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Gene Regulation by a Novel Two-Component System Conserved Among Gammaproteobacteria

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GENE REGULATION BY A NOVEL TWO-COMPONENT SYSTEM CONSERVED AMONG
γ-PROTEOBACTERIA

By

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ABSTRACT

GENE REGULATION BY A NOVEL TWO-COMPONENT SYSTEM CONSERVED AMONG

γ-PROTEOBACTERIA

By

Kristin M. Jacob

Two-component systems are common gene regulatory pathways seen in bacteria. Gene expression is controlled by these systems through a series of phosphorylation events between two proteins; a membrane bound sensor kinase and a cytoplasmic response regulator. Activation of a two-component system can be caused by biological or environmental stimuli, resulting in altered gene expression. A conserved two-component system was recently discovered in entomopathogenic bacteria, including our model organisms *Pseudomonas entomophila* and *Pseudomonas aeruginosa,* and is homologous to the recently identified two-component system, CrbRS, in *Vibrio cholerae.* In *V. cholerae* CrbRS regulates acetate metabolism and controls virulence. The aim of this study is to determine whether a homologous two-component system present within our model organisms regulates similar genes within diverse organisms. Experiments by Hang *et al.*, together with the current study, indicate that the deletion of this two-component system results in the down regulation of the gene *acsA* in *V. cholerae, P. entomophila* and *P. aeruginosa*. This study utilized further shows that this two-component system plays an important role in acetate metabolism by regulating expression of *acsA* in *P. aeruginosa* and *P. entomophila*. However, unlike that seen in *V. cholerae,* it does not regulate the virulence of these organisms.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS & ABBREVIATIONS

acsA: Gene encoding an acetyl Co-A synthetase in *Pseudomonas aeruginosa* and *Pseudomonas entomophila*.

actP: Gene encoding an acetate permease in *Pseudomonas entomophila.*

BLASTn: Basic Local Alignment Search Tool (nucleotide).

CLP: Cyclic lipopeptide

CTx: Cholera Toxin

IMD: Immune deficiency.

IPTG: Isopropyl-β-D-1-thiogalactopyranoside.

ORF: Open reading frame.

PA3271: Gene encoding a putative sensor kinase in *Pseudomonas aeruginosa.*

PA3234: Gene encoding a putative acetate permease in *Pseudomonas aeruginosa.*

PA3604: Gene encoding the response regulator ErdR in *Pseudomonas aeruginosa*

PSEEN1405: Gene encoding a putative sensor kinase in *Pseudomonas entomophila.*

PSEEN1455: Gene encoding the acetate permease ActP in *Pseudomonas entomophila*.

PSEEN4122: Gene encoding a putative response regulator in *Pseudomonas entomophila*.

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

REC: Receiver Domain

TCP: Toxin Co-regulated Pilus

TCS: Two-Component System

Δ: Indicates a bacterial strain containing a deletion.

Δ1405RD: Mutant strain of *Pseudomonas entomophila* containing a deletion in the receiver domain region of the sensor kinase.

Δ3271RD: Mutant strain of *Pseudomonas aeruginosa* containing a deletion in the receiver domain region of the sensor kinase.

Δ3604RD: Mutant strain of *Pseudomonas aeruginosa* containing a deletion in the receiver domain region of the response regulator.

Δ4122RD: Mutant strain of *Pseudomonas entomophila* containing a deletion in the receiver domain region of the response regulator.

INTRODUCTION

Bacteria live in environments that can experience rapid changes in factors such as pH,

temperature, and nutrient availability. One of the ways that bacteria sense and respond

to environmental changes is through two-component systems (TCS), regulatory

mechanisms which control gene expression through two conserved proteins, a histidine

sensor kinase and a response regulator. When an environmental signal interacts with

the membrane bound sensor kinase, it activates a cytosolic response regulator protein

which functions as a transcription factor to activate or repress the expression of specific

genes (13, 36). TCS have been identified in many bacteria species and play important

roles regulating the expression of a diverse array of genes such as those involved in

energy metabolism and cell division (13). These systems can also be utilized by many

types of pathogenic bacteria to control the expression of specific virulence factors

and/or other allelic mechanisms that are detrimental to a host (13). Understanding how

two-component systems regulate gene expression could be beneficial in health care

research and biocontrol of pests. Bioinformatic analysis of the large numbers of fully

sequenced bacterial genomes currently available indicates that most bacteria contain

genes encoding dozens of TCSs (13). New TCSs can be found in many well studied

bacteria, including the model organisms in this study *P. entomophila* and *P. aeruginosa*

(45)*.*

Recently a novel two-component system, CrbRS, was identified in *Vibrio cholerae*, and

homologs of this TCS were also are found in *P. entomophila* and *P. aeruginosa.* Hang *et*

al. utilized RNAseq analysis to identify genes regulated by this system in *V. cholerae* and found 25 genes that were regulated by this TCS. Among the genes identified, 16 were distinguished as being important for virulence, and multiple genes were found to be involved in transport and utilization of carbohydrates and amino acids (14). Some of the genes regulated by this TCS in *V. cholerae* also have homologs in *P. entomophila* and *P. aeruginosa*. Homologs of the genes most highly regulated by this TCS in *V. cholerae*, such as those involved in virulence or carbohydrate utilization, were chosen as a focus for further study as potential genes regulated by this TCS in *P. entomophila* and *P. aeruginosa.* The aim of this study was to determine what genes are regulated by this novel two-component system. We determined whether this TCS has the ability to regulate virulence in *P. entomophila* and *P. aeruginosa,* as well as its potential to regulate other important genes such as *acsA*. *acsA* is an important gene to explore due to its importance in carbon utilization (20), as well as being identified as one of the most highly regulated genes by this TCS in *V. cholerae* (14).

Previous studies have indicated this TCS may be important in the regulation of virulence in *V. cholerae* (14)*.* Further exploration of genes controlled by this system showed *acsA* to be highly regulated in *V. cholerae* (14), therefore its regulation was also explored in *P. entomophila* and *P. aeruginosa*. Our work has shown that *acsA* is also highly regulated by the homologous TCS in these bacteria. *acsA* encodes an acetyl-CoA synthetase (ACS) which catalyzes the conversion of acetate to acetyl-CoA. During the tricarboxylic acid cycle acetyl-CoA is utilized to generate energy and results in the generation of cell components via a glyoxylate shunt (20). Evidence suggests that *acsA* may be important

in *P. entomophila* and *P. aeruginosa* to utilize acetate from its environment as a carbon

source (20).

LITERATURE REVIEW

Vibrio cholerae

Vibrio cholerae, the etiological agent of cholera, causes perfuse diarrhea in patients that contract the pathogen and has a high mortality rate. It is a Gram-negative, curved rod shaped bacteria belonging to the family γ-Proteobacteriaceae found in many aquatic environments including brackish and salt water (9, 16). One of the most intriguing epidemiological findings is that this bacteria causes epidemics in a seasonal pattern, normally following the wet seasons where monsoons occur and results in the mixing of waste runoffs into the drinking and bathing water in underdeveloped countries (9). This phenomenon has led to the characterization of *V. cholerae*'s complete lifecycle, resulting in five distinct life stages; a free-swimming form, a symbiont of phytoplankton, a commensal of zooplankton, a sustainable but non-culturable state, and a stage in which *V. cholerae* can attach to abiotic or chitinous surfaces with the aid of biofilm formation (9, 34).

It is likely that *V. cholerae's* tolerance for these many life stages and many environments is due to its unique genome which contains two circular chromosomes with asymmetrical distribution of essential genes (34, 40). The larger of the two chromosomes (chI) is about 2.9 Mb in size and encodes the majority of the genes that are required for growth and pathogenicity, including the genes encoding the virulence

factors cholera toxin (CTx) and toxogenic co-regulated pilus (TCP) that are associated

with pathogenic *V. cholerae* (16, 34). In contrast, the smaller of the two chromosomes

(chII) is about 1.07 Mb in length and encodes genes associated with essential metabolic

and regulatory pathways. Most notably, chII encodes integron islands (34), or

superintegrons, which are chromosome-bound gene expression elements that convert

open reading frames (ORFs) into functional genes and can carry over 20 gene cassettes

(distinct units of circularized DNA) utilized for activating expression of encoded genes

(26, 32). Three recognizable islands found in the *V. cholera* genome encodes gene

products that could be involved in drug resistance. These gene products include possible

haemagglutinin and lipoprotein virulence factors, as well as three genes whose products

are similar to "host addiction" proteins which are used by plasmids. Though these are

general associations of gene distribution, there have also been incidences of both

chromosomes having copies of the same gene, which may be associated with the

increase in diverse environments *V. cholerae* can inhabit (16, 34).

There are pathogenic and non-pathogenic strains of *V. cholerae*. Pathogenic *V. cholerae*

possess CTx, TCP, the regulatory protein ToxR, while non-pathogenic *V. cholerae* do not

express these toxins (9). It was previously believed that all pathogenic strains were

classified in the serogroup 0:1, as all strains that caused epidemics and endemics

contained the 0 antigen 01. These strains could be further classified into biotypes as

either classical or El Tor based on biochemical properties and bacteriophage

susceptibility (9). The first 6 pandemics have resulted from strains from either of these

two biotypes. *V. cholerae* strains that did not cause cholera, or did so on rare occasions,

were classified as non-01 serogroup (9). A 1993 pandemic was the result of a strain of *V. cholerae* that was identified from the non-01 serogoup and contained the O antigen 0139. Presently, pathogenic *V. cholerae* is distinguishable based on encoding the previously listed virulence factors, as well as classified as having either a 01 or 0139 O antigen (9).

With a long history of pandemics, it has been important to understand the properties of *V. cholerae* that allow it to be virulent in humans. With many studies focused on regulation of the main virulence factors CTx and TCP, scientists have recently identified additional virulence factors and their regulatory mechanisms. Using transposon mutagenesis, Hang *et al*. identified a novel two-component system (TCS) important for virulence of *V. cholerae* in a *Drosophila* model of infection. This TCS, which was termed CrbRS, was shown to regulate acetate utilization through the "acetate switch," a known survival transition found within many genera of bacteria (47). This regulation occurs through controlling transcription of *acs1*, a gene encoding acetyl Co-A synthase-1, an enzyme that converts acetate to acetyl-CoA which is utilized in multiple metabolic pathways (14, 47). It was observed that CrbRS and *acs1* in wild-type *Vibrio cholerae* disrupted host insulin signaling pathway (IIS), a signaling pathway important for glycogen synthesis and lipid storage. This disruption results in decreased lipid accumulation in the fat body and intestinal steatosis, or the deposit of fat into intestinal cells, which played a role in the lethality of *V. cholerae* in a *Drosophila* model of infection (14). Abnormal accumulation of lipid deposits within cells presumably interferes with normal cell function. By deleting either the sensor kinase or response

regulator of this TCS, expression of *acs1* is significantly reduced, thus rendering *V. cholerae* non-pathogenic in *Drosophila* (14). The observation that acetate metabolism can be utilized as a virulence factor by *V. cholerae* is important as it has not previously been seen. Though this is an exciting discovery, it is important to note that this is not the only virulence factor found in *V. cholerae* and that this TCS does not regulate *V. cholerae's* well-known virulence factors, Ctx and TCP. These results led to the question: does this novel TCS regulate virulence through the acetate switch in other microorganisms belonging to the γ-Proteobacteriaceae family?

Drosophila melanogaster as an Infection Model

Drosophila melanogaster, more commonly known as the fruit fly, has been used as a model organism in scientific research for more than 80 years. This organism is a favorable model due to its high reproductive rate, ease of use, and inexpensive maintenance (1, 41). With the ability to utilize this model in both forward and reverse genetic analysis, *Drosophila* allows for more invasive research when studying host-pathogen interactions than mammalian models (43). *Drosophila* also provides a unique invertebrate model when studying opportunistic pathogens such as *Pseudomonas aeruginosa*, as flies possess effective pathogen defense systems. Among various cellular and humoral immune responses, *Drosophila* encode Toll and immune deficiency (IMD) signaling pathways similar to those of mammalian innate immune responses (43). With its long history in scientific research and many positive qualities in the study of host-pathogen interactions, *Drosophila* can be utilized in answering novel questions involving

virulence capabilities and mechanisms of virulence regulation in insect and mammalian pathogens.

Two-component systems

Two-component systems (TCS) are common regulatory pathways found primarily in prokaryotic organisms, but are also observed in a few eukaryotic organisms (13). These systems allow for an organism to sense and respond to environmental changes (36). On average, bacteria encode for 50-60 TCS, though some species can encode more than 250 TCS in their genome, still others may not have any TCS. The vast differences in number of TCS are caused by the environment and physiochemical conditions in which that bacteria reside (13). For those that encode TCS, signals that initiate these systems can be environmental (abiotic), chemical, or biological in nature. These can also be produced by the host or by the bacteria themselves (15). These systems regulate many aspects of an organism's cellular functions including sporulation systems, virulence factors (in pathogenic organisms), cell division and differentiation, and metabolic processes with evidence for a role in "cross-talking" between organisms in symbiotic relationships (13, 36).

TCSs are characterized by two multi-domain proteins with amino acids specific to prokaryotic or eukaryotic organisms (13). Prokaryotic TCS are regulated by two major proteins, a sensor kinase with an associated histidine kinase domain and a response regulator with an associated aspartate residue that acts as a phosphor-receptor. Although rare, eukaryotic TCS also include the same major proteins (a sensor kinase and

response regulator) similar to as those found in prokaryotes, but include serine, threonine, and tyrosine as signaling amino acid residues instead of aspartate and histidine within the signaling domains of TCSs (36). The focus of my research is on a specific prokaryotic TCS. The sensor kinase is known as a "transmitter domain," due to its role in allowing the system to sense an environmental signal, bind ATP and transfer a phosphoryl group from an associated histidine residue to an aspartate residue (13). This domain can be separated into two regions based on the role it plays in inducing a response. The first is an N-terminal sensing region which is the portion of the protein domain that is embedded into the cell membrane and includes transmembrane helices to allow for a stimulus to be sensed in both the periplasmic and extracellular space. The second region is a C-terminal catalytic kinase core that is located in the cytoplasm and has two distinct domains, a dimerization, histidine phosphotransfer domain (DHp), and a catalytic ATP binding domain (CA) (13). These domains are essential for the sensor kinase to bind ATP and phosphorylate the histidine residue in response to the stimulus. The sensor kinase protein is referred to as a "transmitter domain" due to its ability to transmit the signal that activated it to the response regulator domain (13).

The response regulator is a transcription factor that is referred to as a "receiver domain." This protein includes two distinct regions, the N-terminal receiver (REC) domain and C-terminal effector domain (13). Among TCS, the REC is responsible for enzymatic signaling activity and is associated with the aspartate residue that receives a phosphate group from the histidine residue of the sensor kinase. The effector domain is

responsible for DNA binding, and producing the response to the stimuli that activated the system which will result in a change in expression for a specific gene(s) (13).

Once the sensor kinase protein of a TCS is activated by an environmental stimulus, three distinct reactions occur to produce a change in gene expression (Figure 1). In the first reaction, an autophosphorylation event occurs, causing a molecule of ATP to phosphorylate the histidine residue of the kinase domain. This phosphorylation then allows for a phosphotransfer event between the histidine residue and the aspartate residue on the response regulator (36). Once the phosphate is bound to the aspartate residue, the DNA-binding domain of the response regulator drives the activation or repression of specific genes, completing the TCS signaling pathway. Finally, the response regulator domain dephosphorylates, shutting down the TCS (13). A generalized example of a TCS is depicted in Figure 2(a). More complex systems will include a transfer of a phosphate from a histidine residue to an aspartate residue (Figure 2(b)), resulting in a conformational change in a terminal response regulator that can then activate or repress gene expression.

