RENEWAL PROGRESS REPORT

Project Title:
Role of Type I Secretion in the Pathogenicity of *Xylella Fastidiosa.*

Reporting period: The results reported here are for work conducted from December 15, 2008 to March 15, 2009. Funding was delayed by four months on this project.

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Objectives of Proposed Research

1. Determine the specific effect of individual **Temecula hemolysins** PD0143, PD0282, PD0536, calcium binding hemolysin-type proteins PD1506, PD0305, PD2094, and calcium binding protein PD2097 in eliciting PD symptoms of leaf scorch and PCD on *V. vinifera* plants and in enhancing growth in *V. vinifera* plants of Xf biological control strain EB92-1. A novel aspect of the use of EB92-1 as a recipient strain is to determine the effect that an unexpected horizontal transfer event from Temecula to EB92-1 might have on the virulence of EB92-1, or any other Xf strain that may be widely used for biological control. Specifically:

a) use our published pBBRMC5 vector system (Reddy et al., 2007) to move each of these hemolysin genes, expressed by either their native or the *lacZ* promoter into Xf biological control strain EB92-1 (Hopkins, 2005; described above), and inoculating *V. vinifera* plants. (Year I).

b) if any hemolysins cause PD symptom expression, determine how much additional growth, if any, is provided by this ability to elicit PD symptoms (Year I).

c) by transient expression assays of select hemolysin genes in grape leaves using a CaMV promoter and *A. tumefaciens* GV2260 delivery (Year 2).

2. Determine the effect of biocontrol strain **EB92-1 colicins** on multiple PD strains and in eliciting phytotoxic symptoms on *V. vinifera* plants. A novel aspect of the use of EB92-1 as a donor strain is the expectation, based on our Type I colicin secretion component knockouts, that its colicin V or bacteriocin genes are determinative of its ability to perform as an effective biological control agent, and the speed with which this hypothesis can be tested. If the hypothesis is correct, it will lead to a potential new control strategy within the two year time frame of this proposal. Specifically:

a) PCR amplify, clone and sequence the homologs of Temecula colicins PD0215, PD0216, PD0217 and bacteriocin PD1427 from biocontrol strain EB92-1, overexpress the EB92-1 homologs individually in *E. coli* and determine the sensitivity of Temecula and at least 10 other PD strains and a variety of other pathogens to the three colicins using plate overlay assays (Year I).

b) if any colicins or the bacteriocin are found from EB92-1 that kill or inhibit growth of all or most PD strains tested, at least one will be selected for transient expression assays in tobacco and grape leaves (Year 2).

c) if, as expected, no phytotoxicity is observed, transgenic tobacco plants will be made in Year 2 and subsequently tested by challenge inoculation for control of PD and/or elimination of inoculated Xf (late in Year 2).

Research Accomplishments & Results:

Objective 1a) Temecula hemolysins expressed in EB92-1. Standard methods published by our group (Reddy et al., 2007), were used to PCR amplify, sequence verify, and clone all proposed Temecula hemolysins: PD0143, PD0282, PD0536, PD1506, PD0305, PD2094, and PD2097. To date, PD0536 and PD0305 have been recloned into into pBBRMC5 using the *lacZ* promoter on the vector to create a transcriptional fusion and taking care to provide a correct Shine-Delgarno (SD) sequence. Electrocompetent cells of EB92-1 have been prepared for use in electroporation. After confirmation of transformation, these transformants will be inoculated into grapevines for symptom testing.

1b) Growth curves of EB92-1 with enhanced pathogenicity. This objective awaits the results of plant inoculations using EB92-1 transformants from Objective 1a.

1c) Transient Expression Assays of Temecula Hemolysins. To date, colicin PD0215 and hemolysins PD0536 and PD0305 were recloned without a bacterial promoter or SD sequence in pYD40.2 (based on pBI221, Clontech), such that they were operably fused with the CaMV plant promoter. These strains were moved into *A. tumefaciens* by electroporation and inoculated into tobacco. No symptoms were observed in these plants in repeated tests. As a control, the Beta-glucuronidase (GUS) gene was recloned in a similar fashion into this vector, and GUS expression was detected by staining. Recloning of the other genes into pYD40.2 is in progress.

