ABSTRACT:

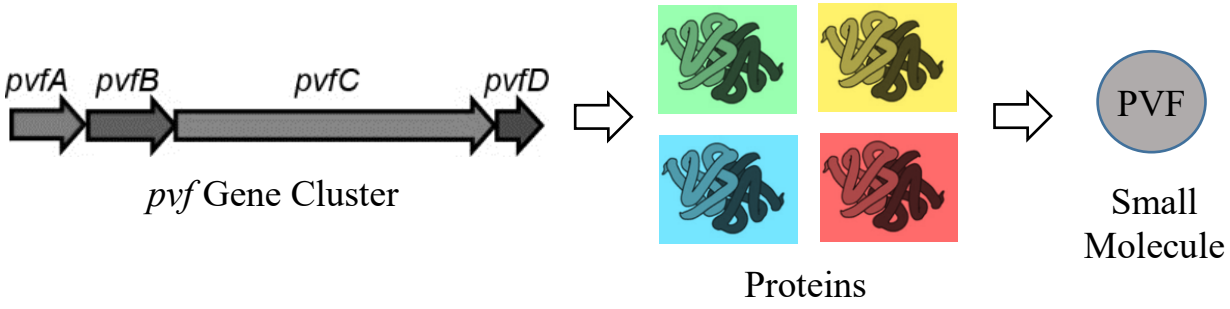
Pseudomonas virulence factor (PVF) is a signaling molecule synthesized by proteins encoded in the *pvf* biosynthetic gene cluster of the *Drosophila melanogaster* pathogen *Pseudomonas entomophila*. Previous research has shown that when these genes are mutated, the ability of this bacterium to persist in a eukaryotic host and have virulent effects is greatly reduced.[1] Furthermore, the Li lab has identified homologues of the *pvf* gene cluster in over 300 strains of bacteria with a variety of different hosts that span the domain, Eukarya. While its ubiquity and importance to bacterial virulence has been established, little is actually known about the signaling pathway PVF initiates in neighboring *P. entomophila* cells. The goal of this project was to identify the genes involved in this **novel signaling pathway**, starting from the reception of the PVF signaling molecule at the cell membrane to the initiation of transcription. To this end, Tn5 mutagenesis was used to disrupt individual genes in a reporter strain of *P. entomophila* containing a PSEEN0973::*lacZ* insertion and a reporter strain of *P. entomophila* containing a PSEEN5493::*lacZ* insertion. Cells that took up the Tn5 plasmid were selected by antibiotic resistance and screened using a blue/white screen. Resultant white colonies exhibited disruptions in genes vital to the signaling pathway as they were not able to produce β-galactosidase, whereas blue colonies had fully functional pathways. These white colonies were further screened with quantitative assay that measured the conversion of *ortho*-nitrophenyl-β-galactosidase to *ortho*-nitrophenol, a reaction catalyzed by β-galactosidase. A total of 8,500 colonies were screened, and 86 white colonies were identified and quantitatively screened. Arbitrary PCR was used to investigate the locations of the Tn5 insertions in the genome. With the aid of bioinformatics, we have identified a putative histidine kinase and methylesterase that are likely involved in the PVF-initiated signaling pathway.

INTRODUCTION:

In the U.S., 2 million people experience antibiotic-resistant infections each year, and over 20,000 will die from those infections.[2] Those who are immunocompromised are particularly at risk for these aggressive infections, and the rise of antibiotic resistance over the past thirty years has made this threat an ever-present reality for these populations.[2] As a result, it has become increasingly important to keep up with the evolution of bacterial species and gain a deeper understanding of their mechanisms of infection so that new drug targets may be discovered. This project aims to advance current knowledge by examining the mechanism of infection initiated by the signaling molecule Pseudomonas Virulence Factor (PVF).

As demonstrated in Figure 1, the small molecule PVF is synthesized by proteins encoded by the *pvf* gene cluster of *Pseudomonas entomophila*.

Figure 1: *Synthesis of PVF*[3]

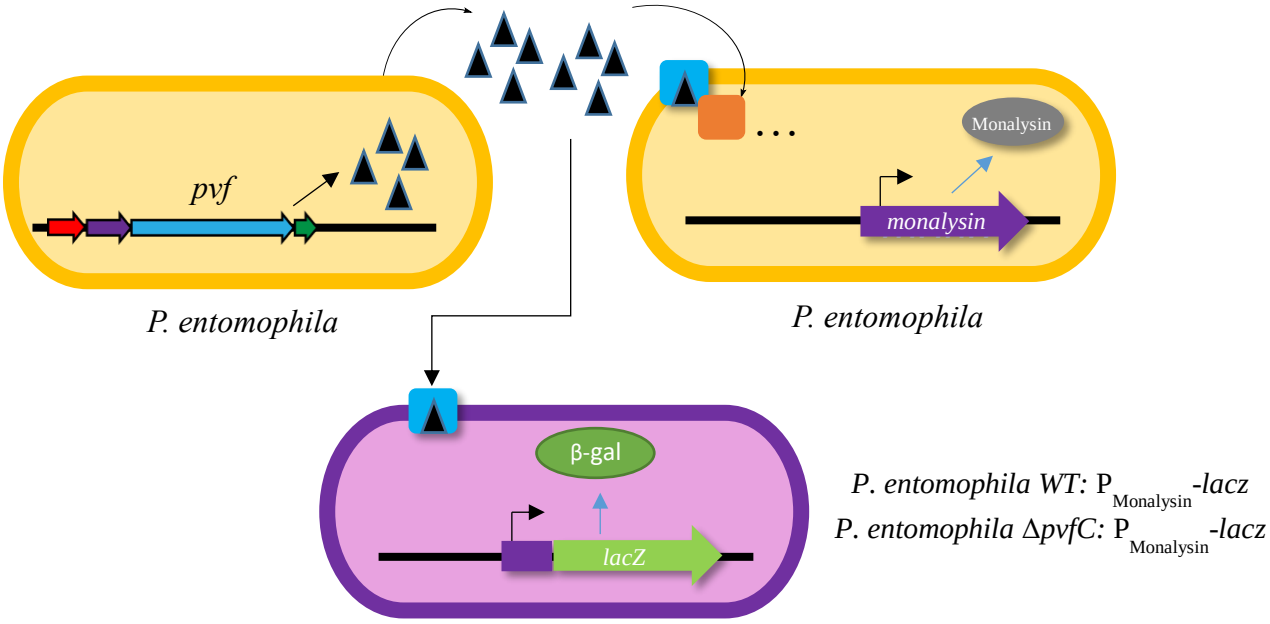


When these genes are mutated, the ability of this bacterium to persist in a eukaryotic host and inflict damage is greatly reduced, indicating that the *pvf* gene cluster, and as an extension PVF, are essential for bacterial virulence.[1] Furthermore, the Li Lab has identified homologs of the *pvf* gene cluster in over 300 strains of bacteria with a variety of different hosts across the Eukarya domain. One example is *Burkholderia cenocepacia*, an opportunistic human pathogen responsible for multi-drug resistant infections in cystic fibrosis patients.[4] The PVF-mediated signaling pathway is believed to be highly conserved among the bacteria that possess it, and thus, information learned from *P. entomophila* can provide insight into the virulence mechanisms of other bacterial strains with the *pvf* gene cluster, including those that are multi-drug resistant.

