AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2016 – 2018

TITLE: Suppressing Virulence Factor Production by the Plant Pathogen Pseudomonas syringae Using Natural Plant-Derived Metabolites

RESEARCH LEADER: Jeff Anderson

EXECUTIVE SUMMARY: *Pseudomonas syringae* is a bacterium that causes disease on many crops important to Oregon agriculture such as blueberry and pear. *P. syringae* infects host plants by producing dozens of toxins that suppress the plant immune system. One class of these toxins, referred to as "effectors", are directly injected into host plant cells by the bacterium using a syringe-like apparatus called the type III secretion system. In recent work, the PI discovered that specific chemicals released by plants into their immediate environment are signals for *P. syringae* to begin producing its effectors and type III secretion system. While researching these virulence-promoting signals, the PI also found that several other plant-derived metabolites, namely the amino acids β-alanine, leucine and valine, are potent inhibitors of effector production in *P. syringae* at micromolar quantities. These simple compounds are ubiquitous in nature, non-toxic to humans and are unlikely to have non-specific antimicrobial activity. The overall goal of this project is to investigate if these naturally-occurring and non-toxic chemical compounds may be effective at specifically interfering with *P. syringae* infection processes. Results from this work could provide important insights into the underlying mechanisms of host perception by *P. syringae*, and may lead to novel strategies to control infection of crop plants by this pathogen.

OBJECTIVES:

Objective 1) Investigate molecular mechanisms of how plant-derived metabolites inhibit effector production in *P. syringae*.

Objective 2) Determine if the inhibitory metabolites broadly act against different *P. syringae* isolates including those found on diseased plants in Oregon.

Objective 3) Investigate if treatment of plants with the inhibitory metabolites can prevent disease caused by *P. syringae*

PROCEDURES:

Objective 1) Generate a collection of several thousand *P. syringae* DC3000 mutants using Tn5 transposon mutagenesis. Screen this collection for mutants that are not inhibited by β-alanine using a reporter plasmid consisting of an effector promoter sequence fused to a gene encoding a green fluorescent protein (GFP). Identify transposon insertion sites in mutants that are no

longer inhibited by β-alanine, as they may disrupt genes that encode for receptors as well as other proteins necessary for the inhibitory effects of this metabolite.

Objective 2) Test a collection of *P. syringae* isolates that are infectious on a broad range of host plants for effector production in the presence of inhibitory metabolites using the same fluorescent protein reporter system as in Objective 1. This work will involve introducing the effector$_{promoter}$:GFP reporter plasmid into each isolate, incubating the plasmid-containing bacteria in conditions that induce effector production in the presence or absence of β-alanine, and measuring the resulting changes in fluorescence as an indirect measure of effector production.

Objective 3) Infect tomato plants with *P. syringae* isolate DC3000 in the presence or absence of inhibitory metabolites. Assess impacts on disease by counting visible speck symptoms on leaves and by serial dilution plating of bacteria from infected tissue. If effective, additional pathogen assays to assess effects of inhibitory metabolites on disease caused by isolates tested in Objective 2 will be pursued.

SIGNIFICANT ACCOMPLISHMENTS: To meet the goals of **objective 1**, we developed a high throughput assay to monitor GFP fluorescence in Tn5-mutated *P. syringae* colonies growing on nitrocellulose filters. Developing this method required the optimization of several washing steps to remove residual media from the filters, as well as establishing conditions for imaging GFP fluorescence from colonies using a stereomicroscope. Using this assay, we successfully screened 20,000 *P. syringae* colonies for loss of GFP fluorescence in response to bioactive metabolites that induce effector production, and from this screen we identified approximately 200 mutants that show partial or complete loss of response. We identified essentially all of the Tn5 transposon insertion sites in this collection of mutants by high-throughput sequencing, and are now characterizing the genes disrupted in these mutants for their function in perceiving plant signals. In regards to objective 1, we used a similar strategy to screen for *P. syringae* mutants in which effector expression is not inhibited by citrate, a metabolite that, similar to β-alanine, inhibits effector production when incubated with *P. syringae* at milli-molar quantities. We chose to investigate citrate rather than β-alanine because it has an interesting bimodal effect on bacteria responses, with low concentrations able to stimulate effector production, whereas higher concentrations are inhibitory. Unfortunately, we were unable to identify any mutants that showed significant suppression of citrate inhibition, indicating that the genes involved are either redundant or required for viability of bacteria. Nevertheless, this experiment was successful in that we developed methods to rapidly screen an entire library of 20,000+ mutants in less than a weeks' time, and we are now re-tooling our efforts to repeat the screen for mutants that are recalcitrant to effects of other inhibitory compounds such as β-alanine. For **objective 2**, we introduced an effector promoter:GFP reporter plasmid into different strains of *P. syringae* and tested for the ability of β-alanine to inhibit expression of effectors using GFP expression as a readout for activity. Figure 1 shows effector production in four different strains of P. syringae (collected from either tomato or bean plants) in the presence or absence of β-alanine. Although striking differences in the magnitude of response to bioactive metabolites fructose and aspartic