1. Autophosphorylation: HK-His + ATP $\Leftrightarrow$ HK-His~P + ADP
2. Phosphotransfer: HK-His~P + RR-Asp $\Leftrightarrow$ HK-His + RR-Asp~P
3. Dephosphorylation: RR-Asp~P + H_2O $\Leftrightarrow$ RR-Asp + P_i

Image adapted from: Stock *et al.*, Annual Reviews Biochemistry. 2000. 69: 183-215

Figure 1: Chemical process in which a two-component system is activated via an autophosphorylated histidine residue, associated with sensor kinase protein, and transferred phosphate to a phosphor-accepting aspartate residue of the response regulator protein. The two-component system deactivates during dephosphorylation of aspartate associated to the response regulator.
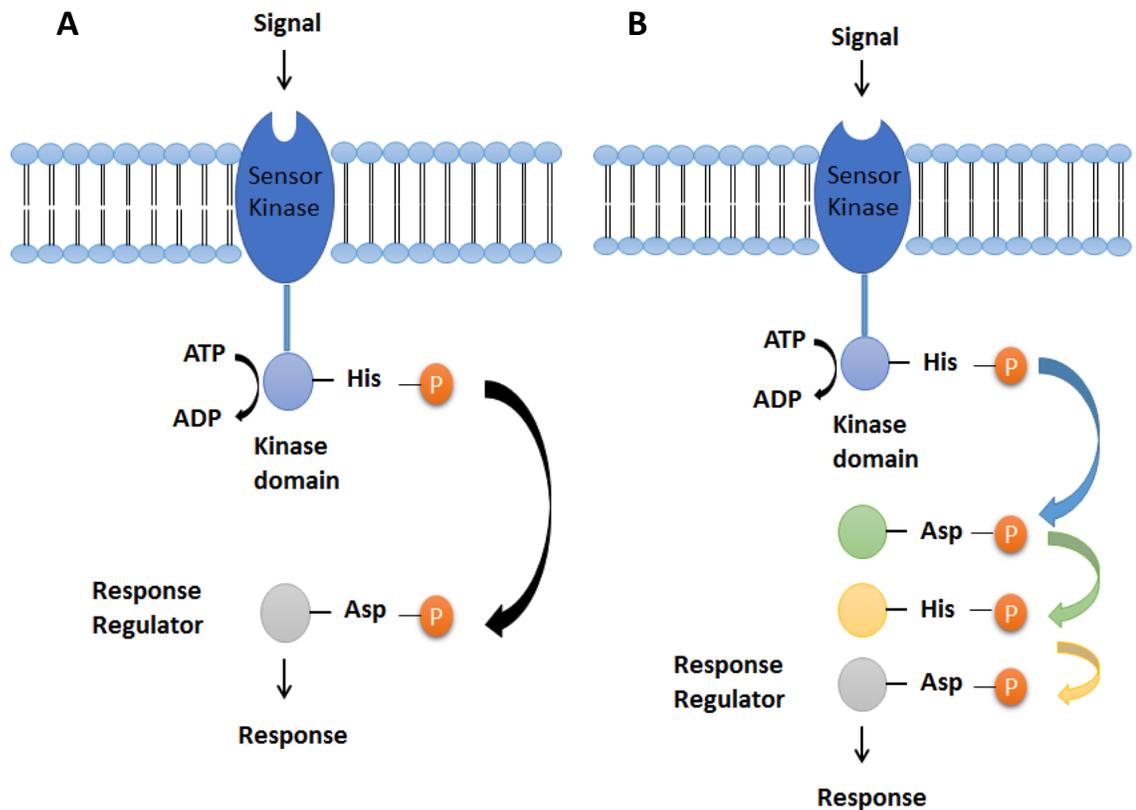
Image adapted from: Bretl D J et al. Microbiol. Mol. Biol. Rev. 2011;75:566-582

Figure 2: Diagram showing activation of autophosphorylation when a signal attaches to the sensor kinase of a TCS (A) and a mutli-component system (B). A: in a TCS, autophosphorylation results in a phosphor-transfer event between the histidine residue of the sensor kinase and the aspartate residue of the response regulator resulting in expression of regulated genes. B: in a multi-component system, autophosphorylation results in an phosphotransfer event between the histidine residue of the sensor kinase and additional histidine and aspartate residues located on proteins other than the sensor kinase and response regulator. The last phosphotransfer event in a multi-component system results in the phosphate interacting with the aspartate residue on the response regulator, activating gene expression.

<p style="text-align:center">Pseudomonas species</p>

Pseudomonas species are Gram-negative, rod- shaped bacteria and are a part of the γ-Proteobacteriaceae family. *Pseudomonas* sp. are a diverse set of organisms which inhabit a large subset of environments (terrestrial to aquatic) and infect hosts such as plants, insects and animals. Pseudomonads can also range from commensal (*P. flourescens* and plant roots) to pathogenic (*P. aeruginosa*) (35). This study will focus on two pathogenic species, *P. aeruginosa* (human pathogen) and *P. entomophila* (insect pathogen)*.*

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a metabolically versatile, Gram-negative, rod-shaped bacterium. It inhabits a wide range of environmental niches including soil and aquatic environments, plants, insects and various mammals (37). Part of *P. aeruginosa*'s ability to inhabit diverse niches is due, in part, to its ability to utilize diverse sources of carbon. Additionally, approximately 20% of the *P. aeruginosa* genome encodes proteins that regulate gene expression (37), which allows *P. aeruginosa* to regulate the expression of specific genes to adapt to fluctuations of environmental conditions (including human hosts or soil/aquatic environments) (35, 37). *P. aeruginosa* has the ability to persist in marginal environments which contains minimal nutrients, as the bacteria only needs acetate (carbon source) and ammonia (nitrogen source) to proliferate. These attributes add to the difficulties in eradicating this organism from hospital environments (8).

Though *P. aeruginosa* can sometimes be found as microflora within the intestine, it is also an opportunistic pathogen which causes various community acquired diseases and nosocomial diseases in immunodeficient humans. Among these are patients with HIV, burn wounds and or cystic fibrosis (33, 35, 37). These diseases can either be acute (e.g. nosocomial ventilator-associated pneumonia) or chronic (e.g cystic fibrosis) (33). Pathogenicity of this organism is attributed to its numerous potential virulence factors and to its intrinsic resistance to a wide range of antibiotics. Additionally, acquisition of resistance plasmids from other bacteria (through conjugation) and multiple encoded efflux pumps (35, 38) makes this pathogen especially difficult to manage. As *P. aeruginosa* cannot easily colonize healthy, intact epithelial cells, its pathogenicity is associated with epithelial cell damaged caused by hospital instruments (i.e. ventilator tubes). Enhanced pathogenicity of the bacteria has also been attributed to damage caused by its many virulence factors, which allows for increased adhesion and colonization (8, 33).

The severity of *P. aeruginosa* infections within individual patients is dependent on an array of expressed virulence factors (33). Virulence factors range from the expression of genes to aid in the initial adherence and colonization to cross-talking capabilities (quorum-sensing). This range allows *P. aeruginosa* to not only elude the immune system, but also compete against other invading bacteria for nutrients, allowing for persistent infection. Major virulence factors influencing infection are surface components aiding in adhesion/colonization process (pili and flagella), *P. aeruginosa*'s

ability to form biofilms and type III and IV secretion systems utilized to elude the host immune system.

Polar flagella allow for bacterium motility and, along with polar pili, aid in the attachment of *P. aeruginosa* to epithelial cells which subsequently activates the initial stages of infection. The flagellar proteins bind to toll-like receptor (TLR) 5 on the apical surface of epithelial cells found in host airways which, in turn, activates interleukin (IL-)-8 production. The pili proteins bind to the GalNacβ-4 gal moiety, associated with glycolipids that lack sialic acid and activates NF-κB and proinflammatory responses (33). Once adhered, *P. aeruginosa* can form biofilms, or "surface-attached microbial communities with characteristic architecture and phenotypic and biochemical properties distinct from their free-swimming, planktonic counterparts" (25). Biofilms, such as those formed by *P. aeruginosa*, benefit the bacterial community as demonstrated by patients with lung infections associated with cystic fibrosis patients. The benefit includes increased resistance to many antimicrobials (those administered clinically and naturally by other bacteria), protect from protozoan predation, and protection from host defenses (24, 25, 28).

Acute infection by *P. aeruginosa* gives rise to activity of the T3SS which increases injection of toxins directly into a host cystol. T3SS activation is dependent on bacterial-epithelial cell contact (32). *P. aeruginosa* T3SS secretes four effector proteins (ExoS, ExoT, ExoU and ExoY) into the host epithelium. These proteins encode for ADP-ribosyltransferase domains (ExoS and ExoT), adenylate cyclase (ExoY) and a cytotoxin phospholipase A2 (ExoU) which results in cell death due to the interruption of cellular

processes and the degradation of the cell membrane (33). The type VI secretion system (T6SS), which is associated with *P. aeruginosa*, is a specialized system that is associated with virulence, involved with biofilm formation and quorum-sensing of the bacteria which encodes this system (17). Most notably, this system is involved in the secretion of the toxin Tse2 which is involved in the arrest of growth in both eukaryotic and prokaryotic cells and has a high propensity for killing bacteria within its own environment (17). This type of system is a potentially important defense mechanism causing the reduction of competing bacterial species, while also functioning as an effective virulence factor promoting pathogenic process in a host. *P. aeruginosa* has numerous additional virulence factors including secreted proteases and hemolysins (37). Understanding how these virulence factors are regulated could aid in the development of novel treatments for *P. aeruginosa* infections.

Pseudomonas entomophila

Pseudomonas entomophila is a recently identified rod shaped, Gram-negative entomopathogenic (insect infecting) bacterium. This organism inhabits soil, aquatic and rhizosphere (soil associated with plant roots) environments (42, 45). Upon ingestion, *P. entomophila* infects adult and larval *Drosophila melanogaster*, causing destruction of gut epithelial cells and ultimately death. Though *D. melanogaster* is the most characterized host organism for *P. entomophila*, it is also pathogenic to multiple insect orders as well (42, 45).

With a genome size of 5.9 Mb, *P. entomophila* encodes a vast number of transporter-encoding genes, virulence genes, and remarkably, over 300 genes encoding transcriptional regulators. The large number of encoded regulatory genes suggests that this organism can readily adapt to fluctuating habitats (35, 45). *P. entomophila*'s genome also encodes a number of virulence factors including proteases, insecticidal toxins, and secreted hemolytic toxins (22, 42, 45). Though *P. entomophila* virulence is multi-factorial, the GacS/GacA two-component system is a master regulator of virulence in this organism. Importantly, it has been shown that GacS/GacA regulates the secreted protease AprA and hemolytic activity among Pseudomonads (22, 37, 42).

Following oral infection of *Drosophila* by *P. entomophila*, the fly innate immune system is triggered and activates multiple cellular and humoral responses, such as production of antimicrobial peptides (AMPs) through Toll and immune deficiency (Imd) pathways, phagocytosis of microorganisms in blood, and blood coagulation (22, 44). Gram-negative bacteria, such as *P. entomophila,* activate the Imd-pathway following recognition of diaminopimelic acid (DAP)-type peptidoglycans by the peptidoglycan recognition protein (PGRP), PGRP-LC, resulting in activation of AMPs (41, 44). The metalloprotease AprA aids *P. entomophila* in avoidance of the Imd-mediated immune response during an infection by degrading the AMP *Diptericin*, which is induced by the activation of the Imd-pathway (22, 41, 42). AprA has also been correlated with the cessation of food-uptake by adult and larval *Drosophila* upon ingestion of *P. entomophila*. The cessation of food-uptake and capability of inhibiting AMPs activity points to the importance of AprA in the pathogenicity and persistence of *P. entomophila* in the gut of *Drosophila* (22).

Secreted exotoxins, such as hemolysins, which damage blood cell membranes causing cells to rupture, are an additional virulence factor expressed in *P. entomophila* (42). A specific hemolytic factor was identified as a cyclic lipopeptide (CLP), a molecule produced by other Gram-negative bacteria (including other Pseudomonads). These toxins have antimicrobial, cytotoxic and surfactant properties. The secretion of hemolysin by this organism is linked to the production of the CLP entolysin. Entolysin is regulated by three nonribosomal peptide synthetase (NAPRs) *etlA, etlB,* and *etlC*, whose expression is regulated through the GacS/GacA two-component system (42). Unlike *P. aeruginosa*, a human pathogen, *P. entomophila* does not express many of the same virulence factors needed for mammalian infection such as a T3SS and pyocyanin production (45). Knowledge gained from studying the pathogenicity and regulatory mechanisms of virulence factors in organisms such as *P. entomophila*, could aide in the development of novel biocontrol techniques, such as innovative pesticide treatments which target specific TCS.

This study aims to answer the following questions: 1. what genes are regulated by a novel TCS found in both *P. entomophila* and *P. aeruginosa*? 2. Does this novel two-component system regulate similar genes in different bacteria species? The characterization of a novel two-component system in *P. entomophila* and *P. aeruginosa* could allow for a greater understanding of how genes are regulated specifically by this system and other TCS in general. Better understanding of gene regulation in *P. entomophila* could potentially allow for advancement in pest control. Understanding these regulatory mechanisms in human pathogens, such as *P. aeruginosa*, could also aid

in the development of novel treatments to reduce virulence of human pathogens. If

established as an important factor for virulence, then manipulation of this TCS could be

used to inhibit disease caused by pathogens found in health care facilities, especially if

they have a similar or identical two-component system as the one studied in *V.*

cholerae, *P. entomophila* and *P. aeruginosa.*

EXPERIMENTAL DESIGN

Deletion mutants were created that are missing either the sensor kinase (PSEEN1405 and PA3271) or response regulator (PSEEN4122 and PA3604) of a novel two-component system (TCS) in both *P. entomophila* or *P. aeruginosa*, respectively, through allelic exchange as described in the methods section below. *P. entomophila* and *P. aeruginosa* strains containing the desired gene deletion were verified via colony PCR analysis. After confirming the deletions of the genes encoding the TCS, fly survival experiments were conducted by a collaborator, Dr. Alexandra Purdy, at Amherst College to assess the virulence phenotypes of the mutants. Dr. Purdy's laboratory assessed fly death versus survival after ingestion of mutant strains compared to that of wild-type.

Potential genes regulated by the TCS of interest in *P. entomophila* and *P. aeruginosa* were identified by comparing genes that are homologous in *V. cholera* and were regulated in the CrbRS TCS. The goal is to find at least one or more homologous genes as TCS regulated targets for further regulation studies. Once potential TCS-regulated genes were identified in *P. entomophila* and/or *P. aeruginosa*, we bioinformaticly assessed which of these genes, played a role in virulence. To validate target gene regulation by this TCS, quantitative real time PCR (qRT-PCR) was used to measure specific transcript levels of genes. If a specific gene was expected to be regulated by this TCS, then we predicted that qRT-PCR analysis would indicate that expression of the transcript was up-regulated or down-regulated when comparing wild-type and TCS mutant strains. After

validation by qRT-PCR, we assessed if a particular gene is involved in virulence. Specific gene deletions were made through allelic exchange and virulence of phenotypes were analyzed through fly survival assays by our collaborator at Amherst College. If a particular gene is important for virulence, we predicted that a specific deletion in that gene would result in an avirulent strain when compared to the wild-type bacteria in a *Drosophila* model of infection. This portion of the study has been completed by our colleague at Amherst College.

After RNAseq analysis, the following genes were identified as being highly regulated by this TCS in *V. cholerae*: *acs-1* (VC0298), and *sssA* (VC2705). *acs-1* and *sssA* are down regulated by 39.86 fold and 13.31 fold, respectively. These genes have been shown to be important in acetate utilization in *V. cholerae.* Therefore, regulation of the homologs genes were the focus for further study in *P. entomophila* and *P. aeruginosa.* The homologous genes were identified as *acsA* (PSEEN3888 and PA0887), a homolog of *acs-1*; the genes PSEEN1455 and PA3234 were identified as a homolog of *sssA* in *P. entomophila* and *P. aeruginosa*, respectively. qRT-PCR was utilized to measure the transcript levels of *acsA*, PSEEN1455, and PA3234 in TCS mutant strains of *P. entomophila* and *P. aeruginosa*. If the function of this TCS is conserved among *V. cholerae*, *P. entomophila* and *P. aeruginosa*, then it would be expected that these homologous genes will be down regulated in TCS mutant strains of *P. entomophila* and *P. aeruginosa*.

Because AcsA is an acetyl-CoA synthase that is important for acetylating CoA, the role *acsA* plays in the utilization of acetate as a carbon source in *P. entomophila* and *P. aeruginosa* were investigated by assessing growth on minimal media containing acetate as a sole carbon source. If *acsA* is important for utilizing acetate as a carbon source, mutations in *P. entomophila* and *P. aeruginosa* that decrease *acsA* expression will result in strains that have inhibited growth on minimal media in which acetate is the sole source of carbon.