Previous research from the Li Lab suggests that PVF has a regulatory function, and operates in an analogous way to the quorum sensing (QS) molecules used by other strains of bacteria. As demonstrated in Figure 2, QS molecules are secreted by bacteria, and once a concentration threshold is reached, these molecules stimulate receptors on the cell membrane that initiate a signaling pathway in the cell and activate the virulent pathways of bacteria.[5] This QS mechanism allows bacteria cells to conserve their resources until there are enough cells to compete with the immune system of the host.[5] Targeting the pathways responsible for the activation of bacterial virulence rather than killing the bacteria will allow non-lethal inhibition of virulence and reduce selection of antibiotic resistance, providing a potential solution for rising antibiotic resistance.[6]

Figure 2 also demonstrates that when a signal, such as PVF, is received by receptors on the cell membrane, a cascade of interacting proteins is triggered, ultimately leading to the activation of a promoter in the DNA of the cell. The activation of this promoter results in the transcription of particular genes and expression of the proteins they encode. Previous studies have identified which promoters have altered transcription in *pvf* knock-outs, distinguishing those that are activated in this signaling pathway.[1] Some of these promoters include the monalysin promoter, the uncharacterized PSEEN0973 promotor, and the uncharacterized PSEEN5493 promoter. These promoters can be utilized in the making of specialized reporter strains of *P. entomophila*, which contain an extra insertion in their genome made up of one of the aforementioned promoters and a gene whose expression can easily be monitored. One example would be the gene *gfp*, which will produce the fluorescent molecule Green Fluorescent Protein (GFP). When the PVF-initiated signaling pathway is functional, the proteins in this pathway will also initiate the transcription of the reporter and the production of GFP, whose fluorescence can be monitored. Another example would be the gene *lacZ*, which will produce β-galactosidase (β-gal). Although β-galactosidase is not fluorescent, its concentration in the cell can be monitored through a number of techniques and assays. Figure 2 provides a summary of these processes for a reporter containing the monalysin promoter and *lacZ* ($P_{Monalysin}$*-lacz)*.

Figure 2: *Summary of $P_{Monalysin}$-lacZ reporter system*[7]



This paper will utilize Tn5 mutagenesis in both a PSEEN0973::*lacZ* reporter strain and a PSEEN5493::*lacZ* reporter strain of *P. entomophila*[1] to examine the genes involved from the reception of the PVF signaling molecule at the cell membrane to the initiation of transcription in the genome.

MATERIALS/METHODS:

Bacterial strains and plasmids – These are listed in Table 1.

Table 1: *Bacterial strains and plasmids*

Strain	Relevant Properties	Source or Reference
E. coli		
RHO3_miniTn5kan	pUT-mini-Tn5-kan plasmid	Cotter Lab
P. entomophila		
L48_145	WT: $P_{Monalysin}$-*gfp*	Li Lab
L48_151	Δ*pvfC*: $P_{Monalysin}$-*gfp*	Li Lab
L48_146	WT: $P_{Monalysin}$-*lacZ*	Li Lab
L48_152	Δ*pvfC*: $P_{Monalysin}$-*lacZ*	Li Lab
L48_182	WT - PSEEN5493::*lacZ*	Dr. Vallet-Gely
L48_183	Δ*pvfC* - PSEEN5493::*lacZ*	Li Lab
L48_184	WT - PSEEN0973::*lacZ*	Dr. Vallet-Gely
L48_185	Δ*pvfC* – PSEEN0973::*lacZ*	Li Lab
L48_75	WT- No reporter	Li Lab

Culture media and growth conditions – The *Escherichia coli* strain was grown at 37ºC and the *Pseudomonas entomophila* strains were grown at 28ºC, both in LB broth. Solid medium contained agar (7.5 g L^{-1}) and lysogeny broth (LB) (12.5 g L^{-1}). All solid media was grown at 28ºC. When required, 2,3-diaminopropionic acid (DAP) (400 µg mL^{-1}), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µg mL^{-1}), and/or kanamycin (Kan) (50 µg mL^{-1}) were added to solid media or LB broth.

Conjugation – Donor cells (RHO3_miniTn5kan*)* (5 mL) and recipient cell cultures (L48_182 or L48_184*)* (5 mL) were grown at their respective growth conditions for 24 hrs. Mating spots were plated on LB DAP agar plates with equal portions of donor and recipient (10 µL) and grown 24 hrs.

Blue/white screening – Two mating spots were collected, spun down at 3500 x *g*, and re-suspended in $MgCl_2$ (10 mM) to an OD_{600} of 0.5. This cell suspension was plated (200 µL) on Kan X-gal plates and grown for 48 hrs. The total number of colonies per plate was recorded. White colonies were streaked out on Kan X-gal plates. Those that produced uniformly white colonies were saved as glycerol stocks and those that produced uniformly blue colonies were discarded. White colonies from mixed plates were selected and re-streaked a second time to obtain plates with uniformly white colonies. The colonies from these second plates were then saved as glycerol stocks.

B-gal Activity Assay – When initially testing the activity of the *lacZ* reporter systems, three cultures (50 mL) of each *P. entomophila* strain were inoculated and allowed to grow 25-60 hours

with 2 aliquots (1 mL) taken from each culture at various time points. When testing the activity of the white colonies from the blue/white screen, three cultures (1 mL) of each white colony were inoculated and grown for 24 hours. Abs_{600} was measured for each time point aliquot and for the white colony samples after 24 hours in a Greiner 96-well flat transparent microplate using a Tecan Infinite M1000 PRO UV-Vis plate reader. The time point aliquots and white colony cultures were spun down at 3500 x *g* and re-suspended in Z-buffer (60 mM $Na_2HPO_4.7H_2O$, 40mM $NaH_2PO_4.H_2O$, 10mM KCl, 1mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) (1 mL). Half of this cell suspension (500 µL) was removed and diluted by half with Z-buffer. Cells were lysed with chloroform (100 µL) and sodium dodecyl sulfate (SDS) (0.1%, 50 µL), thoroughly vortexed, and allowed to incubate at room temperature for 5-15 minutes. *ortho*-Nitrophenyl-β-galactoside (ONPG) (4 mg mL^{-1}, 200 µL) was added, beginning the reaction. This reaction was allowed to progress until the solution was visibly yellow, after which the reaction was quenched with Na_2CO_3 (1 M, 500 µL). OD_{420} and OD_{550} were both then recorded using a spectrometer. Using the following equation, the Miller Units for each sample was calculated.

$$\text{Miller Units} = \frac{1000 * (OD_{420} - (1.75 * OD_{550}))}{\text{Volume (mL)} * Abs_{600} * \text{Reaction Time (s)}}$$

GFP Fluorescence – When initially testing the activity of the *gfp* reporter systems, three cultures (50 mL) of each *P. entomophila* strain were inoculated and allowed to grow 10-24 hours with 2 aliquots (1 mL) taken from each culture at various time points. Fluorescence was measured (excitation 485 nm, emission 535 nm) in a Nunclon 96-well flat black microplate using a Tecan Infinite M1000 PRO UV-Vis plate reader. The fluorescence value for each sample was divided by the Abs_{600} to provide a normalized fluorescence for each sample.