Objective 2a) Sensitivity of Temecula to EB92-1 colicins. Orthologs of the Temecula colicins PD0215, PD0216 and PD0217 were identified and cloned in pGemT from biological control strain EB92-1. These sequences have not previously been available, and will be deposited in GenBank, once we have ruled out potential PCR artifacts (only two independent PCR clones of each colicin were sequenced). One of these EB92-1 colicins was only 87% identical at the amino acid level with its ortholog in Temecula, while the other two were 100% and 99% identical. These 3 new colicins have been cloned into pET27b (Novagen) and operably fused with a pelB leader. Induction of expression of the Temecula colicins was lethal to *E. coli*, but not instantaneous. Induction of expression of the EB92-1 colicins will be initiated shortly. Initial tobacco plant inoculation experiments using the induced cell cultures of Temecula colicins yielded no detected effects. The EB92-1 clones will be overexpressed in *E. coli* and their potential effect on Temecula studied, with particular focus on the one that is only 87% identical.

b) **Transient Expression Assays of EB92-1 colicins**. This objective is dependent upon the results of 2a.

c) **Transgenic tobacco**. This objective is also dependent upon the results of 2a.

Publications or Reports Resulting From This Project

Flores-Cruz, Z., Reddy, S., Hopkins, D., and Gabriel, D.W., 2009. Potential offensive role of the Type I Secretion System in *Xylella fastidiosa*. Abstract submitted to the XIV International Congress of the International Society for Molecular Plant Microbe Interactions, Quebec, Canada.

Research Relevance Statement

Our previous work resulted in the following discoveries: 1) *tolC* is absolutely required not only for pathogenicity, but also for survival of Temecula in *Vitis vinifera* grapevines; 2) that the loss of multi-drug resistance (MDR) efflux (presumably, of phytoalexins and possibly of antibiotics also) through the Type I secretion system was the primary reason that *tolC*$^{-}$ Temecula mutants could not survive in grapevines; and 3) that the vector pBBRMC5 served as a stable complementation vector in Temecula and pathogenicity of the *tolC*$^{-}$ mutant was restored using wild type *tolC* cloned in this vector (Reddy et al., 2007). Our latest results provide evidence that Type I secretion is, in fact, used not only for defense but also offensively: 1) to condition pathogenicity, most likely through the action of hemolysins in eliciting programmed cell death (PCD) and 2) possibly to exclude other bacterial cells from colonization of the same xylem niche, most likely through the action of colicins.

Combined with the Gilchrist lab's discovery that programmed cell death (PCD) in *V. vinifera* grapes is elicited by PD strains and can be suppressed by anti-apoptotic gene function, our data strongly implicates Type I secretion generally and hemolysins in particular as potential elicitors of both PD symptoms and PCD. We hypothesize that the large number of **hemolysins** (PD0143, PD0282 and PD0536) and **calcium binding hemolysin-type proteins** (PD1506, PD0305, PD2094 and PD2097) indicates a redundant and possibly additive role for at least some of these proteins in elicitation of symptoms of PCD and PD. This work is designed to test this hypothesis.

We also hypothesize that the colicins, which are also Type I secreted, may be important in initial Xf colonization for clearing the xylem of competing bacterial strains, and that loss of this ability indirectly results in reduced pathogenicity. As a testable corollary to the hypothesis that colicins are important at least in initial Xf colonization of grape, we believe that the biocontrol strain EB92-1 is effective against PD strains primarily because of its colicins. This work is designed to test these hypotheses utilizing colicins cloned from EB92-1.

Summary in Lay Terms

We discovered that a specialized bacterial secretion system was not only absolutely required for Xf to cause PD, but that both known components of the system were required. The first component of the secretion system was found needed for defense against natural antimicrobial compounds produced by grapevines. The second component of the secretion system was found needed for offense—to cause disease in grapevine. The compounds known to

be secreted by this bacterial system for offensive purposes are usually proteins. This work is an attempt to experimentally determine exactly which proteins are used to cause the offensive effects. In addition, this offensive system may also be used by bacteria to attack other, closely related bacteria that may compete to colonize the same ecological niche. Part of this work is an attempt to determine if some Xf bacteria produce proteins are used to kill other Xf bacteria that may compete in the grapevine xylem niche for nutrients. For that purpose, an Xf strain known to be effective as a biological control agent against Xf strains that cause PD is being examined to determine if proteins can be identified that effectively kill PD bacteria. If such a protein could be identified, it may be useful in transgenic grape rootstock to kill PD bacteria found in grape scions.

Status of Intellectual Property

It is possible that a novel antimicrobial protein may be discovered that could be useful against PD strains infecting grapevine. No patents have been filed to date.

Literature cited:

Duan, Y.P., Castaneda, A., Zhao, G., Erdos, G.W. and Gabriel, D.W. 1999. Expression of a single, host-specific gene in citrus cells elicits division, enlargement and cell death. Molec. Plant-Microbe Interact. 12:556-560

Kapila, J., R. De Rycke, M. Van Montagu, and G. Angenon. 1997. An *Agrobacterium*-mediated transient gene expression system for intact leaves. Plant Science 122:101-108.