To confirm the target genes are being regulated by a TCS, mutant strains of *P. entomophila* and *P. aeruginosa* were constructed in which the receiver domain of the predicted sensor kinase (PSEEN1405 and PA3271) and response regulator (PSEEN4122 and PA3604) have been deleted. The receiver domains of TCS proteins are important for receiving the phosphate molecule in order to enable the phophotransfer event between the sensor kinase and response regulator which results in the activation or repression of regulated genes (13). The transcript levels of *acsA* was measured by qRT-PCR. If *acsA* is regulated by this TCS, receiver domain mutants should have decreased *acsA* expression. These results were expected to be identical to those found in the qRT-PCR analysis of original TCS mutants of *P. entomophila* and *P. aeruginosa*, performed earlier in this study. Finally, minimal media assays in which acetate was the sole carbon source (quantitative and qualitative) were utilized to determine if there are differences in growth rates of mutant strains of *P. entomophila* and *P. aeruginosa,* when compared to wild-type parent strains.

METHODS

Bacterial Strains, Fly Strains, and Media

Pseudomonas entomophila L48 and *Pseudomonas aeruginosa* PA01 were used as the wild-type parent strains for construction of mutant strains. All plasmid manipulations were performed in *Escherichia coli* DH5α. *E. coli* SM10 is a strain that expresses conjugation pili that is utilized to transfer plasmids into *P. entomophila* and *P. aeruginosa.* Mutant strains used during the course of the study included *P. entomophila* Δ*gacA* (negative control)*, P. entomophila* Δ*acsA* (positive control)*, P. entomophila* L48 Δ1405, *P. entomophila* L48 Δ4122*, P. entomophila* L48 Δ1405 receiver domain, *P. entomophila L48* Δ4122 receiver domain, *P. aeruginosa* PA01 Δ3271, *P. aeruginosa* PA01 Δ3604, *P. aeruginosa* PAO1 Δ3271 receiver domain, and *P. aeruginosa* PAO1 Δ3604 receiver domain. Fly survival experiments were performed utilizing the model host *Drosophila melanogaster yw,* where *D. melanogaster yw* were fed either wild-type or mutant strains previously listed. *P. entomophila* strains were cultured, and fly survival experiments were conducted using Luria Bertani Miller (LBM) media (Fisher Scientific) supplemented with a lower concentration of sodium (per liter; 15 g agar (as needed), 10 g casein peptone, 3 g sodium chloride and 5 g yeast extract). *P. aeruginosa* strains were cultured and fly survival experiments were conducted using Luria Bertani (LB) media (Fisher Scientific) (per liter; 15 g agar (as needed), 10 g tryptone, 10 g sodium chloride and 5 g yeast extract). When antibiotic selection was required the appropriate media was supplemented with 15 μg/ml gentamycin (for *E. coli*), or 30 μg/ml gentamycin (for

Pseudomonas spp.). Minimal media experiments utilized M63 Minimal Media (VWR)
(per liter; 15 g agar (as needed), 2 g $(NH_4)_2SO_4$, 34 g KH_2PO_4, 0.5 mg $FeSO_4.7H_2O$, pH
adjusted to 7.0 with NaOH, 1 ml $MgSO_{4)}$, isopropyl β-D-1-thiogalactopyranoside (IPTG)
(1 mM), gentamycin (30 μg/ml), and supplemented with acetate (5 mM) and glucose (5
mM) (as needed)). For experiments requiring expression of PA3604 or PSEEN4122 from
plasmids, IPTG was added to a final concentration of 1 mM to induce expression of
these genes.

Deletion Mutant Construction

Two-Component System Deletion Mutants

Two-component system protein deletions were constructed as follows: Mutants were
constructed from parental strain *P. entomophila* L48 by allelic exchange utilizing
Escherichia coli SM10. pEXG2 is an allelic exchange plasmid that can replicate in *E. coli*
and confers gentamycin resistance. pEXG2 plasmids containing desired deletion
constructs were conjugated into *P. entomophila* using *E. coli* SM10 essentially as
described in (5). Deletion constructs for the PSEEN1405 and PSEEN4122 genes were
generated by amplifying 1 kilobase regions flanking the gene to be deleted by PCR and
then splicing the flanking regions together by overlap extension PCR. The deletions were
in frame and contained the following linker sequences: 5'-GAATTC-3' and 5'-AAGCTT-3',
respectively. The resulting PCR products were cloned into plasmid pEXG2 (5), yielding
plasmids pEX-ΔPSEEN1405 and pEX-ΔPSEEN4122. These plasmids were then used to
create strains *P. entomophila* ΔPSEEN1405 and ΔPSEEN4122 containing in-frame
deletions of the PSEEN1405 and PSEEN4122 genes by allelic exchange. The allelic

exchange was performed essentially as described in (5). Target gene deletions were

confirmed by colony PCR. This protocol was utilized to construct deletion mutants

ΔPA3604 and ΔPA3271 from parental strain *P. aeruginosa* PA01.

Two-Component System Receiver Domain Deletion Mutants

Deletion mutants of *P. entomophila* and *P. aeruginosa* were constructed by deleting the

receiver domains of either the sensor kinase or response regulator of the two-

component system. Mutants were constructed from either parental strain *P.*

entomophila L48 or *P. aeruginosa* PA01 through allelic exchange, as previously

described. The *P. entomophila* mutants had deletions of nucleotides 3118-3462 in the

sensor kinase (PSEEN1405), or nucleotides 19-369 in the response regulator

(PSEEN4122). *P. aeruginosa* mutants had deletions of nucleotides 3124-3468 in the

sensor kinase (PA3271) or nucleotides 19-368 in the response regulator (PA3604).

Construction of Expression Plasmids Encoding the PSEEN 4122 and PA3604

The PSEEN4122 gene was amplified by PCR from *Pseudomonas entomophila* L48

chromosomal DNA. This gene was amplified between a 5' EcoRI restriction enzyme site

and a 3' HindIII restriction enzyme site. The PCR amplified PSEEN4122 gene was

digested with EcoRI and HindIII and ligated into the pPSV38 plasmid expression vector

digested with same restriction enzymes as described in (31). Plasmid p4122 expresses

the 4122 gene (PSEEN4122) from *P. entomophila* strain L48 under the control of the

IPTG-inducible lacUV5 promoter. Plasmid pPSV38 is a derivative of pPSV35 (31) that

contains the IPTG-inducible lacUV5 promoter flanked by two lac operators. pPSV38-

4122 drives expression of the 4122 gene and confers resistance to gentamycin. Identical

PCR processes were utilized for PA3604, though this gene was amplified by PCR from *Pseudomonas aeruginosa* PA01 chromosomal DNA.

Construction of Expression Plasmids Encoding PSEEN1405 and PA3271

We attempted to construct PSEEN1405 and PA3271 expression plasmids as described previously (*Construction of Expression Plasmids Encoding PSEEN4122 and PA3604*). After numerous attempts to amplify each gene by PCR in *P. entomophila* L48 and *P. aeruginosa* PAO1, respectively, we were unable to amplify the target sequence using multiple primer sets, PCR conditions, and DNA polymerase enzymes.

Complementation Experiments, Allowing for Plasmid Based Expression of Deleted Genes PSEEN4122 and PA3604

Complementation experiments were performed using wild-type and mutant strains. Wild-type or mutant strains of *P. entomophila* or *P. aeruginosa* were grown in 5 ml of LBM or LB media, respectively, overnight with shaking at 200 rpm at 30°C or 37°C respectively. Overnight cultures were used to make competent cells (6). Competent cells were transformed by centrifuging 1 mL of designated culture (wild-type or mutant) at maximum speed (~12000 g) (Brushless Microcentrifuge 260D, Denville Scientific) after which the supernatant was discarded. One mL of 300 mM sucrose was added to the cell pellet and vortexed to resuspend. This mixture was centrifuged at max speed for one minute, and supernatant was discarded. A second sucrose rinse was repeated as described above. The cell pellet was then resuspended in 200 μl of 300 mM sucrose.

Forty microliters of resuspended cells were transferred to an electroporation cuvette with a 0.2 cm gap (USA scientific). One microliter of either the pPSV38 plasmid, pPSV38-

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4122 plasmid or pPSV38-1405 plasmid at a starting concentration of approximately 80 ng/µl was added to each sample and then shocked in a MicroPulser (Bio-Rad) using one pulse at 2.5 kV. One milliliter of LBM-LS broth was added immediately after shocking the bacteria samples. Shocked samples were transferred to a 1.5 mL microcentrifuge tube and incubated at 30°C for 1-1.5 hours. Seventy microliters of desired sample was plated on an LBM-LS agar containing 30 µg/mL gentamycin and 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). *P. entomophila* cultures were incubated at 30°C for 24 hours and *P. aeruginosa* cultures were incubate at 37°C for 24 hours. A single colony from each *P. entomophila* culture was used to inoculate 5 mL of LBM-LS broth containing 30 µg/mL gentamycin and incubated for 24 hours, in a 30°C incubator with agitation at 200 rpm. *P. aeruginosa* cultures were used to inoculate 5 mL of LB broth containing 30 µg/mL gentamycin and incubated for 24 hours in a 37°C incubator with agitation at 200 rpm. Overnight cultures were used to inoculate initial cultures to a starting OD_{600nm} of 0.03. *P. entomophila* cultures were grown at 30°C on a shaker set at 200 rpm until an OD_{600nm} of 0.500 was achieved. *P. aeruginosa* were grown at 37°C on a shaker set at 200 rpm until an OD_{600nm} of 0.500 was achieved. After desired absorbance was reached, 10 mL of each culture was transferred to a 15 mL centrifuge tube and centrifuged at 3220 g (Eppendorf 5810 R) for 10 minutes at 4°C. Cells were then resuspended in 1 mL of RNAzol (Molecular Research Center) and incubated at 60°C for 10 minutes. RNA isolation was conducted as essentially described in (11). cDNA synthesis was conducted essentially as described in (48).

Colony PCR to confirm desired deletions in mutant strains

Single colonies of wild-type and mutant strains of *P. entomophila* or *P. aeruginosa* were

transferred and resuspended in 50 µl of nuclease free water in 1.5 mL microcentrifuge

tubes using sterilized pipette tips. A master mix was prepared with 2x Xtreme Buffer,

dNTPs (2 mM ea.), nuclease free water 10 pmol/µl primers and KOD Xtreme enzyme.

Five microliter of template DNA was mixed with 45 µl of master mix in a 0.2 mL PCR

tubes. PCR parameters: polymerase activation was achieved at 92°C for 2 minutes and

denaturing was conducted at 98°C for 30 seconds. Annealing was performed at the

lowest primer melting point (Tm°C) for 30 seconds and extension time was dependent

on amplicon size (1 minute per kilobase pair) at 68°C. Denaturing, annealing and

extension were repeated 32 times. PCR reactions were then electrophoresed on a 1%

agarose gel containing ethidium bromide for a visual confirmation of desired deletions

in each sample. A 10 kb and 1 kb ladder (Denville Scientific) were used to determine the

size of PCR product generated. Primers constructed for deletion confirmation can be

found in appendix I (Table 5).

qRT-PCR to Evaluate Transcript Abundance

RNA was isolated from wild-type and mutant strains of *P. entomophila* and *P.*

aeruginosa essentially as described in (11). Extracted RNA was used for cDNA synthesis

essential as described in (48). A Nano-Drop 200c spectrophotometer (Thermo Fischer)

was used to check the concentration and purity of the resulting cDNA isolated from

mutant and wild-type strains. The abundance of target transcripts relative to *clpX*

transcripts were demonstrated by quantitative real-time PCR using the iTaq SYBR Green

kit (Bio-Rad) and MyIQ Single-Color Real-Time PCR Detection System (Bio-Rad).

Transcript expression data were determined utilizing the ΔΔCt method as described in

(23). Experiments were performed in duplicate. Real-time PCR primers were tested for

amplification efficiency. Only those primers with a single amplicon and amplification

efficiencies greater than or equal to 90% with an R^2 values of 0.9 or higher were utilized

for quantification of gene expression. Primers utilized during this experiment are listed

in appendix I (Table 6).

Statistical Analysis of Target Transcript Levels

Statistical analysis of target transcript levels measured by qRT-PCR in this study were

performed with the Prism 3 program (GraphPad Software, Inc.). Statistical significance

was measured through one-way ANOVA utilizing the Bonferroni multiple comparison

test (29). Statistical analysis compared the mean cycle threshold (CT) values of qRT-PCR

transcript analysis, standard error of the mean (SEM) values and the total number of CT

values (n) per bacterial strain used in an individual qRT-PCR transcript analysis, where all

values were previously analyzed through PRISM prior to one-way ANOVA analysis (29).

Statistical significance was defined as those comparisons with a P-value less than 0.05

when comparing mutant strains to the wild-type parent strain of either *P. aeruginosa* or

P. entomophila. A P-value greater than 0.05 indicated that there was no statistical

significance between mutant strains compared to wild-type strain for either *P.*

aeruginosa or *P. entomophila*.

Fly Survival Assays (Performed at Amherst College)

Virulence of wild-type and mutant strains were tested using fly survival assays using

Drosophila melanogaster yw. In each assay, wild-type and mutant strains of *P.*

entomophila were cultured overnight in LBM-LS broth, and LBM-LS broth alone was

used as a control. Two milliliters of desired culture was added to a cellulose acetate

plug. Ten flies then ingested one of the following: a mutant strain containing a deletion

of either the gene PSEEN1405 or gene PSEEN4122, wild-type P. *entomophila* or control

media from a cellulose acetate plug. *D. melanogaster yw* survival was then monitored

twice a day for 48-60 hours. Three replications of survival assays were performed.

Survival at each time point was quantitated by averaging surviving flies and plotting data

using a log-based scale to compare virulence of wild-type and mutant strains. An

identical protocol was utilized for wild-type *P. aeruginosa* and its PA3271 and PA3604

deletion mutant strains. Fly survival assays were performed in the lab or Dr. Alexandra

Purdy at Amherst College.

Role of *acsA* in Acetate Metabolism Utilizing M63 Minimal Media

P. entomophila and *P. aeruginosa* two-component system mutants were analyzed for

their ability to utilize acetate as a sole carbon source using M63 minimal media (VWR)

supplemented with 5 mM sodium acetate (VWR), which we will refer to as M63-acetate

agar or broth. The M63-acetate agar or broth was utilized for qualitative and

quantitative assays, respectively. As a control, qualitative and quantitative assays were

also performed utilizing M63 minimal media supplemented with 5 mM sodium acetate

and 5 mM glucose (VWR), which will be identified as M63-acetate/glucose agar or broth.

Qualitative M63 Minimal Media Assay

Qualitative assays were performed as follows: wild-type *P. entomophila*, and *P. entomophila* mutants ΔPSEEN1405, ΔPSEEN4122 and receiver domain mutants were plated from frozen stock cultures on LBM low sodium agar and incubated for 24 hours at 30°C. One colony from each culture was inoculated into 5 mL of LBM low sodium broth and incubated at 30°C while shaking at 200 rpm for 24 hours. Cultures were used for transformations by electroporation with either an empty vector plasmid (pPSV38) or a plasmid expressing the response regulator (pPSV38-PSEEN4122) as previously described (6). Cultures were plated as a lawn on LBM low sodium agar supplemented with 30 μg/μL of gentamycin. Cultures were incubated at 30°C for 24 hours. A colony of each transformed culture was inoculated on either M63-acetate or M63-acetate/glucose agar supplemented with 1 mM IPTG or 30 μg/μL gentamycin. Cultures were incubated at 30°C for 96 hours. Wild-type *P. aeruginosa* and *P. aeruginosa* mutants ΔPA3271, ΔPA3604 and receiver domain mutants were also prepared as described above for *P. entomophila* with the following modifications: LB agar or LB agar supplemented with 30 μg/μL of gentamycin in place of LBM low sodium media, transformation with plasmid pPSV38 or a plasmid expressing the response regulator (pPSV38-PA3604) and were incubated at 37°C rather than 30°C. Any differences in growth on M63-acetate agar compared to M63-acetate/glucose agar were recorded

every 24 hours. All cultures were plated on a 150 mm X 15 mm agar plate (VWR). These experiments were repeated in duplicate.