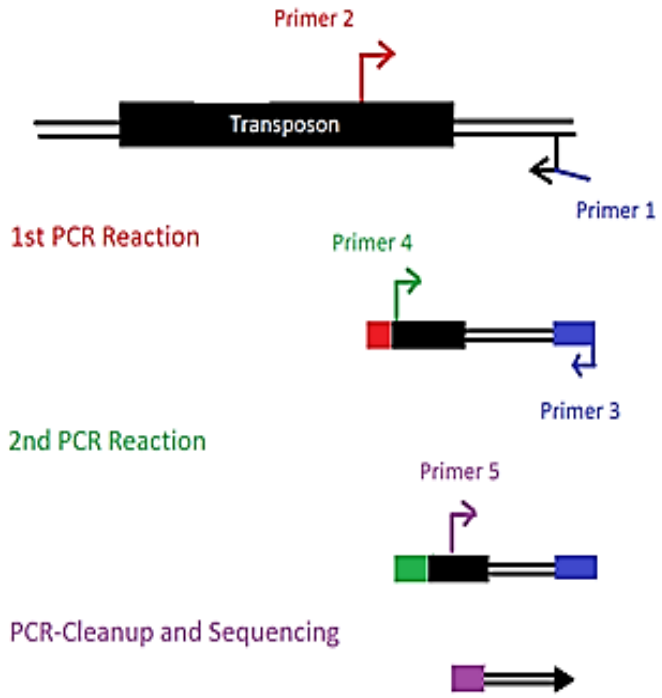
Arbitrary PCR – See Table 2 for a summary of the primers used. In primer #1, the bolded sequence represents the overhang of the primer.

Table 2: *Arbitrary PCR primers*

Primer #	Primer Sequence
1	**GGCCACGCGTCGACTAGTAC**NNNNNNNNNNGATAT
2	CAAGCGCGAGATGTTCACCGACCC
3	GGCCACGCGTCGACTAGTAC
4	CCACGCAGATGGGCCGGC
5	GCACTTGTGTATAAGAGTCAG

Genomic DNA for each white colony was acquired by boiling a small sample of the glycerol stock in water (10 µL) to lyse the cells, and spinning the sample down at 20,000 x *g*. Genomic DNA was first amplified with primers 1 (2 µM) and 2 (0.2 µM) in One*Taq* 2X Master Mix with

Figure 3: *Arbitrary PCR summary*



Standard Buffer as directed. The product of this reaction was subsequently treated with ExoSAP-IT™ PCR Product Cleanup Reagent. The clean product was amplified a second time with primers 3 (0.4 µM) and 4 (0.2 µM) in One*Taq* 2X Master Mix with Standard Buffer as directed. See Figure 3 for a generalized summary of the arbitrary PCR steps. A small portion of the first round arbitrary PCR product and the final product were run on an agarose gel (1%) for confirmation of product bands. A PCR clean-up was performed on each sample that produced only one band for the final product. Those that did not produce a band for the final product but produced at least one dominant band after the first round of arbitrary PCR also underwent a PCR clean-up.

Sequencing- Each successful arbitrary PCR sample was sent for sequencing to EtonBio with primer 5. See Figure 3 for an explanation of primer alignment. Sequencing results were submitted to the BLAST database for sequence identification.

RESULTS:

Reporter System – A number of reporter systems were tested prior to choosing the PSEEN0973::*lacZ* and PSEEN5493::*lacZ* reporter strains of *P. entomophila.* All of the *gfp* reporters examined failed to produce any significant difference in fluorescence between the reporter strain and the wild-type strain, suggesting that the *gfp* reporters were not effectively producing GFP. This could be for a number of reasons, including the cells degrading GFP before the fluorescence could be measured or improper folding of the GFP protein. Figure 4 illustrates the difference in fluorescence of the wild-type reporter strain and ΔpvfC reporter strain for the monalysin promoter, as well as the fluorescence of the wild-type strain without the reporter. This figure is a representative example of the data obtained for each of the *gfp* reporter strains.

The *lacZ* reporter systems proved to be more effective as all of the *lacZ* reporter strains examined produced a significant difference in the production of β-galactosidase between the reporter strain and the wild-type strain. Furthermore, all of the reporter strains had a significant difference in the production of β-galactosidase from the Δ*pvfC* strain. The Δ*pvfC* strain represents the level of β-galactosidase produced when the PVF-initiated signaling pathway is

non-functional as no useful PVF can be synthesized without *pvfC*. Therefore, all of the *lacZ* reporter strains would be useful in identifying whether or not the signaling pathway was functional in a given sample. Figures 5, 6, and 7 illustrate the difference in production of β-galactosidase between each of the *lacZ* reporter strains and their respective Δ*pvfC* strain. According to Figures 5, 6, and 7, the monalysin promotor, gene 0973 promoter, and gene 5493 promoter systems produced an approximately 6-fold, 35-fold, and 8-fold difference, respectively, in the production of β-galactosidase between the wild-type reporter strain and the Δ*pvfC* strain. The PSEEN0973::*lacZ* reporter strain was chosen initially due to the exceptionally low activity of the Δ*pvfC* strain, which is useful for ensuring a definitive white colony phenotype in the blue/white screening. Later rounds of Tn5 utilized the PSEEN5493::*lacZ* reporter strain due to inconsistencies later identified with the PSEEN0973::*lacZ* reporter.