acid was observed between the four strains, in all cases β-alanine was able to significantly suppress effector production (Figure 1). These data are important because they suggest β-alanine is effective at inhibiting recognition of host metabolites signals by strains that are adapted to grow on very different host species, suggesting potential broad efficacy at limiting bacterial virulence. β-alanine did not inhibit bacterial growth in these experiments (data not shown), indicating that the inhibitory activity of this compound is specific to effector production.

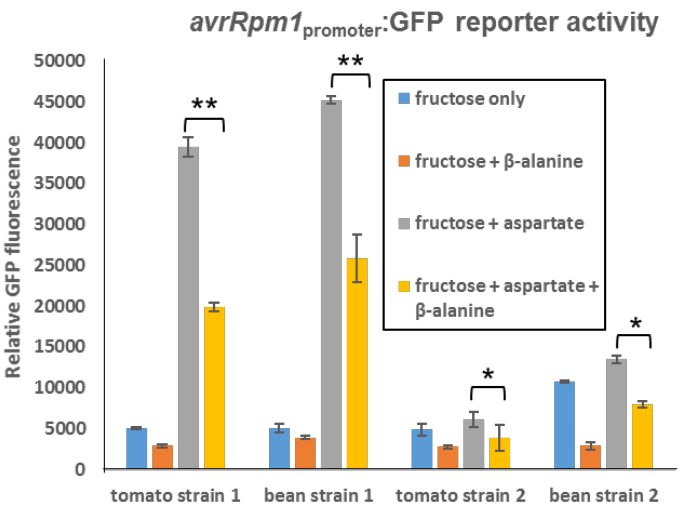

Figure 1. β-alanine inhibits effector production in *P. syringae* strains isolated from diverse plant hosts.

Funds from this grant supported Conner Rogan, a first-year PhD student in my lab. Conner is working to identify additional plant-derived compounds that, similar to β-alanine, inhibit effector production *in P. syringae*. Conner has developed a protocol for isolating metabolites from the interior spaces of Arabidopsis and tomato leaves, and has found that these leaf samples can strongly inhibit effector production by *P. syringae*. We are now working to identify the bioactive compounds in these leaf samples, with the future goal of testing these compounds for biocontrol of crop diseases. These results are potentially transformative, as few plant-derived metabolites that are effective at inhibiting *P. syringae* are known.

BENEFITS & IMPACT: Data from this project provide further evidence that the commercially-available compound β-alanine can inhibit the production of virulence-promoting effector proteins by the plant pathogenic bacterium *Pseudomonas syringae*. Furthermore, this compound showed inhibitory activity towards strains adapted to different host plant species, suggesting potential broad efficacy as a virulence inhibitor. We also developed more rapid methods for screening population of *P. syringae* mutants for altered responses to inhibitory metabolites, opening the door for future genetic screens to understand inhibition mechanisms. Last, our work to identify additional inhibitory compounds from plants could lead to the development of new disease control strategies.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:

Title: Regulation of *Pseudomonas syringae* Virulence by Plant-derived Chemical Signals
Source: National Science Foundation
PI: Jeffrey Anderson, OSU
Duration: 7/15/16-7/15/19
Total Funds to OSU: $646,724

FUTURE FUNDING POSSIBILITIES: Results generated from this funding will improve the competiveness of future funding requests from federal programs such as USDA AFRI and NSF.