Quantitative M63 Minimal Media Assay

Quantitative measures of the effect of this two-component system on growth rates in media containing acetate as the sole carbon source were performed as follows: Wild-type and mutant strains of *P. entomophila* listed under "Qualitative M63 Minimal Media Assay" were inoculated from frozen stock cultures on LBM low sodium media and grown at 30°C for 24 hours. Overnight cultures were made by inoculating one colony into 5 mL of LBM low sodium broth and incubated at 30°C, shaking at 200 rpm for 24 hours. These cells were utilized for transformations with either an empty plasmid vector (pPSV38) or a plasmid expressing the response regulator (pPSV38-PSEEN4122), as previously described (6) and plated on LBM low sodium agar supplemented with 30 μg/μL of gentamycin. After 24 hours, transformed cultures were placed at 4°C overnight. Two colonies of each transformed culture were inoculated into individual broth tubes containing 5 mL LBM low sodium broth supplemented with 30 μg/μL of gentamycin and incubated at 30°C, shaking at 200 rpm for 24 hours. Overnight cultures were then transferred into 15 mL centrifuge tubes and centrifuged (Eppendorf 5810 R) at 3220 g for 10 minutes at 4°C to pellet cells. Supernatant was removed with sterilized pipettes and cells were resuspended in 5 mL of M63-acetate or M63-acetate/glucose and centrifuged for an additional 10 minutes at 3220 g at 4°C. Supernatant was removed with sterilized pipettes and cells resuspended in 5 mL M63-acetate or M63-acetate/glucose. Initial cell densities were analyzed utilizing a UV-spectrophotometer 20

(Thermo Scientific) at an OD_{600nm}. A starting concentration at OD_{600nm} of 0.03 was calculated for inoculation into 50 mL of M63-acetate or M63-acetate/glucose broth supplemented with 1 mM IPTG and 30 μg/μl of gentamycin. Cultures were incubated at 30°C, shaking at 200 rpm for 26 hours. Assays using *P. aeruginosa* strains previously listed under "Qualitative M63 Minimal Media Assay" were performed similar to that of *P. entomophila* though it was grown utilizing LB media in place of LBM low sodium and was incubated at 37°C rather than 30°C. The increase in the optical density over time was measured for all cultures at 600 nm by spectrophotometry and recorded every 2 to 4 hours for the first 12 hours, and again at 22 and 26 hours. These experiments were performed in duplicate and any differences in growth rate between the wild-type and mutant strains were recorded.

Comparison of the novel two-component system, CrbRS, in *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Pseudomonas entomophila*

CrbRS is a novel two-component system recently identified within *V. cholerae* and is involved in the regulation of virulence within *Drosophila melanogaster* (14). To elucidate if this TCS is conserved among other Gram-negative organisms, a BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, 3) through NCBI was performed using default parameters to identify genes with highest similarity to the sensor kinase, CrbS, and the response regulator, CrbR. Two Pseudomonads, *P. aeruginosa* and *P. entomophila* were identified as having homologous genes to the CrbRS TCS. Homologous genes of CrbS and CrbR were identified as PA3271 and PA3604, respectively, in *P. aeruginosa.* The genes PSEEN1405 and PSEEN4122 of *P. entomophila* were identified as homologs to CrbS and CrbR, respectively. Sequence alignments were performed utilizing global alignment through ClustalW2 programing on the European Bioinformatics Institutes (ETI) website (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using default parameters (12, 21, 27). Protein sequences of either the sensor kinase, response regulator or receiver domain regions of either the sensor kinase or response regulator of each organism were aligned and percent identities were determined.

Through the alignment of the protein sequences encoding the sensor kinase of each organism (Figure 3) percent identities of either *P. aeruginosa* or *P. entomophila* to *V.*

cholerae showed 50% and 49% identity, respectively. A 79% identity was observed when comparing sensor kinase sequences from the two Pseudomonads to one another (Table 1). Alignment of protein sequences encoding the response regulator of *P. aeruginosa* and *P. entomophila* to *V. cholerae* (Figure 4) indicated a 50% and 52% identity, respectively. The comparison of response regulator sequences of the two *Pseduomonas* spp. yielded a percent identity of 82% (Table 2). The alignment of protein sequences encoding the receiver domain region of the sensor kinase (Figure 5) of *P. aeruginosa* and *P. entomophila* showed a percent identity of 44% each when compared to *V. cholerae,* and an 86% identity when compared to each other (Table 3). Finally, the alignment of the receiver domain portion of the response regulator region (Figure 6) for *P. aeruginosa* and *P. entomophila* yielded a percent identity of 50% and 49%, respectively, when compared to *V. cholerae*. When compared to one another, these Pseudomonads showed a percent identity of 86% for sequence encoding this region (Table 4). These results indicate that the genes encoding this TCS has high homology between the two studied *Pseudomonas* spp., but low homology when comparing either *P. aeruginosa* or *P. entomophila* to *V. cholerae*.

Analysis of Virulence Regulation through Fly Survival Assays and Measurements of Hemolytic or Protease Activity

<u>Effect of this novel two-component system on virulence of *P. aeruginosa* and *P. entomophila* in a fly model of infection</u>

CrbRS has been shown to alter the pathogenicity of *V. cholerae* within *Drosophila* through regulation of *acsA* expression*,* a gene which encodes an acetyl-CoA synthetase (14). Fly survival assays were performed in which *Drosophila* ingested either wild type or

Δ*crbS* and Δ*crbR* deletion mutant. The assay indicated a significant increase in fly

survival when ingesting Δ*crbS* or Δ*crbR* strains compared to wild-type *V. cholerae*, (14).

Homologs of this TCS were identified within two Pseudomonads, *P. aeruginosa* and *P.

entomophila*. Genes encoding sensor kinase of *P. aeruginosa* and *P. entomophila* were

determined to be PA3271 and PSEEN1405, respectively. The response regulator was

determined to be encoded by the genes PA3604 and PSEEN4122 for *P. aeruginosa* and

P. entomophila, respectively. We hypothesized that this system would play a role in

virulence of the identified Pseudomonads through the regulation of *acsA* expression,

similar to that observed in *V. cholerae*.

To determine the role this TCS plays within *P. aeruginosa* and *P. entomophila* virulence,

fly survival assays were performed by placing either wild-type or deletion mutant strains

containing deletions of the genes encoding either the sensor kinase or response

regulator of *P. aeruginosa* or *P. entomophila*. No significant difference in fly survival was

observed when comparing the Δ3271 and Δ3604 deletion mutants to the wild-type

strain of *P. aeruginosa* (Figure 7). Similarly, there was no significant difference in fly

survival observed when comparing Δ1405 and Δ4122 deletion mutants to the wild type

strain of *P. entomophila* (Figure 8). These results indicate that unlike the effects of CrbRS

seen in *V. cholerae,* this TCS does not play a role in the pathogenicity of either *P.

aeruginosa* or *P. entomophila.*

<u>Effect of this novel two-component system on hemolytic and protease activity in *P. aeruginosa* and *P. entomophila* in a fly model of infection</u>

Secreted hemolysins and proteases are two known virulence factors identified in both

Pseudomonas sp. being studied (37, 45). The production of hemolysins and protease can

be easily assayed on agar media. We predicted that if this TCS is involved in the

regulation of virulence in *P. aeruginosa* and *P. entomophila*, then hemolytic and

protease activity of either organism might be inhibited when either the sensor kinase or

response regulator was deleted. To determine if this TCS had an effect on the hemolytic

or protease activity of either *P. aeruginosa* or *P. entomophila*, overnight broth cultures

of wild-type, sensor kinase and response regulator deletion mutant strains were plated

on either blood agar or casein (milk protein) plates and incubated for 24 hours. No

significant difference in hemolytic activity on blood agar was observed when comparing

the haloing effect (β-hemolysis), of Δ3271 and Δ3604 deletion mutants to the wild-type

strain of *P. aeruginosa* (Figure 9A). When plated on casein plates, no significant

difference was seen in the haloing effect (proteolytic degradation of casein), in either

Δ3271 or Δ3604 when compared to wild type (Figure 9B). Similarly, no significant

differences were observed in hemolytic or protease activity of *P. entomophila* when

comparing either Δ1405 or Δ4122 to wild-type strains plated on blood agar (Figure 10A),

or casein plates (Figure 10B), respectively. These results indicate that this TCS is not

involved in the regulation of hemolytic and protease activity in either *P. aeruginosa* or *P.

entomophila*. These results are congruent with the identification of the master regulator

GacS/GacA on the regulation of virulence in *P. aeruginosa* and *P. entomophila* (37, 45).

Effect of this novel two-component system on the expression of *acsA* in *P. aeruginosa* and *P. entomophila*

As previously mentioned, CrbRS was shown to regulate *acsA* in *V. cholerae*. We

hypothesized that the homologs of this TCS within *P. aeruginosa* and *P. entomophila*

would regulate *acsA* expression as well. To determine this TCS's involvement in the

regulation of *acsA*, quantitative real-time PCR (qRT-PCR) was utilized to measure the

transcript abundance, or expression, of *acsA* within either the sensor kinase or response

regulator mutants of *P. aeruginosa* or *P. entomophila*, and compared to wild-type

strains of these organisms in each analysis. Relative abundance of *acsA* transcript levels

were normalized to the transcript levels of the housekeeping gene *clpX* for both

organisms. Preliminary qRT-PCR was performed utilizing wild-type *P. aeruginosa* or *P.

entomophila* with their respective sensor kinase or response regulator deletion mutants

to measure transcript abundance of *acsA* prior to the full exploration of this TCS's

involvement in the regulation of *acsA* expression (Appendix II). The deletion mutant

strains utilized for preliminary studies did not contain a complementation vector.

qRT-PCR analysis of *P. aeruginosa* showed about a 31-fold and a 16-fold decrease in

acsA expression within Δ3271 and Δ3604, respectively, compared to wild-type (Figure

11A-B). Surprisingly, both mutant strains were rescued with a complementation plasmid

expressing PA3604. Statistical analysis using one-way ANOVA with a Bonferonni's

multiple comparison test through Prism 3 programing (GraphPad Software, Inc., 29)

resulted in P-values less than 0.001 for all deletion mutant strains, indicating

significance in decreased *acsA* expression of either Δ3271 or Δ3604. The qRT-PCR

analysis of *acsA* in *P. entomophila* showed similar results to that of *P. aeruginosa*, where

Δ1405 and Δ4122 showed a 2-fold and 4-fold decrease, respectively, in the expression of

acsA compared to that of wild-type (Figure 12A-B). Both mutant strains were rescued

with a complementation plasmid expressing PSEEN4122. Surprisingly, statistical analysis

using one-way ANOVA with a Bonferonni's multiple comparison test (29) showed Δ1405

to have a P-value greater than 0.5 when compared with wild-type *P. entomophila*,

indicating no statistical significance in the decrease of *acsA* expression. Δ4122 showed

significance with a P-value less than 0.001. These results indicate that this TCS is

important in the regulation of expression for *acsA* only in *P. aeruginosa*, but not *P.*

entomophila.

To explore the role this TCS has in the regulation of *acsA*, transcript abundance was

measured through qRT-PCR in mutants where deletions were made in the receiver

domain region of either the sensor kinase or response regulator of the model

organisms. This region has been shown to be important for signaling between the

sensor kinase and response regulators of TCSs (36). Sensor kinase receiver domain

mutants are depicted as Δ3271RD and Δ1405RD for *P. aeruginosa* and *P. entomophila,*

respectively, and Δ3604RD and Δ4122RD for deletion of this region in the response

regulators. We predicted that if this TCS is involved in the regulation of *acsA*, then we

would see a decrease in gene expression in mutants containing a deletion of the

receiver domain of either the sensor kinase or response regulator similar to results

observed in full TCS gene deletions previously performed.

qRT-PCR analysis of the relative transcript abundance of *acsA* in Δ3271RD was,

surprisingly, 7-fold higher compared to wild-type *P. aeruginosa* (Figure 11C). In

correlation to results previously observed, Δ3604RD showed about a 15-fold decrease in the relative abundance of *acsA* compared to wild-type, where expression of *acsA* in a Δ3604RD mutant was rescued by complementation of PA3604 (Figure 11D). Similarly, qRT-PCR analysis in *P. entomophila* showed about a 2-fold increase and a 3-fold decrease in the relative abundance of *acsA* in Δ1405RD and Δ4122RD, respectively, compared to wild-type (Figure 12C-D). Expression of *acsA* was rescued in a Δ4122RD mutant through the complementation of PSEEN4122. Statistical analysis of all receiver domain deletion mutants of both *P. aeruginosa* and *P. entomophila* was performed using a one-way ANOVA with a Bonferonni's multiple comparison test (29). This analysis showed P-values less than 0.001, indicating statistical significance of qRT-PCR results. These results show that by deleting this TCS, or by deleting the receiver domain region of the response regulator, interruption of *acsA* expression occurs in *P. aeruginosa,* indicating this novel TCS is involved in the regulation of *acsA* in this organism. Statistical analysis of the qRT-PCR results indicates that interruption of *acsA* expression with statistical significance is seen only in the response regulator and receiver domain region of the response regulator in *P. entomophila.* Therefore, we cannot confidently determine that this TCS plays a role in regulating *acsA* in this organism. It is important to note, additional research should be conducted regarding the deletion of the sensor kinase within *P. entomophila*, and the receiver domain region of the sensor kinase within both Pseudomonads to further understand its importance as a signaling mechanism within the sensor kinase of this TCS.

Effect of this novel two-component system on the expression of acetate permease homologs in *P. aeruginosa* and *P. entomophila*.

In natural conditions, acetate is a charged molecule and cannot cross the bacterial cell membrane (39). Therefore, acetate permeases are proteins that are involved in the uptake and transport of acetate into the cell (10). Upon entering the cell, acetate can then be metabolized in other processes, such as conversion to acetyl Co-A, within an organism. RNAseq analysis within *Vibrio cholerae* indicated an acetate permease, *sssA*, had the second highest degree of regulation by this TCS (Unpublished Data, Alexandra Purdy Amherst College). A BLASTn (3) search produced three possible homologues to *sssA* in *P. aeruginosa* and *P. entomophila*, where only the gene with the highest similarity, or percent identity, within each organism was chosen for further study. Therefore, PA3234 and PSEEN1455 (*actP*) were selected from *P. aeruginosa* and *P. entomophila*, respectively for further analysis of this TCS's involvement in the regulation of acetate permease expression. We predicted that if this TCS plays a role in the regulation of acetate permeases, then we would see a decrease in expression of the identified *sssA* homologs in deletion mutants of *P. aeruginosa* and *P. entomophila.*

qRT-PCR transcript analysis was first performed utilizing deletion mutants containing full deletions of the sensor kinase or response regulator in *P. aeruginosa* and *P. entomophila*. Analysis of *P. aeruginosa* produced about a 16 and 8-fold decrease in relative abundance of PA3234 in Δ3271 and Δ3604, respectively, compared to wild-type (Figure 13A-B). Unlike the results observed in *P. aeruginosa*, qRT-PCR transcript analysis of *P. entomophila actP* revealed about a 175-fold decrease in relative abundance of *actP*

in Δ4122 compared to wild type (Figure 14A). Surprisingly, analysis in Δ1405 showed

about a 2-fold increase in the relative abundance of *actP* compared to wild-type (Figure

14B). The increase of *actP* transcript abundance in Δ1405 was not statistically

significant. Statistical analysis performed using the Bonferonni's multiple comparison

test through Prism 3 programing (GraphPad Software, Inc., 29) showed P-values less

than 0.001 for all deletion mutant strains in *P. aeruginosa* and in the response regulator

and receiver domain region of sensor kinase deletion mutants in *P. entomophila*,

indicating results of the qRT-PCR analysis are significant. P-values greater than 0.05

were seen in the sensor kinase and receiver domain region of the response regulator

deletion mutants in *P. entomophila*, indicating no significance in the qRT-PCR expression

results. These results indicate variation in the regulation of expression for the homologs

of *sssA* by this TCS when comparing *P. aeruginosa* to *P. entomophila.* Results from the

analysis performed with *P. aeruginosa* indicate this TCS plays a role in the regulation of

PA3234. In contrast, the variation of results in *P. entomophila* indicate that this TCS may

not play a role in the regulation of *actP.* Further research will be needed to investigate

this TCS's role in regulating expression of acetate permeases within Pseudomonads.

Though the acetate permase expression results in strains with deletions of either the

sensor kinase or response regulator varied, qRT-PCR analysis of the *sssA* homologs was

still performed with receiver domain deletion mutants of *P. aeruginosa* and *P.*

entomophila. qRT-PCR transcript analysis showed about a 9-fold increase and 3-fold

decrease in transcript abundance of PA3234 in Δ3271RD and Δ3604RD, respectively,

compared to wild-type (Figure 13C-D). The results seen in Δ3604RD strain were not

statistically significant. *P. entomophila* qRT-PCR analysis showed about a 4-fold increase

and 3-fold decrease in the transcript abundance of *actP* of Δ1405RD and Δ4122RD,

respectively, compared to wild-type, figure 14C-D. Again, statistical analysis of receiver

domain deletion mutants in both *P. aeruginosa* and *P. entomophila* using one-way

ANOVA with a Bonferonni's multiple comparison test (29) showed a P-value less than

0.001, indicating significance of qRT-PCR analysis results. These results confirm the

presence of variation in transcript abundance levels of *sssA* homologs when deleting

either the sensor kinase or response regulator of this TCS in *P. entomophila*, but not *P.*

aeruginosa. Due to the results, we cannot determine that this TCS is involved in the

regulation of *actP* within *P. entomophila.* However, we can conclude that this TCS is

involved in the regulation of PA3234 in *P. aeruginosa.* It is important to note that further

studies should be performed utilizing transcript abundance of not only the previously

selected *sssA* homologs, but also the additional two similar genes identified through the

BLASTn search.