Figure 4: *$P_{Monalysin}$-gfp Reporter*

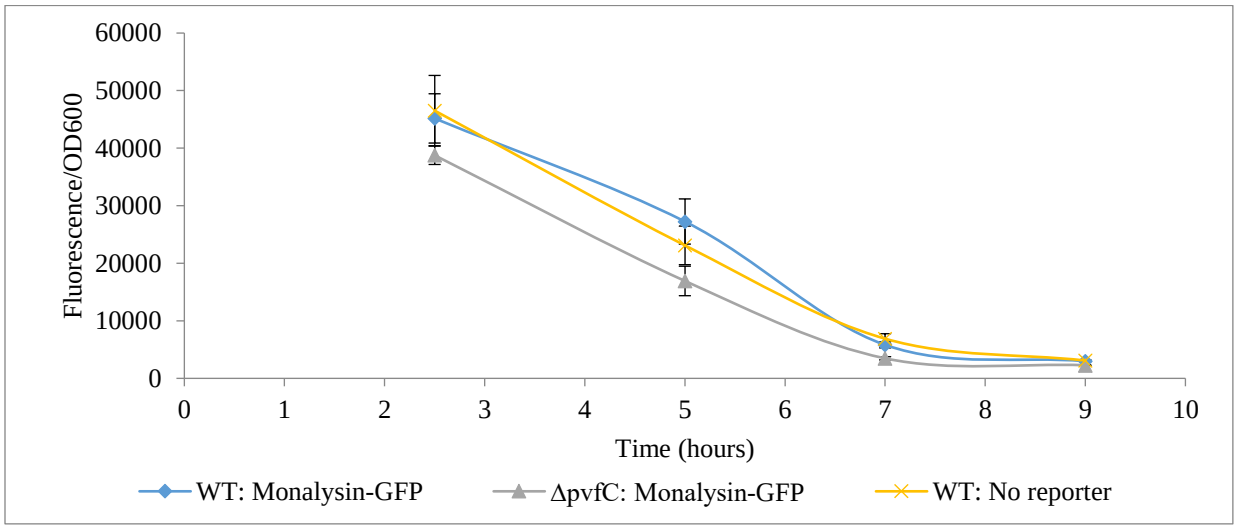


Figure 5: *$P_{Monalysin}$-lacZ Reporter*

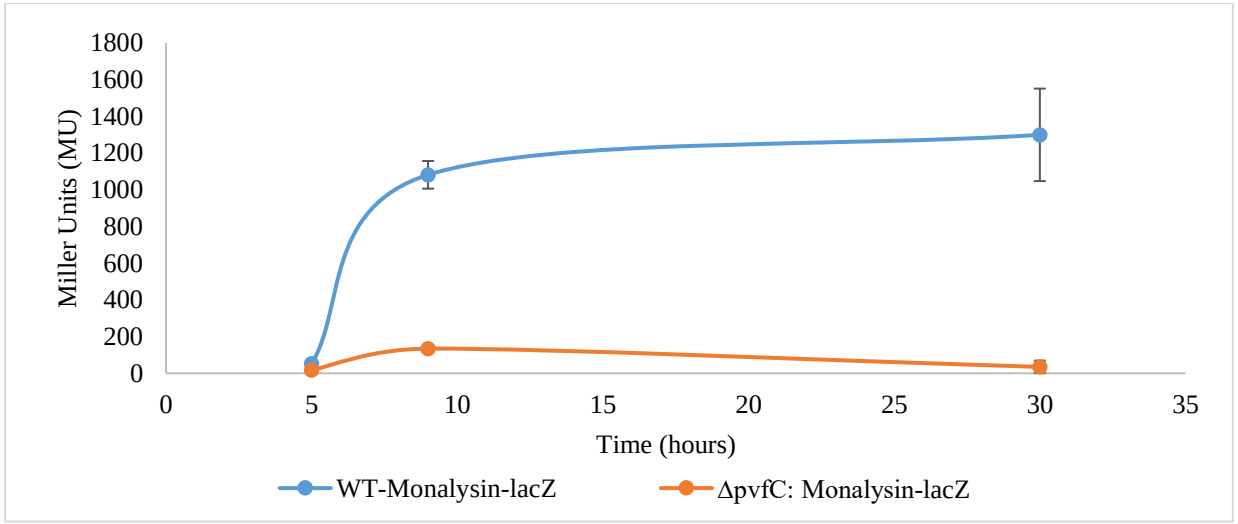


Figure 6: *PSEEN0973::lacZ Reporter*

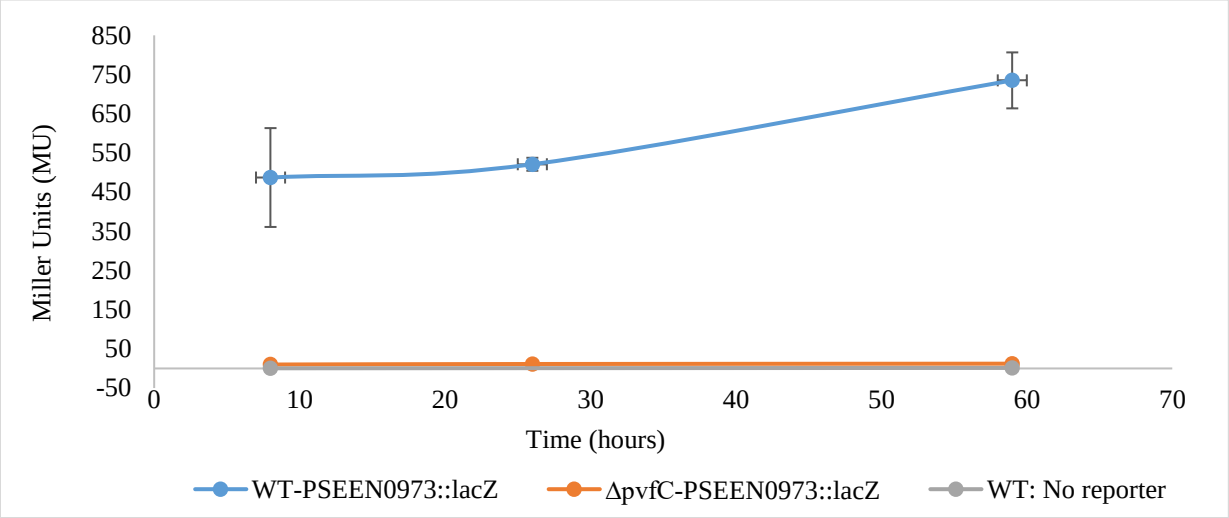
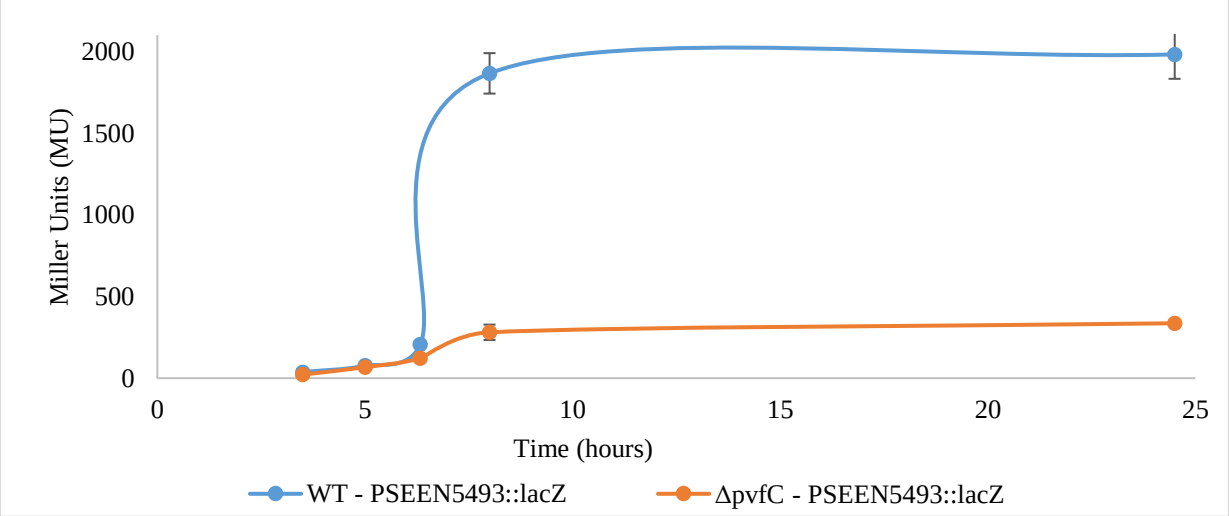


