooled to 50˚C, poured into sterile tubes, and aliquots of the stock solutions of each phytochemical used were mixed at various concentrations before solidifying in Petri dishes. Dilution series were initially 1:10, followed by 1:2 dilutions to more accurately determine the minimum inhibitory concentrations (MICs). Cultures of Temecula and M1 were streaked from freezer storage onto PD3 agar plates 14 days prior to use. Loopfuls of Temecula and M1 were collected from these plates and used to streak PD3 plates containing different concentrations of chemical. Each plate was divided into two halves. Temecula was streaked on one half of the plate, and M1 was streaked on the other half. Plates were incubated at 30˚C. Colony growth results were recorded 14-21 days after streaking. Results were as follows:

. Minimum inhibitory concentrations (MICs) of four antimicrobial plant chemicals.

Chemical	MIC (ug/ml)		Fold Difference
	WT	$tolC^-$	
Berberine	25	.02	1000x
Genistein	5	0.5	10X
Resveratrol	12.5	12.5	1X
Rhein	50	.05	1000X

Clearly, the tolC mutant was strongly and differentially affected by several phytochemicals, demonstrating that multi-drug efflux is critical to survival of Temecula.
Grape sap was extracted by cutting ca. 3 inches off the tops of well watered, 18 inch high grapevines in pots and collecting ca. 1.5 ml of sap with a micropipette. The sap was filter sterilized using a 0.2 µm filter, and kept on ice until used. Fresh sap was collected and used for each experiment. The sap pH ranged from 5.5 to 6.0.

Crude plant extract was made by cutting four internodes from one plant using a sterile scalpel at a 5-cm distance beyond the inoculation site on either side. These four pieces were surface sterilized by rinsing with 95% ethanol, immersed in 10% chlorox for 10 min, and then soaked in sterile water for 10 minutes. Approximately 2-cm was cut from each end of each sterilized stem piece using a sterile scalpel (to remove any excess chlorox that entered the cut tips of the stems) and each inoculated internodal piece was ground in 1 ml sterile SCP buffer using a mortar and pestle.

A loopful of each PD strain to be tested was scraped from freshly grown (7 day old) PD3 agar plate cultures and suspended in 100 uL of PD3 medium. 10µL of each bacterial suspension was mixed with 90µL of grape sap. After ten minutes, a 10X dilution series was made, using SCP buffer (trisodium citrate, 1g L^{-1}; disodium succinate, 1g L^{-1}; $MgSO_4 \cdot 7H_2O$, 1g L^{-1}; K_2HPO_4, 1.5g L^{-1}, KH_2PO_4, 1g L^{-1}, pH 7.0) as the diluent. Five µL of each dilution was pipetted onto PD3 plates. Replicate plates were made for each treatment. As controls, 10 µL of each of the liquid suspensions were pipetted into individual tubes containing either 90 µL of SCP Buffer or PD3 medium, and after incubation for 10 minutes, serial dilutions were made and plated in the same manner as above.

The results from each of four replicated experiments were that _*grape sap per se, collected in the spring, had no detected effect on survival of the tolC mutants*_. However, when crushed internodal segments of grape stem were ground up and used in a manner that duplicated the extraction of bacteria from the grape stems, the *tolC* mutants did not survive beyond two hours in the resulting crushed plant extract.

This portion of the project was successful and is included in the following publication:

Reddy, J. D, S. L. Reddy, D. L. Hopkins, and D. W. Gabriel. 2007. TolC is required for pathogenicity of *Xylella fastidiosa* in *Vitis vinifera* grapevines. Molec.Plant-Microbe Interact. 20 (4): (April issue, in press).

Conclusions

Despite our experience in enriching aptamer libraries for peptides that bind to a given target sequence, we were largely unable to enrich for peptides that bind to four peptides specifically selected from the region of TolC that is most likely to be exposed through the LPS outer membrane. Furthermore, we were unable to raise antibodies that reacted to presence of TolC, even when the TolC protein was abundantly overexpressed in *E. coli*. It is possible that our selection of the four synthetic peptides used to enrich for aptamers (seven separate panning experiments) or to raise antibodies (three separate experiments using two mice each) was unlucky or somehow very poorly considered or executed. We doubt that, simply based on our previous experience with both biopanning of aptamers and in raising polyclonal and monoclonal antisera. It is also possible that the "exposed" region of TolC is folded, formed or buried in such a way as to avoid or escape easy protein to protein recognition based on short portions of the "exposed" region. If so, our results may indicate that use of the entire TolC protein, or at least a large overexpressed fragment consisting of the entire "exposed" region might be more rewarding of results.