**Growth effects of two-component system mutants on minimal media were acetate is
the sole carbon source.**

As we have shown, this TCS is important for the regulation of *acsA* in *P. aeruginosa* and

P. entomophila. Next we wanted to identify how this regulation affected these

organisms' ability to utilize acetate as a carbon source. We hypothesized that the

deletion of this TCS would reduce these organisms' ability to utilize acetate from the

environment. We predicted that if this TCS affected *P. aeruginosa* and *P. entomophila's*

ability to metabolize acetate, and then reduced growth, or no growth, would be

observed in mutants strains compared to wild-type strains on a minimal media where

acetate is the sole carbon source. To identify this TCS's role in regulating acetate

metabolism, a qualitative minimal media assay was performed. Wild-type and mutant

strains containing deletions of either the sensor kinase, response regulator or receiver

domain regions of either the sensor kinase or response regulator of *P. aeruginosa* or *P.

entomophila* were streaked on M63 minimal media plates. The M63 minimal media was

supplemented with either 5 mM acetate or 5 mM acetate and 5 mM glucose as

available carbon sources. *P. aeruginosa* showed reduced growth of some, but not all,

mutant strains on M63 minimal media supplemented with 5 mM acetate compared to

wild-type (Figure 15). Mutant strains Δ3271, Δ3604, and the mutant strain containing

Δ3604RD showed reduced growth, andΔ3271RD grew comparable to wild-type *P.

aeruginosa.* Δ3604 and Δ3604RD were rescued when PA3604 was complemented back

on a plasmid expression vector, producing comparable growth with wild-type.

Interestingly, Δ3271 was also rescued when PA3604 was complemented on a plasmid

expression vector. When grown on M63 minimal media supplemented with both 5 mM

acetate and 5 mM glucose (a secondary carbon source that all strains should be able to

metabolize), all mutant strains of *P. aeruginosa* grew comparable to wild-type (Figure

16). Similar results were produced when Δ1405, Δ4122, Δ1405RD, Δ4122RD and wild-

type *P. entomophila* were plated on M63 minimal media supplemented with 5 mM

acetate (Figure 17), or M63 minimal media supplemented with both 5 mM acetate and

5 mM glucose (Figure 18). All strains were rescued by complementation of PSEEN4122.

Interestingly, the additional mutant, Δ*acsA*, showed visible growth, though reduced

compared to wild-type *P. entomophila,* when plated on M63 minimal media supplemented with 5 mM acetate. These results indicate that by deleting this TCS and disrupting the expression of *acsA*, the ability to utilize acetate as a carbon source is significantly reduced in *P. aeruginosa* and *P. entomophila*. One possible reason for the observation of reduced growth, rather than absence of growth, could result from additional genes that encode additional acetate metabolism genes that may not be regulated by this TCS. It is shown that both *P. aeruginosa* and *P. entomophila* encode an additional acetyl-CoA synthetase gene, annotated as *acsB* and *acs-2*, respectively (46). Proteins encoded by these genes could be metabolizing acetate, though less efficiently than the primary AcsA pathway, resulting in the reduced growth. Regulation of these genes by this TCS should be explored.

To further study this TCS role in acetate metabolism and confirm results seen in the qualitative minimal media assays, quantitative growth assays were performed in which *P. aeruginosa* and *P. entomophila* strains were inoculated into 50 mL of M63 minimal media supplemented either solely with 5 mM acetate or 5 mM acetate and 5 mM glucose as sources of carbon. Growth assays utilizing wild-type and mutant strains of *P. aeruginosa* showed a significant decrease in growth of Δ3271, Δ3604 and Δ3604RD mutants compared to wild-type. All deletion strains were rescued with the complementation of PA3604. Similar to the results observed in the qualitative assay, Δ3271RD showed growth similar to that of wild-type *P. aeruginosa* (Figure 19). When grown in M63 minimal media supplemented with both 5 mM acetate and 5 mM glucose,

all mutant strains of *P. aeruginosa* showed no significant growth difference compared to the wild-type strain (Figure 20).

Growth curves of *P. entomophila* showed similar results to those of *P. aeruginosa*. In these growth curves, Δ1405, Δ4122 and Δ4122RD mutants showed a significant reduction in growth compared to wild-type and were rescued by the complementation of PSEEN4122. Δ1405RD grew comparable to wild-type *P. entomophila*, as was seen in the qualitative assays. In this growth assay, Δ*acsA* showed significant reduction in growth compared to wild-type, but differed from that of the other deletion mutants studied. When compared to Δ1405, Δ4122 and Δ4122RD, there was an increase in growth seen after 12 hours post inoculation in the Δ*acsA* mutant of *P. entomophila* (Figure 21). It is important to note that this phenomenon was further studied utilizing additional plate assays and colony PCR, which ruled out contamination and confirmed correct deletion of the Δ*acsA* gene (Appendix III). When grown in M63 minimal media supplemented with 5 mM acetate and 5 mM glucose, all mutant strains showed no significant difference in growth when compared to wild-type (Figure 22). These results confirm those observed in the qualitative assay, further strengthening the indication that this TCS plays an important role in the regulation of acetate metabolism in both *P. aeruginosa* and *P. entomophila.*

```
Pseudomonas_aeruginosa    MSLSIGLIAVVALVYMAILFAIAFYGDRRRTPLSPRLRAWVYSLSLAVYC 50
Pseduomonas_entomophila   MSLSSGLIAVVALAYMAIMFAIAFYGDRRSTPLPPRLRAWVYSLSLAVYC 50
Vibrio_cholerae           --MQGWLVIPVSLAYLGVLFLIAWYGDRQTRWLAN-WRPWIYSLSIAVYC 47
                            :.   *:   *:*.*:..:* **:****:    *.    *.*:****:****

Pseudomonas_aeruginosa    TSWTFFGAVGQATDQLWSFLPIYLGPVLLMLFAPWVLQKMIMISKQENIT 100
Pseduomonas_entomophila   TSWTFFGAVGQAAEQLWAFLPIYLGPMLLLIFAPWVVQKMVLISKQQNIT 100
Vibrio_cholerae           TSWTFYGTVGQASVNPWSFLPIYIAPILMFVFGWRVLARLILIAKREHIT 97
                          *****.*:****: . *:*****.*.*:::.*   *:  ::::*:*:::**

Pseudomonas_aeruginosa    SIADFIAARYGKSQALAVVVALICMVGVLPYIALQLKGIVLGVNLLIGSG 150
Pseduomonas_entomophila   SIADFIAARYGKSQTLAVVVALICLVGVLPYIALQLKGIVLGVNLLIGAN 150
Vibrio_cholerae           SLADFIAARYGKSQGLAVAVTVIAVLGILPYIALQLRGITMGLNIVAPDL 147
                          *.************ *** .*::*..:.*:*********:** .:*:*::

Pseudomonas_aeruginosa    ADSTGTRAQDTALIVSLVLALFTIVFGTRSLDVTEHHRGMVLAIAFESLV 200
Pseduomonas_entomophila   ADATGSRVQDTALVISLVLALFAIVFGTRSLDVTEHHRGMVLAIAFESLV 200
Vibrio_cholerae           MTVYGYQESHVSWFVVLALALFTMLFGTRHIDNTEHHRGMMMAIAFESIV 197
                           *  : ...:  .:  *.****.::**** :* ********:.*******.*

Pseudomonas_aeruginosa    KLTAFLAVGIFATFGLYDGFADLFSQARAAPQLADYWEETVHWPAMLLQT 250
Pseduomonas_entomophila   KLLAFLAVGAFVVFNLYDGFDDLFTQARQSVHLESYWQETINWPSMVVQT 250
Vibrio_cholerae           KLVAFLCVGVFIAYLAWR------TDGLELSTIAASTYQAPNWPTLLIHT 241
                          ** ***.** * .:  :        ::    :         :: :**::::*

Pseudomonas_aeruginosa    GVAMTAIMCLPRQFHVTVVENIEPRDLNLARWVFPIYLVLAALFVVPIAL 300
Pseduomonas_entomophila   AVAMMAIICLPRQFHVTVVENIEPQDMRLARWVFPLYLALAALFVVPIAL 300
Vibrio_cholerae           LLTMIAIVCLPRQFHTMVVENERPQDLHTARWLFPVYLLLMSLFVLPIAW 291
                           .:* **.*********.  **** .*.*:. ***.**:** * :***.***

Pseudomonas_aeruginosa    AGQLHLPAGVMPDSFVISLPLAEAHPALALLAFIGGASAATGMVIVASVA 350
Pseduomonas_entomophila   AGQMLLPGTVISDSFVISLPLAEAHPSLALLAFIGGASAATGMVIVEAVA 350
Vibrio_cholerae           TGQGLLPN-TSPDTFVISLPVFAGAHDIALLAFLGGTSAASGMVIVSTIA 340
                          :**  **     . .*.*******:.  .    :*****.**.***.***** ::*

Pseudomonas_aeruginosa    LSTMVSNDMLLPWLLRRREKDTERPFEAFRHWLLTVRRVSIAVILLLAYV 400
Pseduomonas_entomophila   LSTMVSNDMLLPWLLRR--NNAERPFEAFRHWMLSVRRVTIVVILLLAYV 398
Vibrio_cholerae           LAIMVSNDLVMPLLLRR-MRLSQRTHRHFSGLLVVIRRTLILLLLFGAWG 389
                          *: *****::* ****   . ::*... *   :: :**. * ::*: *:

Pseudomonas_aeruginosa    AYRLLGSSASLATIGQIAFAAIAQLGPAMIGALYWKQANRRGVFAGLAAG 450
Pseduomonas_entomophila   SYRLLGSTASLATIGQIAFAAVTQLTPAMLGALYWKQANRRGVFAGLAAG 448
Vibrio_cholerae           FYLVLDSIPSLSAIGFLSFSAITQFAPAIFGGMYWREGNRKGVYVGLAVG 439
                           * :*.* .**:.:** .:*.*:*: **..*.:**.:.**:.**:**.***.*

Pseudomonas_aeruginosa    SLLWAYTLVLPVVAKGLGWPLERIPGLTWLASNPFGLPIEPLTQGVLISL 500
Pseduomonas_entomophila   IFLWFYTLVLPITAHSLGWSLSLFPGLAWLHGNPLNLPITPLTQGVVLSL 498
Vibrio_cholerae           FTLWLITLMSATDMLAGDASNNVLLWVITPPDWLVALGLKSADWGMLLSV 489
                            **  **:.       .  . . :  :  :  .  . * :.  *:::*:

Pseudomonas_aeruginosa    VGNFALFGLVSVLSRTRVSEHWQASRFIGQEISQRQ-NSRFMLAVQVEDL 549
Pseduomonas_entomophila   AGNFTLFAWVSILSRTRVSEHWQAGRFIGQQTSARP-SSKPLLAVQIDDL 547
Vibrio_cholerae           TLNTLCYIGVSLVTRASLSERLQAAAFVGTPLPENENMSLYQSRVTVGEL 539
                          . *   :   **::.*: :**: **. *.*     .  *     * : .*

Pseudomonas_aeruginosa    LMLAARFVGEERARQSFIRFAYRQGKGFTPNQTANNEWIAHTERLLAGVL 599
Pseduomonas_entomophila   LKLAARFVGEERARQSFIRFAYRQGKGFNPNQNADGDWIEHTERLLAGVL 597
Vibrio_cholerae           EMLASRFVGRTRVRNAFAQYWSQQRETLLPNQQASSALIRHTERVLAGVF 589
                           **.****. *.*::* :: .  :* : : *** *.. * **** ****:
```

```
Pseudomonas_aeruginosa   GASSARAVVKAAIEGREMQVEDVVRIADEASEVLQFNRALLQGAIENITQ 649
Pseduomonas_entomophila  GTSSTRAVVKAAIEGREMQLEDVVRIADEASEVLQFNRALLQGAIENINQ 647
Vibrio_cholerae          GASSAKLVLTSALQGRKMQLEEVATIVDEASELYDFSRGLLQGAIEHIGQ 639
                         *:**:: *:.:*:.**:**:*:*. * *****: :*.*.********:* *

Pseudomonas_aeruginosa   GISVVDQSLRLVAWNHRYLELFEYPDGLIYVGRPIADIIRYNADRGLCGP 699
Pseduomonas_entomophila  GISVVDQNLRLVAWNRRYLELFNYPDGLISVGRPIADIIRYNAERGLCGP 697
Vibrio_cholerae          GIAVVDKQLRLVAWNQRYLELFEFPPGLIQVGRPIADVIRHNAQQGLCGP 689
                         **:***:.*******:******::* *** *******:**:**:.*****

Pseudomonas_aeruginosa   GDPDMHVAKRLYWMRQGTPHTSERLFPNGRVIELIGNPMPGGGFVMSFTD 749
Pseduomonas_entomophila  GEAQVHVARRLHWMRQGRAHSSERLFPNGRVIELIGNPMPGGGFVMSFTD 747
Vibrio_cholerae          GDPEDHVRRRVYHLEQGTSHTSSRVRADGRVIEVQGNPMPGGGFVMSFTD 739
                         *:.: ** :*:: :.** .*:*.*: .:*****: ***************

Pseudomonas_aeruginosa   ITAYREAEQGLKGANESLEQRVQERTQELSQLNQELSEAKSNAEAANQSK 799
Pseduomonas_entomophila  ITPFREAEQALRDSNESLEQRVAERTHELSQLNQALTEAKSRAEAVSQSK 797
Vibrio_cholerae          ITVFREAEQSLKMANETLEERVLQRTLELEKLNKQLVTATQRSERESQSK 789
                         ** :*****.*: :**:**:** :** **.:**: *  *....:*  :***

Pseudomonas_aeruginosa   TRFLAAVSHDLMQPLNAARLFSAALAHQDA-LPGEVRDLVQHLDSSLRSA 848
Pseduomonas_entomophila  TRFLAAVSHDLMQPLNAARLFSAALSQQAEGMNDEAQQLVQHMDSSLRSA 847
Vibrio_cholerae          SRFLAAVSHDLMQPLNAARLFASSLSEVAK--EAEVQKLAHHIESALGAA 837
                         :*******************::.*:.      *.:.*.:*::*:* :*

Pseudomonas_aeruginosa   EDLITDLLDISRLESGRVSAERNPFPLNNLYDALGVEFKALAQEQGLHFR 898
Pseduomonas_entomophila  EELISDLLDISRLENGKVTPDVKPFALNELFDTLGAEFKVLAAEKGLEFR 897
Vibrio_cholerae          EDLISDLLDISRLESGKIDLHPHSFAIMDVLSNLNAEFSALAAKQGVQFS 887
                         *.**.*********.*::  . :.*.: :: . *..**..** ::*:.*

Pseudomonas_aeruginosa   LRGSQLRVDSDIKLLRRILQNFLTNAFRYA-KGHVLLGVRRED-GYLRLE 946
Pseduomonas_entomophila  LRGSRLRVDSDMKLLRRILQNFLTNALRYG-KSPLLLGARRQG-ERLWLE 945
Vibrio_cholerae          LVPSLLWVHSDPKLLRRVIQNFLTNAFRYNPNGKVILGVRRVATGQVRID 937
                         *  * * *.** *****::*********.**  :.  ::**.**    : ::

Pseudomonas_aeruginosa   VWDQGPGIPPDKQKVIFEEFKRLDSHQTRAEKGLGLGLAIADRLCKVLGH 996
Pseduomonas_entomophila  VWDRGPGIADDKLQVIFQEFKRLDSHQTRAEKGLGLGLAIADGLCRVLGH 995
Vibrio_cholerae          VWDNGIGIEQDKQQEIFEEFNRGG--QVRSDQGLGLGLAISKGIAHVLGH 985
                         ***.* **  **. : **:**:* .  *.*::.*********:. ::.:****

Pseudomonas_aeruginosa   PLEVRSWPGKGSVFSVRVPLARQAPPALAN--GHKAEPAQALNGAQVLCV 1044
Pseduomonas_entomophila  QLEVRSWPGKGSVFRVSVPIARSPAPAVA---APVEQAGQPLAGLQVLCV 1042
Vibrio_cholerae          HISMRSWPGQGSVFSITLDRAQPMPSSLTQTVAMVNEKGSELQHLRVLCV 1035
                         :.:*****:****  :  *:  .:::   .     :  .. *   :****

Pseudomonas_aeruginosa   DNEDSILAGMNSLLSRWGCQVWTARSREECATLLDSEMRPQLALIDYHLD 1094
Pseduomonas_entomophila  DNEDSILIGMNSLLGRWGCQVWTARNQAECEALLAEGLRPHLALVDYHLD 1092
Vibrio_cholerae          DNEPDILVGMRDLLERWGCEVKTATDIHGSLKALEGQWIPDVILSDYRLD 1085
                         *** .** **..** ****.* **  .    *    *:.: * **.**

Pseudomonas_aeruginosa   DGETGTQLMAWLRTRLGEPVPGVVISADARPELVAEIHAAGLDYLSKPVK 1144
Pseduomonas_entomophila  DGETGTGLMGWLRARLGEPVPGVVISADGRNETIAMVHAAGLDYLAKPVK 1142
Vibrio_cholerae          NGRTGLEVLQQCRLRLGDCFAGVIISADRNPDILDGIESSGFRFMAKPIK 1135
                         :*.**  ::    * ***: ..**:****  : :  :.:.*: :::*:

Pseudomonas_aeruginosa   PAALRALLSRHLSLRS 1160
Pseduomonas_entomophila  PAALRALLNRHLSLVQ 1158
Vibrio_cholerae          PLKLRALLN-SLS--- 1147
                         * *****. **
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Figure 3: Sensor kinase protein sequence alignment of the model organisms utilized in this study. Alignments were constructed using ClustalW2 where an " * " indicates positions with conserved amino acid residues, a " : " indicates conservation between highly similar amino acid groups, and a " . " indicates conservation of weakly similar amino acid groups. The color of each amino acid residue indicates one of the following properties; small (small + hydrophobic (incl. aromatic – Y)) (Red), acidic (Blue), basic – H (Magenta), Hydroxyl + sulfhydryl +aminie + G (Green), unusual amino/imino acids etc. (Grey) (12, 21, 27).

Table 1: **Percent Identity of the Sensor Kinase Protein Sequences of the Model Organisms.** Percent identity of the sensor kinase protein sequence between the three model organisms utilized in this study. Score indicates the percent identity between the aligned sequences. Results retrieved from a ClustalW2 global alignment (12, 21, 27). Lengths are given as the total number of nucleotides (nt.).

SeqA	Name	Length (nt.)	SeqB	Name	Length (nt.)	Score
1	Pseudomonas_aeruginosa	1160	2	Pseduomonas_entomophila	1158	79.1
1	Pseudomonas_aeruginosa	1160	3	Vibrio_cholerae	1147	50.74
2	Pseduomonas_entomophila	1158	3	Vibrio_cholerae	1147	49.0