Figure 7: *PSEEN5493::lacZ Reporter*[8]



Blue/white screening – Of the plates grown, four PSEEN0973::*lacZ* plates and seven PSEEN5493::*lacZ* plates were used for screening due to their high density of cells. As described above, reporter strains of *P. entomophila* were used that contained an extra insertion in their genome made up of a promoter activated by the PVF signaling pathway and a downstream *lacZ* gene, the gene encoding β-galactosidase. When presented with the X-gal in the agar plate, β-galactosidase will produce a blue dye through the reaction depicted below in Figure 8. White samples on the plate have disruptions in genes vital to the signaling pathway, as the promoter is not activated and no β-galactosidase is produced. On the four PSEEN0973::*lacZ* plates, ~5500 colonies were screened, and out of these, 49 colonies produced uniformly white colony plates. Later attempts at Tn5 with the PSEEN0973::*lacZ* promoter strain proved to be inconsistent and significantly less efficient, producing 50/50 blue and white colonies. This is likely due to

PSEEN0973 being an unreliable promoter and not consistently expressing *lacZ*, resulting in a larger number of false positives. Therefore, subsequent attempts at Tn5 utilized the PSEEN5493::*lacZ* reporter strain instead. Of the seven PSEEN5493::*lacZ* plates, ~3000 colonies were screened, and out of these, 37 colonies produced uniformly white colony plates. An example of a typical plate is shown in Figure 9.

Figure 8: *X-gal Reaction Scheme*

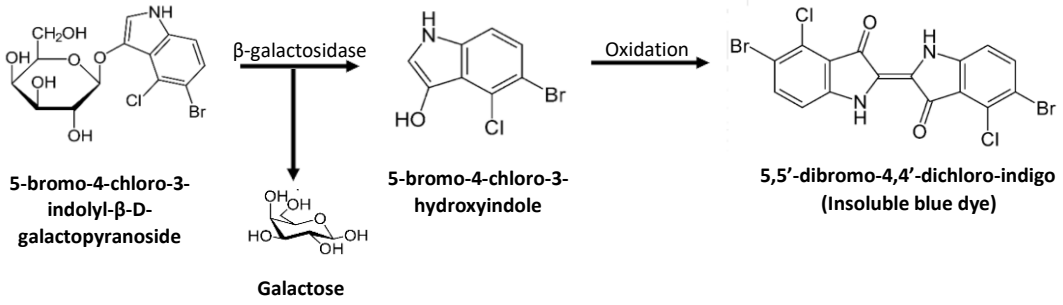
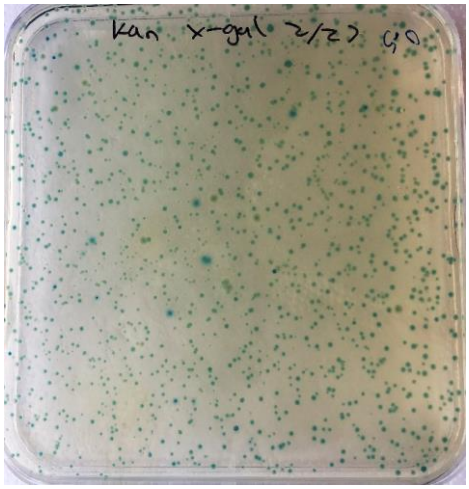
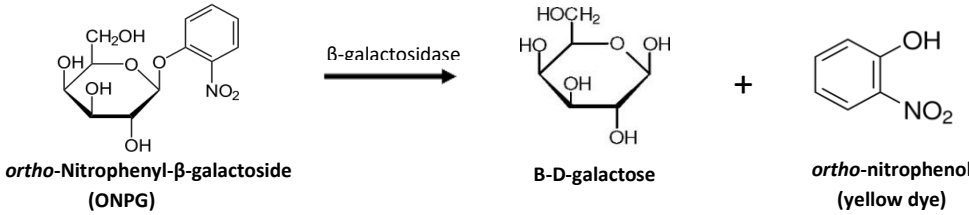


Figure 9: *Blue/white screening*



β-galactosidase assay identified samples with low activity – Each of the 49 PSEEN0973::*lacZ* and 19/37 of the PSEEN5493::*lacZ* white colonies were subsequently screened using the β-galactosidase (β-gal) activity assay. When presented with ONPG, β-galactosidase will produce *ortho*-nitrophenol, as depicted below in Figure 10. *Ortho*-nitrophenol is yellow in color and its concentration in solution can be measured by recording the OD_{420}. Additionally, this assay measures the OD_{550} to help correct for the background noise in solution. Samples that produce activities similar to L48_184 and L48_182, i.e. the wild-type reporter strains, have a fully functional PVF signaling pathway, while those that produce a lower activity similar to L48_185 and L48_183, i.e. the ΔpvfC strains, have a disrupted pathway. A third sample type is expected if the transposon inserts in the reporter gene itself. This will produce the lowest level of activity, similar to the original wild-type strain or L48_75, as it will no longer be possible for the cells to produce any β-gal.

Figure 10: *ONPG Reaction Scheme*



L48_185 will have low levels of β-gal activity as well; however, it will still be able to produce a very limited amount due to the leaky activation of the promoter.

Figures 11-13 illustrate that with the exception of sample 10, the white colony samples from the PSEEN0973::*lacZ* reporter strain all had activity levels similar to L48_185 or significantly lower in activity. While the error bar on sample 10 is very large, suggesting this data is not credible, this error range was due to one erroneous sample out of the three tested. With an activity of 836 Miller Units, this outlier is likely due to improperly re-suspended cells, resulting in a larger number of cells being present in the assay than recorded. The other two trials produced activity levels of 95 and 54 Miller Units, both of which are similar to the activity of L48_184 and far larger than the activity of L48_185 at any time point. Therefore, sample 10 was discarded, and the rest of the samples moved on to further analysis.

Figure 11: *β-galactosidase assay PSEEN0973::lacZ samples 1-8*

Figure 12: *β-galactosidase assay PSEEN0973::lacZ samples 11,13-15, and 17-41*

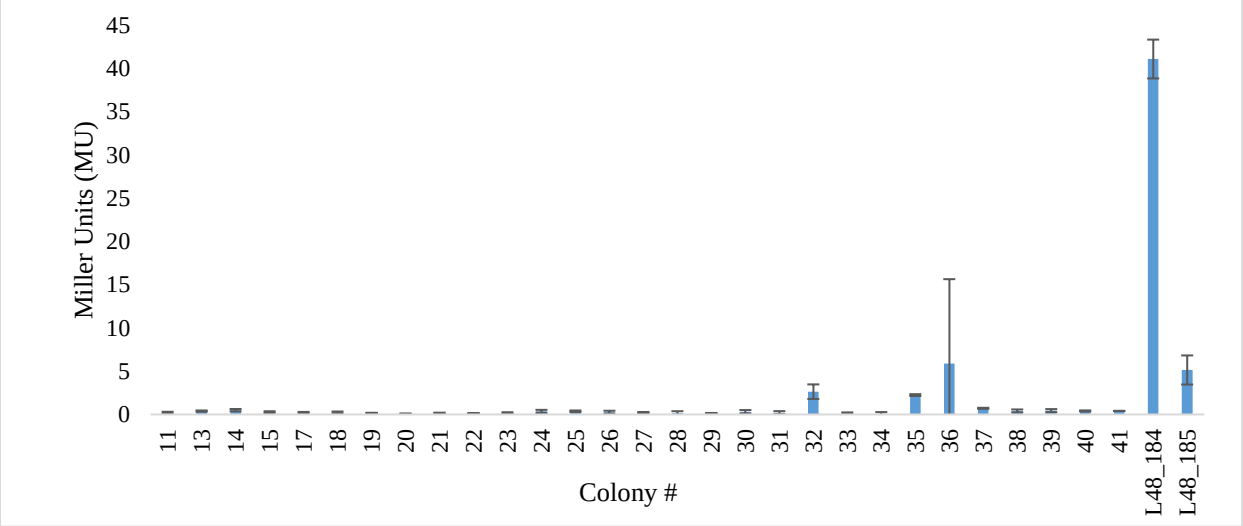


Figure 13: *β-galactosidase assay PSEEN0973::lacZ samples 11,13-15, and 17-41*

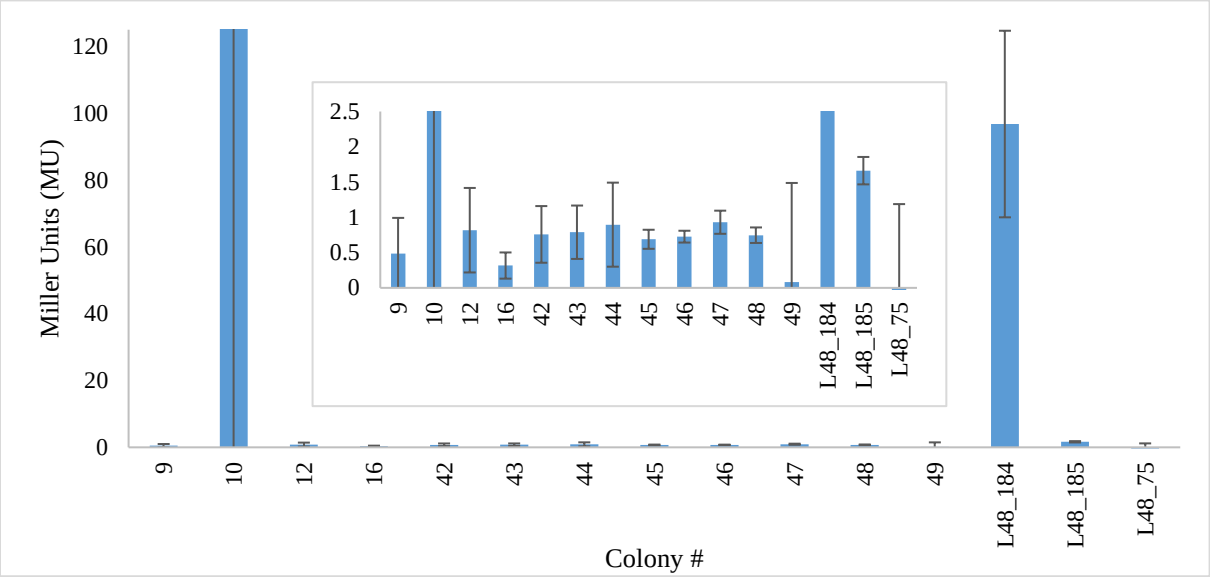
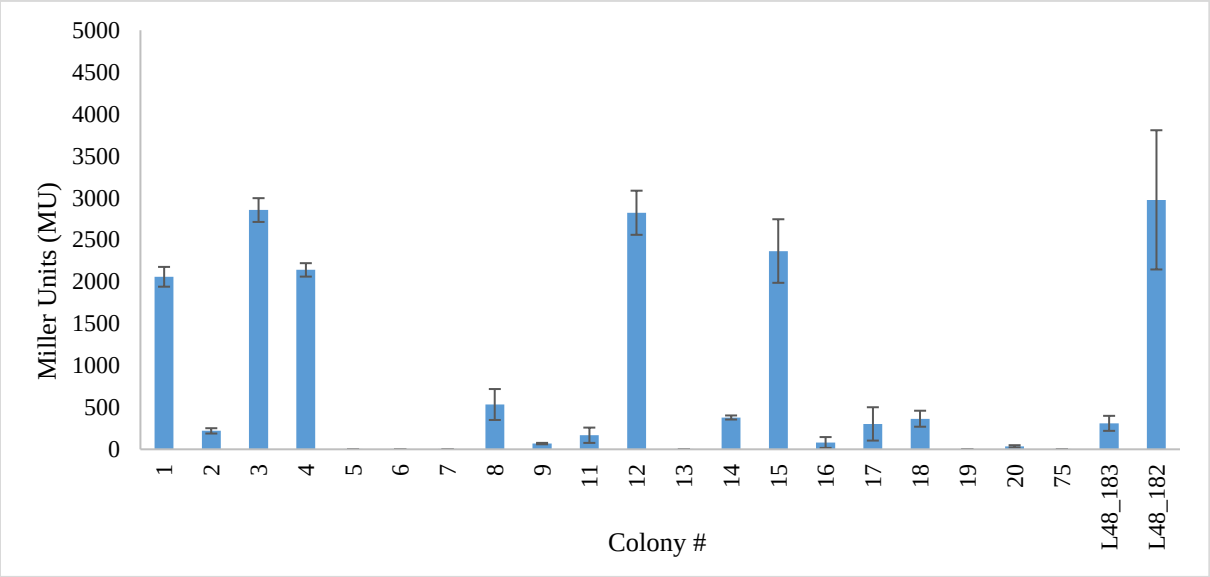


Figure 14 illustrates that with the exception of colonies 1, 3, 4, 12, and 15, the white colony samples from the PSEEN5493::*lacZ* reporter strain all had activity levels similar to L48_183 or significantly lower in activity. Also, with this second reporter system, the division between the three expected levels of activity is more readily observed.