```
Pseudomonas_aeruginosa    -MASYEILIADDHPLFRSALHQALTLGLGPDVRLVEAASIAELEARLNEK 49
Pseduomonas_entomophila   -MATYEILIADDHPLFRSALRQAVTLGLGPDVRLVEVASIAELETSLTEK 49
Vibrio_cholerae           MDSTYTIIIADDHPLFRNALFQSVHMAIS-GANLLEADSLDALLTLLNKE 49
                           :.* *:.*********.** *:: :.:. ...*.*. *:  * : *.::

Pseudomonas_aeruginosa    ADWDLVLLDLNMPGAYGFSGLVLLRGQYPQVPVVMISAQEEAAVVQRSRE 99
Pseduomonas_entomophila   ADWDLVLLDLNMPGAYGFSGLVLLRGQYPQIPVVMVSAQEEAAVVVKSRE 99
Vibrio_cholerae           SEPDLLLLDLKMPGANGMSGLIHLRSEYPDLPIVVISASEEPSVVSQVKS 99
                          :: **:****:**** *:***: **.:**::*:*::**.**.:** : :.

Pseudomonas_aeruginosa    FGASGFIPKSSPLETLQQAVRAVLDGDTWWPPQDAAAEALSEEAKAASAG 149
Pseduomonas_entomophila   FGASGFIPKSSELGKIQEAVRNVLDGDVWWPPQAFEKVDVSAEAKAASEG 149
Vibrio_cholerae           HGAFGFIPKSSDMRSLIAALNQVLNGEPYFPAHLLVDGFVGND---LAEK 146
                          .** ******* : .:  *:. **:*: ::*.:       :. :     :

Pseudomonas_aeruginosa    LASLTPQQFRVLTMVCEGLLNKQIAYDLNVSEATVKAHVTAIFRKLNVRT 199
Pseduomonas_entomophila   LASLTPQQFRVLTMVCEGLLNKQIAYELNVSEATIKAHVTAIFRKLGVRT 199
Vibrio_cholerae           VAALTPQQYKVLGMLSDGLLNKQIAYELNVSEATIKAHMTAIFRKLGVKN 196
                          :*.:*****::** *:..:*********.*******.***.*******.*:.

Pseudomonas_aeruginosa    RTQAALLLQQMETVPASH 217
Pseduomonas_entomophila   RTQAALLLQQLESVSGS- 216
Vibrio_cholerae           RTQAVILLKEMSE----- 209
                          ****.:**:::.
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Figure 4: Response regulator protein sequence alignment of the model organisms utilized in this study. Alignments were constructed using ClustalW2 where an " * " indicates positions with conserved amino acid residues, a " : " indicates conservation between highly similar amino acid groups, and a " . " indicates conservation of weakly similar amino acid groups. The color of each amino acid residue indicates one of the following properties; small (small + hydrophobic (incl. aromatic – Y)) (Red), acidic (Blue), basic – H (Magenta), Hydroxyl + sulfhydryl +amine + G (Green), unusual amino/imino acids etc. (Grey) (12, 21, 27).

Table 2: **Percent Identity of the Response Regulator Protein Sequences of the Model Organisms.** Percent identity of the response regulator protein sequence between the three model organisms utilized in this study. Score indicates the percent identity between the aligned sequences. Results retrieved from a ClustalW2 global alignment (12, 21, 27). Lengths are given as the total number of nucleotides (nt.).

SeqA	Name	Length(nt.)	SeqB	Name	Length(nt.)	Score
1	Pseudomonas_aeruginosa	217	2	Pseduomonas_entomophila	216	84.26
1	Pseudomonas_aeruginosa	217	3	Vibrio_cholerae	209	50.72
2	Pseduomonas_entomophila	216	3	Vibrio_cholerae	209	52.15

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Pseudomonas_aeruginosa    LCVDNEDSILAGMNSLLSRWGCQVWTARSREECATLLDSEMRPQLALIDY 50
Pseduomonas_entomophila   LCVDNEDSILIGMNSLLGRWGCQVWTARNQAECEALLAEGLRPHLALVDY 50
Vibrio_cholerae           LCVDNEPDILVGMRDLLERWGCEVKTATDIHGSLKALEGQWIPDVILSDY 50
                          ******  .** **..** ****.* **  .    .    *     *.: * **

Pseudomonas_aeruginosa    HLDDGETGTQLMAWLRTRLGEPVPGVVISADARPELVAEIHAAGLDYLSK 100
Pseduomonas_entomophila   HLDDGETGTGLMGWLRARLGEPVPGVVISADGRNETIAMVHAAGLDYLAK 100
Vibrio_cholerae           RLDNGRTGLEVLQQCRLRLGDCFAGVIISADRNPDILDGIESSGFRFMAK 100
                          :**:*.**  ::    * ***: ..**:****  . : :  :.::*: :::*

Pseudomonas_aeruginosa    PVKPAALRALLSRHL  115
Pseduomonas_entomophila   PVKPAALRALLNRH-  114
Vibrio_cholerae           PIKPLKLRALLNSL-  114
                          *:**  *****.
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Figure 5: Sequence alignment of receiver domain protein sequences within the sensor kinase of our model organisms. Alignments were constructed using ClustalW2 where an " * " indicates positions with conserved amino acid residues, a " : " indicates conservation between highly similar amino acid groups, and a " . " indicates conservation of weakly similar amino acid groups. The color of each amino acid residue indicates one of the following properties; small (small + hydrophobic (incl. aromatic – Y)) (Red), acidic (Blue), basic – H (Magenta), Hydroxyl + sulfhydryl +amine + G (Green), unusual amino/imino acids etc. (Grey) (12, 21, 27).

Table 3: **Percent Identity of the Receiver Domain Protein Sequences in the Sensor Kinase of the Model Organisms.** Percent identity of the receiver domain protein sequences within the sensor kinase of the three model organisms utilized in this study. Score indicates the percent identity between the aligned sequences. Results retrieved from a ClustalW2 global alignment (12, 21, 27). Lengths are given as the total number of nucleotides (nt.).

SeqA	Name	Length(nt.)	SeqB	Name	Length(nt.)	Score
1	Pseudomonas_aeruginosa	115	2	Pseduomonas_entomophila	114	78.95
1	Pseudomonas_aeruginosa	115	3	Vibrio_cholerae	114	44.74
2	Pseduomonas_entomophila	114	3	Vibrio_cholerae	114	44.74

```
Pseudomonas_aeruginosa    LIADDHPLFRSALHQALTLGLGPDVRLVEAASIAELEARLNEKADWDLVL 50
Pseduomonas_entomophila   LIADDHPLFRSALRQAVTLGLGPDVRLVEVASIAELETSLTEKADWDLVL 50
Vibrio_cholerae           IIADDHPLFRNALFQSVHMAIS-GANLLEADSLDALLTLLNKESEPDLLL 49
                          :**********.** *:: :.:. ...*:*. *:   * : *.:::: **:*

Pseudomonas_aeruginosa    LDLNMPGAYGFSGLVLLRGQYPQVPVVMISAQEEAAVVQRSREFGASGFI 100
Pseduomonas_entomophila   LDLNMPGAYGFSGLVLLRGQYPQIPVVMVSAQEEAAVVVKSREFGASGFI 100
Vibrio_cholerae           LDLKMPGANGMSGLIHLRSEYPDLPIVVISASEEPSVVSQVKSHGAFGFI 99
                          ***.**** *:***: **.:**:.:*:*:*::**.**..:** : :..** ***

Pseudomonas_aeruginosa    PKSSPLETLQQAVRAVL---------- 117
Pseduomonas_entomophila   PKSSELGKIQEAVRNVLDGDVWWPPQA 127
Vibrio_cholerae           PKSSDMRSLIAALNQVL---------- 116
                          ****  : .:   *:.  **
```

Figure 6: Sequence alignment of receiver domain protein sequences within the response regulator of our model organisms. Alignments were constructed using ClustalW2 where an " * " indicates positions with conserved amino acid residues, a " : " indicates conservation between highly similar amino acid groups, and a " . " indicates conservation of weakly similar amino acid groups. The color of each amino acid residue indicates one of the following properties; small (small + hydrophobic (incl. aromatic – Y)) (Red), acidic (Blue), basic – H (Magenta), Hydroxyl + sulfhydryl +amine + G (Green), unusual amino/imino acids etc. (Grey) (12, 21, 27).

Table 4: **Percent Identity of the Receiver Domain Protein Sequences in the Response Regulator of the Model Organisms.** Percent identity of the receiver domain protein sequences within the response regulator of the three model organisms utilized in this study. Score indicates the percent identity between the aligned sequences. Results retrieved from a ClustalW2 global alignment (12, 21, 27). Lengths are given as the total number of nucleotides (nt.).

SeqA	Name	Length(nt.)	SeqB	Name	Length(nt.)	Score
1	Pseudomonas_aeruginosa	117	2	Pseduomonas_entomophila	127	86.32
1	Pseudomonas_aeruginosa	117	3	Vibrio_cholerae	116	50.86
2	Pseduomonas_entomophila	127	3	Vibrio_cholerae	116	49.14