Figure 14: *β-galactosidase assay PSEEN5493::lacZ samples 11,13-15, and 17-41*



Some of the discrepancies in activity levels ing Figures 11-14 can be accounted for by the dependency of the PVF signalling pathway activity on the location of cells in the growth curve. As seen in Figures 5-7, β-gal activity varies with OD_{600}. Therefore, two samples could both have a disrupted PVF signaling pathway, but not have overlapping error bars if they were at different

points in the growth curve. It is important to note, however, that L48_184 and L48_185 produce a ~45-75 fold difference in β-galactosidase activity during their stationary phases and L48_182 and L48_183 produce an 8-fold difference. Therefore, this method can still be used to distinguish between those samples that have functional vs. disrupted PVF signaling pathways. This assay may not be able to reasonably distinguish between L48_185 and L48_75, however, as their activites only produce a ~5-fold difference at stationary phase and their activities are lower than the 0.1-1.0 recommended measurement range of the spectrometer. On the other hand, L48_183 and L48_75 have an ~880-fold difference in activity and L48_183 produces OD_{420} values safely in the recommended measurement range of the spectrometer. Therefore, the PSEEN5493::*lacZ* reporter system may be able to not only distinguish between the Δ*pvfC* and the wild-type reporter, but also between the Δ*pvfC* and the original wild-type strain without a reporter.

Arbitrary PCR and Sequencing – The first PCR reaction of arbitrary PCR was attempted on all of the 48 remaining samples of the PSEEN0973::*lacZ* reporter system. Of these samples, only 26 produced a dominant band after either the first or second round of PCR and were sent for sequencing. Only 19/48 yielded successful sequencing results. These results are summarized in Table 3. The most frequent result achieved was sequence alignment with both a putative histidine kinase and CheB-like methylesterase.

Table 3: *Summary of sequencing results*

Result	Number of occurrences
Putative Hybrid Sensor Histidine Kinase/Response Regulator and CheB-like Methylesterase	10
CheB-like Methylesterase	1
DNA Polymerase III, β Chain	2
Xylulokinase	4
Putative Peptidase	1
Putative NRPS	1

DISCUSSION:

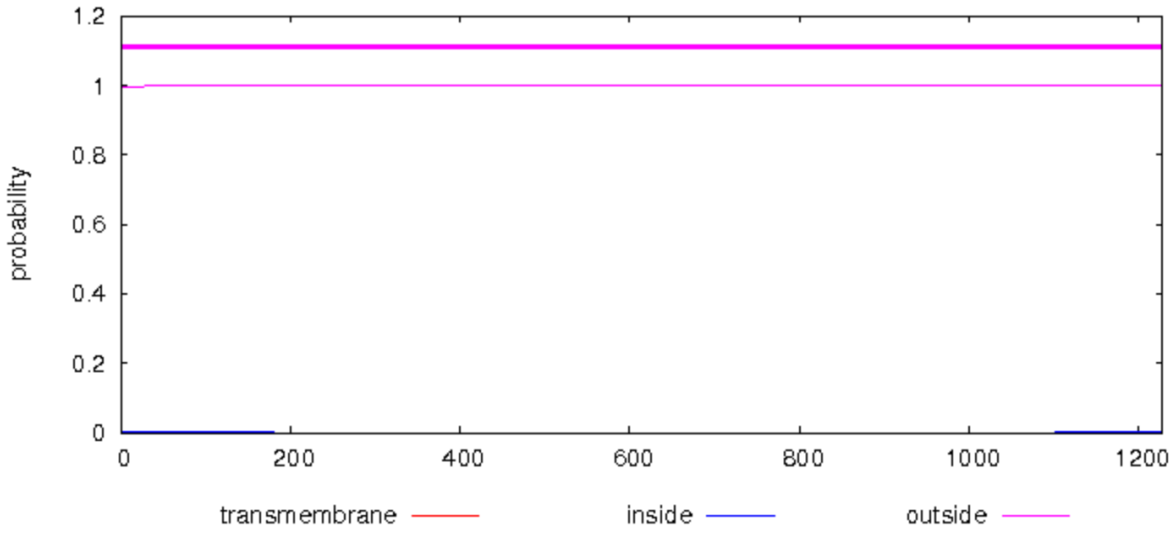
Overall, ~5,500 colonies from the PSEEN0973::*lacZ* reporter strain of *P. entomophila* and ~3,000 colonies from the PSEEN5493::*lacZ* reporter strain of *P. entomophila* were qualitatively screened using a blue/white screen. A total of 49 single white colonies were isolated from the PSEEN0973::*lacZ* reporter plates while 37 single white colonies were isolated from the PSEEN5493::*lacZ* reporter plates. The 49 samples from the PSEEN0973::*lacZ* reporter plates were screened using the quantitative β-galactosidase assay, and all but one of these samples (10) produced activity similar to or less than the Δ*pvfC* strain. Only 19/37 of the white colonies from the PSEEN5493::*lacZ* reporter plates have been screened thus far, and all but five of these samples (1, 3, 4, 12, and 15) produced activity similar to or less than the Δ*pvfC* strain. The

location of the Tn5 insertion was identified in 19/49 of the PSEEN0973::*lacZ* reporter samples through arbitrary PCR and subsequent sequencing, yielding a putative histidine kinase and a putative CheB-like methylesterase as the most frequent sequence alignment results. Of the proteins listed in Table 3, the putative hybrid sensor histidine kinase/response regulator and the CheB-like methylesterase are the most likely to be directly involved in the PVF signaling pathway as relatives of these proteins are known to be involved in the signaling pathways of a number of bacterial species.[9] These specific *P. entomophila* proteins will be referred to as HisK and CheB-L, respectively, for the remainder of the paper.