Fly survival Assay with Comparing Two-Component System Mutants of *Pseudomonas aeruginosa*
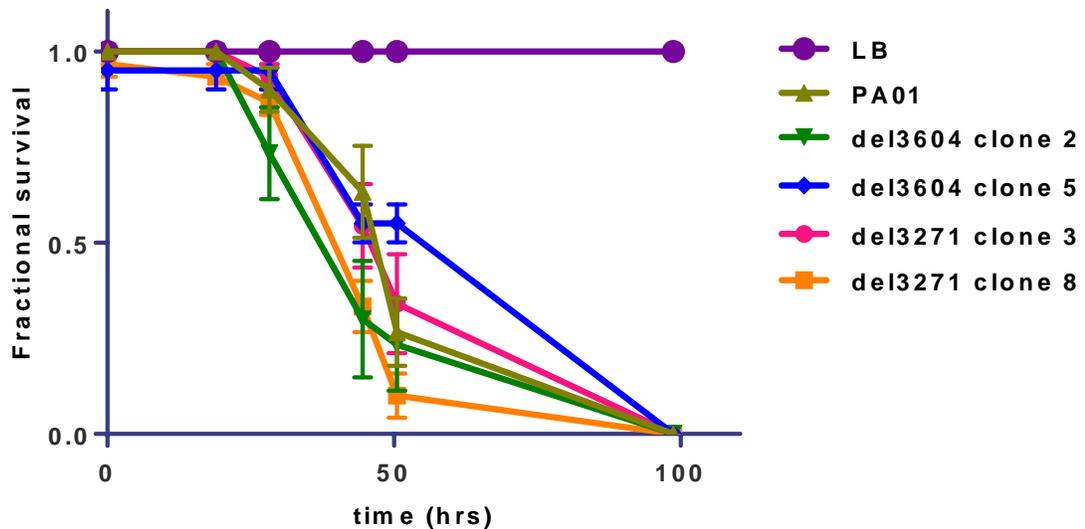
Figure 7: Fly survival curves after ingestion of wild-type (PAO1) or two-component system mutants containing a deletion of either the sensor kinase (PA3271) or response regulator (PA3604) of *P. aeruginosa* over a 68 hour time period. No significant difference in fly survival was observed in either of the two-component system deletion mutants compared to wild-type *P. aeruginosa.*
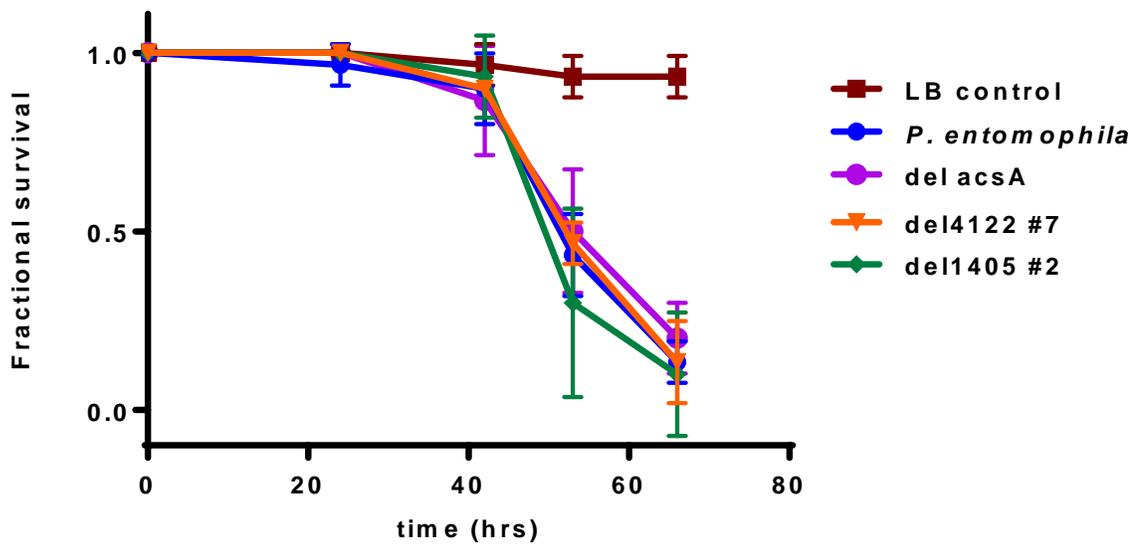
Figure 8: Fly survival curves after ingestion of wild-type *P. entomophila* or two-component system mutants containing a deletion of either the sensor kinase (PSEEN1405) or response regulator (PSEEN4122) over a 68 hour time period. No significant difference in fly survival was observed in either of the two-component system deletion mutants compared to wild-type *P. entomophila*.
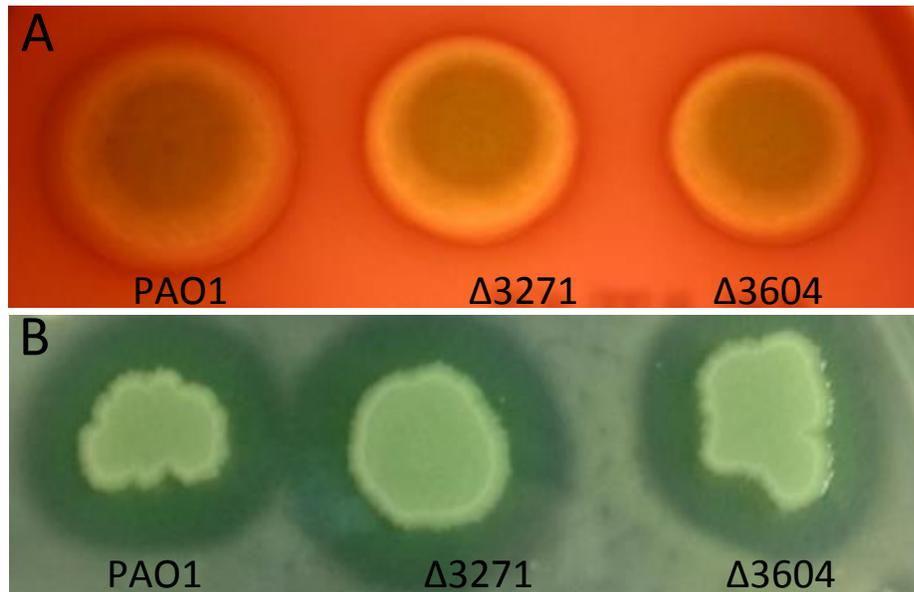
Figure 9: Hemolysin production (A) and AprA protease production (B) of wild-type or mutant strains of *P. aeruginosa* containing deletions of either the sensor kinase (PA3271) or response regulator (PA3604) homologs of the CrbRS TCS, plated on blood agar or LB agar + 5% milk, respectively.
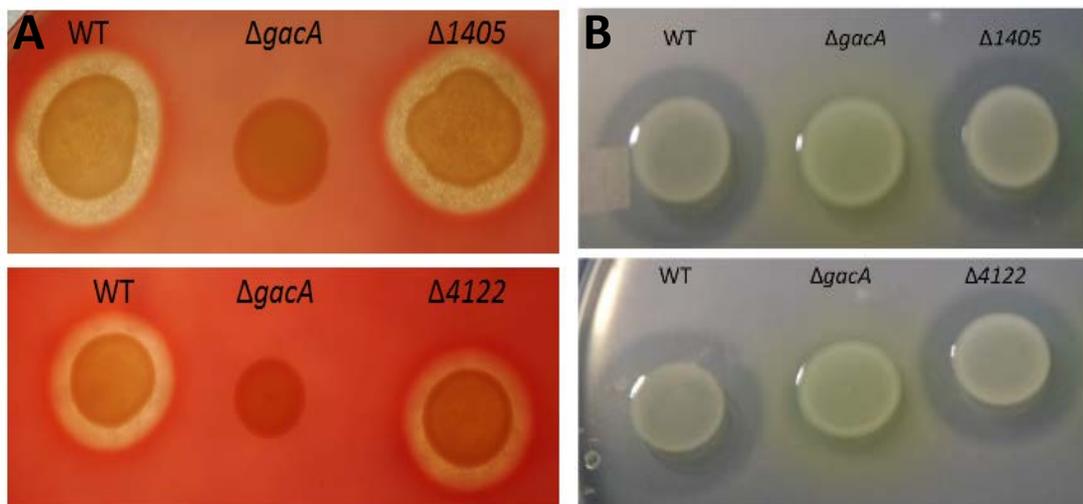
Figure 10: Hemolysin production (A) and AprA protease production (B) of wild-type or mutant strains of *P. entomophila* containing deletions of either the sensor kinase (PSEEN1405) or response regulator (PSEEN4122) homologs of the CrbRS TCS. A *P. entomophila* strain containing a deletion in the response regulator of the GacAS TCS was utilized as a positive control. Strains were plated on blood agar or LBM low sodium agar + 5% milk to assay for hemolytic or AprA protease activity, respectively.
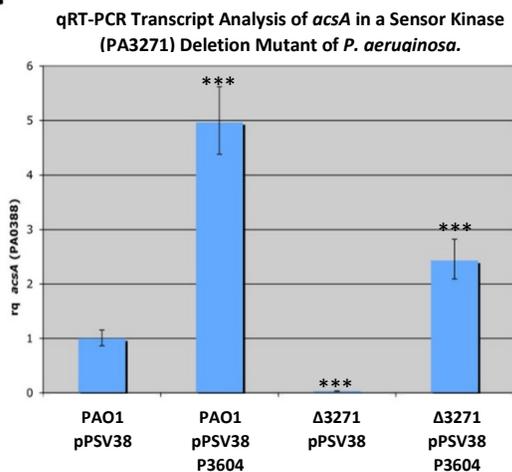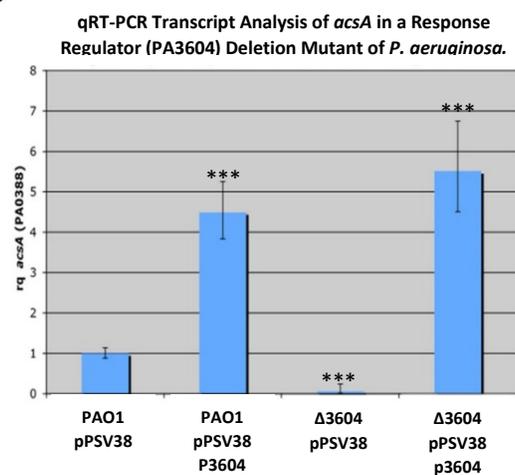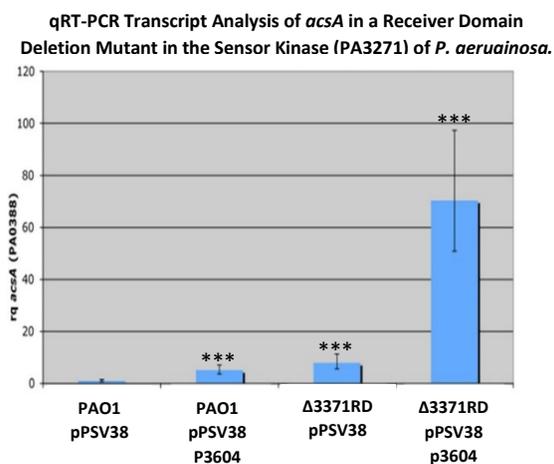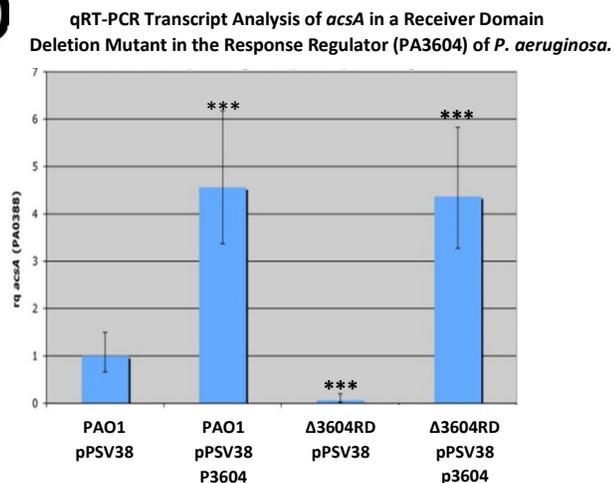
Figure 11: qRT-PCR transcript analysis of *acsA* in a sensor kinase (A), response regulator (B), and receiver domain mutants of either the sensor kinase (C) or response regulator (D) in *Pseudomonas aeruginosa*. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PA3604 complemented back into the strain are denoted by pPSV38_p3604". A decrease in expression of *acsA* was seen in the deletion mutants of the sensor kinase, response regulator and receiver domain region of the response regulator compared to wild-type (PAO1). An increase in expression of *acsA* was seen in the receiver domain mutant of the sensor kinase. Statistical significance (P-value) was determined comparing results of each mutant strain to the wild-type strain. One asterisk (*) denotes a P-value less than 0.05, two asterisks (**) denotes a P-value less than 0.01, and three asterisk (***) denotes a P-value less than 0.001.

A

qRT-PCR Transcript Analysis of *acsA* in a Sensor Kinase (PSEEN1405) Deletion Mutant of *P. entomophila.*

B

qRT-PCR Transcript Analysis of *acsA* in a Response Regulator (PSEEN4122) Deletion Mutant of *P. entomophila.*

C

qRT-PCR Transcript Analysis of *acsA* in a Receiver Domain Deletion Mutant in the Sensor Kinase (PSEEN1405) of *P. entomophila.*

D

qRT-PCR Transcript Analysis of *acsA* in a Receiver Domain Deletion Mutant in the Response Regulator (PSEEN4122) of *P. entomophila.*

Figure 12: qRT-PCR transcript analysis of *acsA* in a sensor kinase (A), response regulator* (B), and receiver domain mutants of either the sensor kinase (C) or response regulator (D) in *Pseudomonas entomophila*. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PSEE4122 complemented back into the strain are denoted by pPSV38_p4122". A decrease in expression of *acsA* was seen in the deletion mutants of the sensor kinase, response regulator and receiver domain region of the response regulator compared to wild-type (L48). An increase in expression of *acsA* was seen in the receiver domain mutant of the sensor kinase. Statistical significance (P-value) was determined comparing results of each mutant strain to the wild-type strain. One asterisk (*) denotes a P-value less than 0.05, two asterisks (**) denotes a P-value less than 0.01, and three asterisk (***) denotes a P-value less than 0.001.

*This experiment was performed in duplicate on two separate occasions

Figure 13: qRT-PCR transcript analysis of PA3234 in a sensor kinase (A), response regulator (B), and receiver domain mutants of either the sensor kinase (C) or response regulator (D) in *Pseudomonas aeruginosa*. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PA3604 complemented back into the strain are denoted by pPSV38_p3604". A decrease in expression of *acsA* was seen in the deletion mutants of the sensor kinase, response regulator and receiver domain region of the response regulator compared to wild-type (PAO1). An increase in expression of PA3234 was seen in the receiver domain mutant of the sensor kinase compared to wild-type. Because of overlapping error bars, the results of the receiver domain deletion mutant in the response regulator were not statistically significant. Statistical significance (P-value) was determined comparing results of each mutant strain to the wild-type strain. One asterisk (*) denotes a P-value less than 0.05, two asterisks (**) denotes a P-value less than 0.01, and three asterisk (***) denotes a P-value less than 0.001.
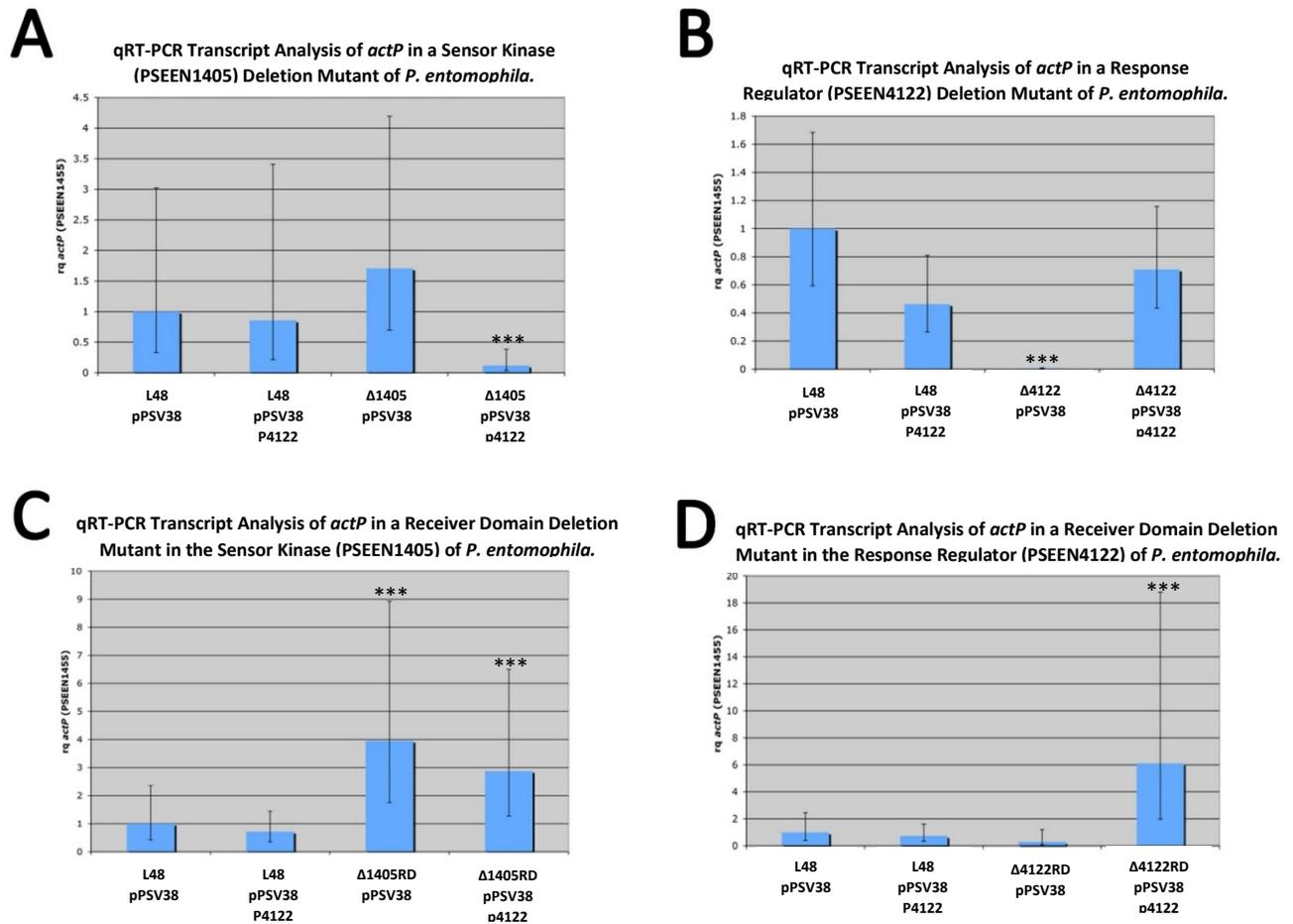
Figure 14: qRT-PCR transcript analysis of *actP* in a sensor kinase (A), response regulator (B), and receiver domain mutants of either the sensor kinase (C) or response regulator (D) in *Pseudomonas entomophila*. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PSEE4122 complemented back into the strain are denoted by pPSV38_p4122". A decrease in expression of *acsA* was seen in the deletion mutants of the sensor kinase, response regulator and receiver domain region of the response regulator compared to wild-type. An increase in expression of *accsA* was seen in the receiver domain mutant of the sensor kinase compared to wild-type (L48). Because of overlapping error bars, the results of the receiver domain deletion mutants are determined to be insignificant. Statistical significance (P-value) was determined comparing results of each mutant strain to the wild-type strain. One asterisk (*) denotes a P-value less than 0.05, two asterisks (**) denotes a P-value less than 0.01, and three asterisk (***) denotes a P-value less than 0.001.
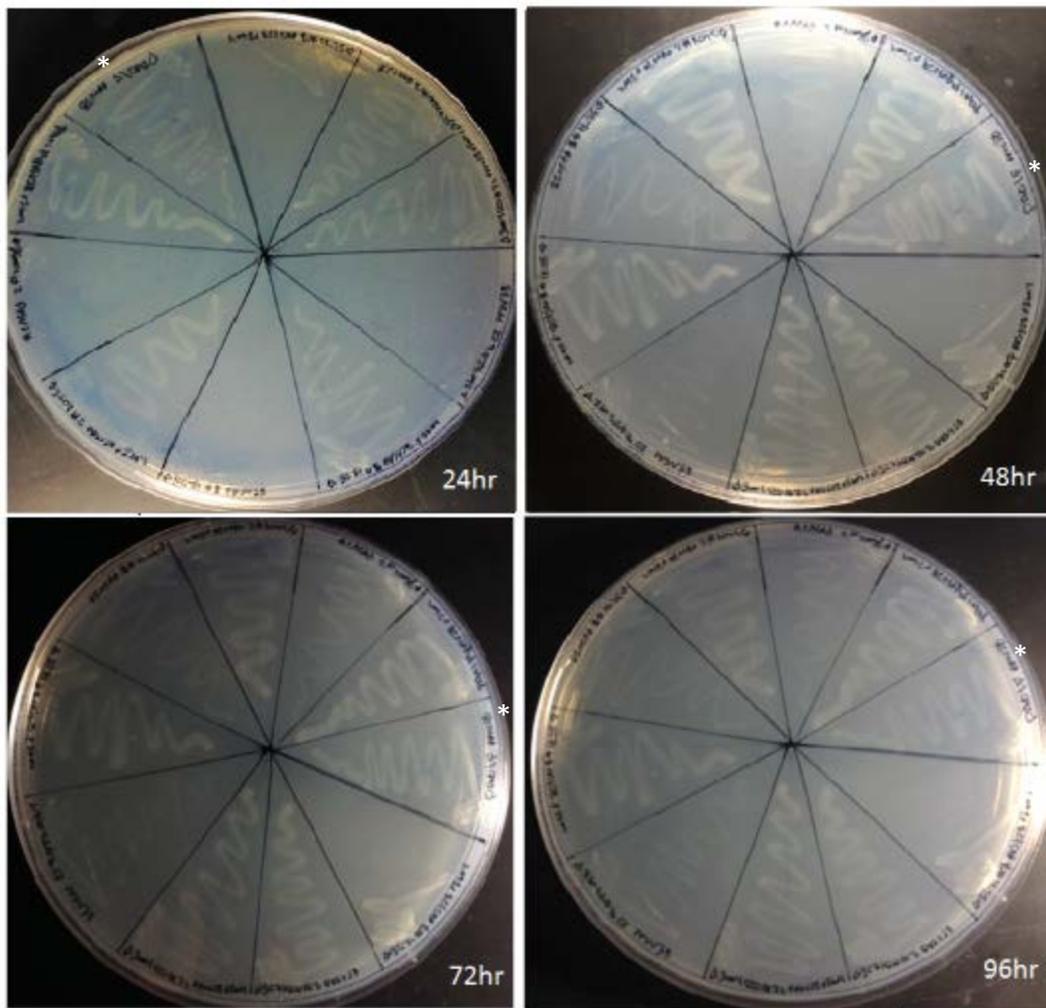
Figure 15: Qualitative growth assay of *Pseudomonas aeruginosa* on M63 minimal media supplemented with 5 mM acetate, 1 mM IPTG and 30 μg/μL Gentamycin. Observations were recorded post-inoculation over a 96 hour period. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PA3604 complemented back into the strain are denoted by pPSV38_p3604". The "*" marks wild-type *P. aeruginosa* pPSV38. Following strains were plated counterclockwise from *P. aeruginosa* pPSV38; *P. aeruginosa* pPSV38_p3604, Δ33271 pPSV38, Δ3271 pPSV38_p3604, Δ3604 pPSV38, Δ3604 pPSV38_p3604, Δ3271RD pPSV38, Δ3271RD pPSV38_p3604, Δ3604RD pPSV38, and Δ3604RD pPSV38_p3604.