*Putative hybrid sensor histidine kinase/response regulato*r- Typically, histidine kinases are transmembrane homodimers that bind the extracellular signaling molecule and subsequently, use adenosine triphosphate (ATP) to auto-phosphorylate a conserved histidine residue.[9] They will usually go on to phosphorylate an aspartate residue on a response regulator molecule that will then continue to progress the signaling pathway.[9] In hybrid histidine kinases, this response regulator is not a separate protein and is actually part of the overall structure of the histidine kinase itself. An example would be VirA, which "controls the expression of *Agrobacterium tumefacians* virulence genes in response to phenolic compounds.[9]" The regulator portion of the molecule typically acts as an inhibitor of the action of the histidine kinase until it receives an extracellular signal.[9] In addition, there are also some hybrid histidine kinases that have no transmembrane component and function in the cytoplasmic portion of the signaling pathway rather than binding the initial extracellular signaling molecule.[9] An example of this would be the FrzE protein in *Myxococcus xanthus*, which is responsible for regulating the gliding motility of the organism.[9] In addition to this relatively conserved mechanism, histidine kinases typically have 5 conserved domains: the H, N, G1, F, and G2 boxes.[9] The role of the N and F boxes are not known, but the H box contains the conserved histidine residue and the G boxes are responsible for nucleotide binding and kinase/phosphatase activity.[9]

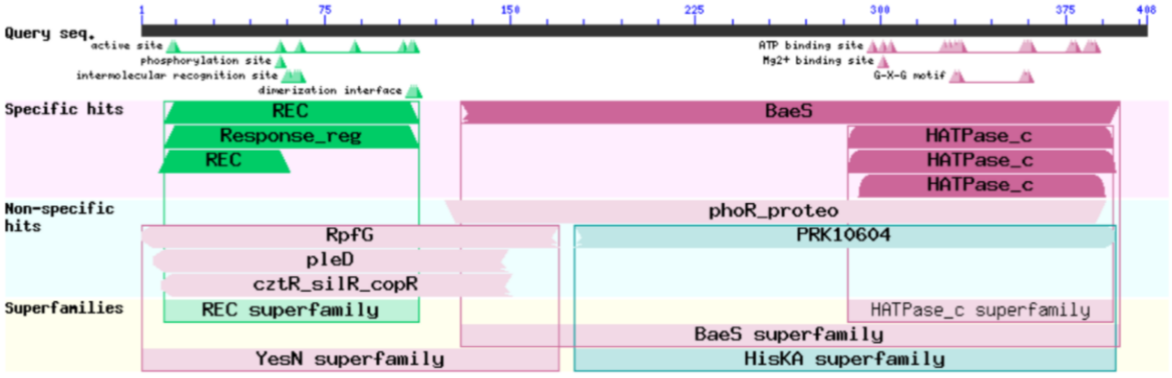
Based on data from the BLAST database, HisK is divided into two main domains: a histidine kinase that is part of the BaeS superfamily and a response regulator. The first distinction that needs to be made regarding HisK vs. other histidine kinases is whether BaeS is a transmembrane protein or confined to the cytoplasm. The response regulator domain will always exist in the cytoplasm so it is the histidine kinase component that raises the question. According to the BLAST database, the closest relatives of HisK, which are present in *P. putida*, *P. mosselii*, and *P. guariconensis* are similarly uncharacterized; however, a moderately close relative of the HisK is present in *P. syringae pv. actinidiae* and *P. fluorescens*, having 72% and 73% sequence identity, respectively. The histidine kinases in both of these organisms are predicted to have two transmembrane regions.[10] On the other hand, the bioinformatics program TMHMM from the Technical University of Denmark predicts that there are no transmembrane segments in HisK, as demonstrated by Figure 15. Therefore, this data suggests that HisK is likely a cytosolic protein rather than a transmembrane one.

Figure 15: *TMHMM predicting transmembrane segments of putative histidine kinase*



Additionally, HisK has a number of conserved motifs, including an ATP-binding site, dimerization interface, and a phosphorylation site. As mentioned above, the two most characterized domains of a histidine kinase are the H and G box. The G box, or the site of ATP-binding, is typically rich in glycine residues, and such a region can readily be observed in Figure 16 as the HATPase region with its G-X-G motif. The H box, containing the phosphorylated histidine residue, is less apparent. There are 9 H residues in HisK; however, this H residue must be one of those between 125 and 408 residues as this range represents the histidine kinase portion of the protein, eliminating four H residues as possibilities (His-4, His-34, His-49, and His-109). For the response regulator, the BLAST search data in Figure 16 identifies Asp-65 as the phosphorylated aspartate residue.

Figure 16: *Domains of putative hybrid histidine kinase*



CheB-like methylesterase- CheB is a well-studied methylesterase in *E. coli*. It is involved in bacterial chemotaxis and helps to adjust the swimming behavior of *E. coli* in the presence of external stimuli.[9] When CheB is phosphorylated it will remove methyl groups from carboxyl

methylated glutamate residues of the receptors that respond to environmental stimuli.[9] In this system, demethylation is an inhibitory mechanism for the signaling pathway as decreased methylation results in less activation of a cytosolic histidine kinase known as CheA.[9] Therefore, it is possible that CheB-L acts in this same capacity with HisK, especially considering HisK is likely also a cytosolic histidine kinase.

As mentioned previously, the PVF signaling pathway serves to activate genes involved in virulence. If PVF acts as a quorum sensing molecule, then it is very important for HisK to be able to differentiate between different concentrations of the signaling molecule and respond only when there is a large enough concentration of PVF so as to indicate a large bacterial population. If the signaling pathway was initiated by low concentrations of PVF, *P. entomophila* would activate the genes important for virulence before there was an adequate bacterial population to compete with the defenses of the host, resulting in easier eradication. One potential mechanism for this signaling pathway would be that CheB-L serves to inhibit HisK until a certain extracellular concentration of PVF is reached, which then overpowers the inhibitory action of CheB-L.

The functions of the other hits from this experiment are far less understood as they are not as obviously involved in the PVF signaling pathway or any other signaling pathway. The PVF signaling pathway needs to be further characterized in order for the role of these other proteins to be identified.

Future directions- Knock-outs of the genes found to be involved in the PVF signaling pathway, such as HisK and CheB-L, would need to be constructed in order to assure that the presence of these genes are important for the PVF signaling pathway. These genes should then be reintroduced through complementation to test for the return of the functional signaling pathway. The activity of the signaling pathway could be measured using the blue/white screen and β–galactosidase assay described in this paper so long as the reporter strain L48_184 or L48_182 was used to construct the knock-outs. Additionally, this experiment only screened ~8500 colonies. For a genome the size of *P. entomophila*, 60,000 colonies would need to be screened so that "a gene 327 bp in length has a 95% chance of being disrupted.[11]" Therefore, more colonies should be screened to say with certainty that all of the proteins involved in the PVF signaling pathway have been disrupted.

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