Figure 16: Qualitative growth assay of *Pseudomonas entomophila* on M63 minimal media supplemented with 5 mM acetate, 1 mM IPTG and 30 μg/μL Gentamycin. Observations were recorded post-inoculation over a 96 hour period. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PSEE4122 complemented back into the strain are denoted by pPSV38_p4122". pPSV38_p4122". The "*" marks wild-type *P. entomophila* pPSV38. Following strains were plated counterclockwise from *P. entomophila* pPSV38; *P. entomophila* pPSV38_p4122, Δ*acsA* pPSV38 (positive control), Δ1405 pPSV38, Δ1405 pPSV38_p4122, Δ4122 pPSV38, Δ4122 pPSV38_p4122, Δ1405RD pPSV38, Δ1405RD pPSV38_p4122, Δ4122RD pPSV38, and Δ4122RD pPSV38_p4122.

Figure 17: Qualitative growth assay of *Pseudomonas aeruginosa* on M63 minimal media supplemented with 5 mM acetate, 5 mM glucose, 1 mM IPTG and 30 µg/µL Gentamycin. Observations were recorded post-inoculation over a 96 hour period. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PA3604 complemented back into the strain are denoted by pPSV38_p3604". The "*" marks wild-type *P. aeruginosa* pPSV38. Following strains were plated counterclockwise from *P. aeruginosa* pPSV38; *P. aeruginosa* pPSV38_p3604, Δ33271 pPSV38, Δ3271 pPSV38_p3604, Δ3604 pPSV38, Δ3604 pPSV38_p3604, Δ3271RD pPSV38, Δ3271RD pPSV38_p3604, Δ3604RD pPSV38, and Δ3604RD pPSV38_p3604.

Figure 18: Qualitative growth assay of *Pseudomonas entomophila* on M63 minimal media supplemented with 5 mM acetate, 5 mM glucose, 1 mM IPTG and 30 µg/µL Gentamycin. Observations were recorded post-inoculation over a 96 hour period. Strains with an inserted empty vector plasmid are denoted by "pPSV38" and strains with the response regulator PSEE4122 complemented back into the strain are denoted by pPSV38_p4122". The "*" marks wild-type *P. entomophila* pPSV38. Following strains were plated counterclockwise from *P. entomophila* pPSV38; *P. entomophila* pPSV38_p4122, Δ*acsA* pPSV38 (positive control), Δ1405 pPSV38, Δ1405 pPSV38_p4122, Δ4122 pPSV38, Δ4122 pPSV38_p4122, Δ1405RD pPSV38, Δ1405RD pPSV38_p4122, Δ4122RD pPSV38, and Δ4122RD pPSV38_p4122.

Figure 19: Growth assays comparing deletion mutant strains to wild-type *P. aeruginosa*. Strains were inoculated in M63 minimal media supplemented with 5mM acetate as the sole carbon source. Growth was observed over a 28 hour period, where cell density was measured at an OD_{600nm} with a UVSpec20 spectrophotometer. Inserted empty vector plasmids are denoted by "pPSV38," and plasmid complementation vector expressing PA3604 are denoted by "p3604". Reduction in growth is seen in the following deletion mutants when compared to wild-type; del3271 #8 pPSV38, del3604 #2 pPSV38, and del3604 rec dom #22 pPSV38.

Figure 20: Growth assays comparing deletion mutant strains to wild-type *P. entomophila*. Strains were inoculated in M63 minimal media supplemented with 5mM acetate as the sole carbon source. Growth was measured over a 28 hour period, where cell density was measured at an OD_{600nm} with a UVSpec20 spectrophotometer. Inserted empty vector plasmids are denoted by "pPSV38," and plasmid complementation vector expressing PSEEN4122 are denoted by "p4122". Reduction in growth is seen in the following deletion mutants when compared to wild-type; del1405 #2 pPSV38, del4122 #3 pPSV38, and del4122 rec dom #4 pPSV38. del*acsA* #2 pPSV38 shows an initial reduction in growth until about 12 hours post inoculation.

Growth Assay Comparing Deletion Mutants to Wild-Type *P. aeruginosa* grown in M63 Minimal Media Supplemented with 5mM acetate and 5mM glucose

Legend:
- *P. aeruginosa* pPSV38
- *P. aeruginosa* p3604
- del3271 *#8 pPSV*38
- del3271 *#8 p3604*
- del3604 #2 pPSV38
- del3604 #2 p3604
- del3271 rec dom #7 pPSV38
- del3271 *rec dom* #7 p3604
- del3604 *rec dom* #22 pPSV38
- del3604 *rec dom* #22 p3604
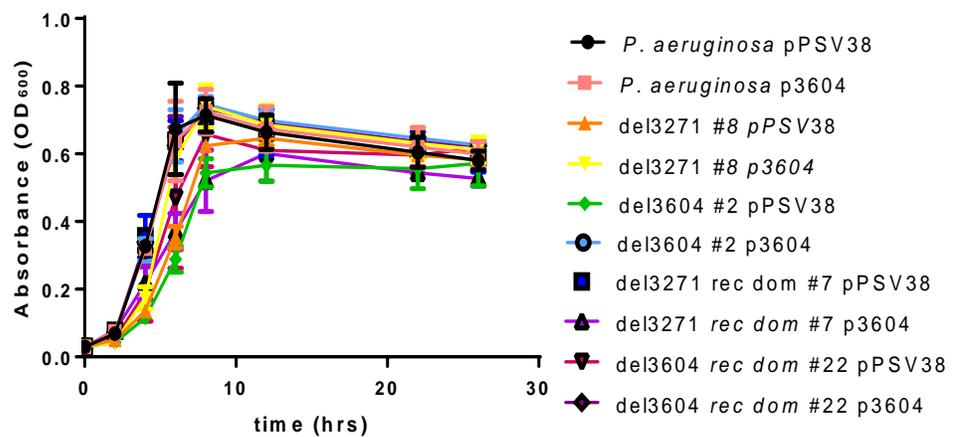
Figure 21: Growth assays comparing deletion mutant strains to wild-type *P. aeruginosa*. Strains were inoculated in M63 minimal media supplemented with 5mM acetate and 5mM glucose as carbon sources. Growth was measured over a 28 hour period, where cell density was measured at an OD_{600nm} with a UVSpec20 spectrophotometer. Inserted empty vector plasmids are denoted by "pPSV38," and a plasmid complementation vector expressing PA3604 are denoted by "p3604". All mutant strains grew comparable to wild-type *P. aeruginosa.*

Growth Assay Comparing Deletion Mutants to Wild-Type *P. entomophila* grown in M63 Minimal Media supplemented with 5mM acetate and 5mM glucose

Legend:
- *P.entomophila* pPSV38
- *P.entomophila* p4122
- del*acsA* #2 pPSV38
- del1405 #2 pPSV38
- del1405 #2 p4122
- del4122 #3 pPSV38
- del4122 #3 p4122
- del1405 rec dom #2 pPSV38
- del1405 rec dom #2 p4122
- del4122 rec dom #4 pPSV38
- del4122 rec dom #4 p4122
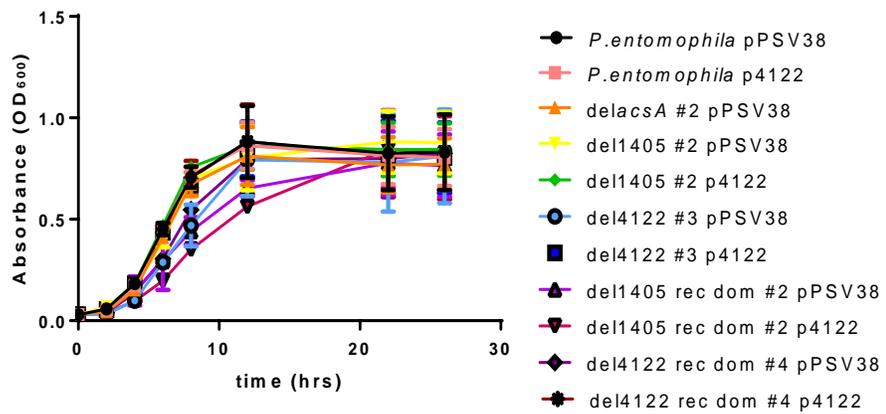
Figure 22: Growth assays comparing deletion mutant strains to wild-type *P. entomophila*. Strains were inoculated in M63 minimal media supplemented with 5mM acetate and 5mM glucose as carbon sources. Growth was measured over a 28 hour period, where cell density was measured at an OD_{600nm} with a UVSpec20 spectrophotometer. Inserted empty vector plasmids are denoted by "pPSV38," and plasmid complementation vector expressing PSEEN4122 are denoted by "p4122". All mutant strains grew comparable to wild-type *P. entomophila*.

DISCUSSION

This study described a novel two-component system, homologous to the recently

identified CrbRS system (14) of *V. cholerae*, within two *Pseudomonas spp.* which is

involved in acetate utilization through a phenomenon known as the acetate switch. This

"switch" has been defined as the moment where acetate dissimilation (excretion)

equals acetate assimilation (uptake) within an organism, and can only be functional

when expression of molecular machinery involved in the "switch" is activated (47). The

occurrence of this "switch" has been shown to occur when an organism depletes its

preferred, acetogenic carbon sources (i.g. D-glucose or L-serine) within its environment

during its transition into stationary phase (47). As CrbRS was shown to be involved in

virulence of *V. cholerae* by regulating the assimilation of acetate through a phenomenon

known as the acetate switch (14), it was thought that similar functionality would be

seen in the homologous systems within *P. aeruginosa* and *P. entomophila*.

Preliminary experiments in this study utilized qualitative real-time PCR to elucidate if

this homologous system was involved in the regulation of *acsA*, a gene known to encode

an acetyl-CoA synthetase in many organisms including those used in this study (37, 45,

47). An acetyl-CoA synthetase is a key enzyme involved in the acetate switch, where it

converts acetate into acetyl-CoA (47). This gene can be utilized for studying this TCS's

involvement in the regulation of acetate metabolism within *P. aeruginosa* and *P.

entomophila*. Experiments in this study indicated that deletion of this TCS in *P.

aeruginosa* resulted in the down regulation of *acsA*, indicating that this TCS regulates

the expression of *acsA*. In contrast, varying results of qRT-PCR analysis combined with statistical analysis, but not results of the growth assay experiments, indicated this TCS may not be involved in the regulation of *acsA* in *P. entomophila* and further analysis is needed.

As this initial data was comparable to results seen in the CrbRS TCS in *V. cholerae* (14), the effects of this change in *acsA* expression on virulence were explored utilizing fly survival assays, where *Drosophila melanogaster yw* was the model organism. Through these assays, it was observed that fly mortality did not change in *Drosophila* that ingested wild-type strains of either *P. aeruginosa* or *P. entomophila* or those that consumed the TCS deletion mutants. These data indicates that though this TCS has an effect on the regulation of *acsA* expression, it does not affect the pathogenicity of either *Pseudomonas spp.* utilized in this study. This is intriguing as, previously mentioned, this TCS was involved in virulence in the model TCS, CrbRS within *V. cholerae*. One possible reason for the differing virulence functionality between homologous TCSs and the model TCS is that *Pseudomonas spp.* have a more diverse group of virulence factors that are relied upon during pathogenicity, including; hemolysin and protease secretion, type III secretion system toxins, pyocyanin production, or secreted insecticidal toxins compared to those seen in *V. cholerae* infections, some of which may be dependent on what organism is being utilized as a host (34, 37, 45). Many virulence factors of *Pseudomonas sp.* have been shown to be primarily regulated by the well-known TCS, GacAS, which is classified as a master regulator of virulence among many Gram-negative bacteria, including *P. aeruginosa, P. entomophila* and *V. cholerae* (34, 37, 45). This is

confirmed through the results of hemolytic and protease plate assay experiments. As these are common virulence factors that are regulated through the GacAS TCS (37, 45), neither was seen to be regulated through this novel, homologous TCS in either *P. aeruginosa* or *P. entomophila*. With extensive knowledge in the regulation of many virulence factors expressed in Pseudomonads, and the results of our fly survival assays, we decided to continue investigating the importance of this novel TCS with the metabolism of acetate rather than relating this system to the pathogenicity of our model organisms.

In addition to exploring the effects of this TCS on the regulation of *acsA*, an additional gene encoding an acetate permease was investigated to also be involved within this system. As previously stated, acetate permeases are involved in the assimilation of acetate from the environment, thus allowing its metabolization by other cellular processes. The predicted acetate permease studied within *P. aeruginosa* is encoded by the gene PA3234, as this gene is annotated as a permease based solely on sequence homology to other permeases, and not experimental evidence (37, 46). In *P. entomophila*, the acetate permease within this study is encoded by PSEEN1455, which has been annotated as *actP* (46). To our surprise, only the probable acetate permease, PA3234, was shown to be regulated through this homologous TCS in *P. aeruginosa. actP* encoded in *P. entomophila* showed varied results indicating this homologous TCS may not regulate this acetate permease. One reasoning for the varied findings is that it is difficult to indicate the substrate that is imported into the cell by a permease based on the nucleic acid sequence encoding a permease alone. Additional experimentation of a

selected permease would be needed to confirm imported substrate, such as acetate (7, 30). Therefore, this study did not continue investigating the involvement of this novel, homologous TCS's in the regulation of acetate permeases.

For the remainder of the study, further experimentation to confirm the involvement of this TCS in the regulation of acetate metabolism was the main focus. Experiments utilized both full gene deletion mutants, as well as mutant strains containing deletions in only the response regulator of the two main proteins in a TCS, the sensor kinase and response regulator, within *P. aeruginosa* and *P. entomophila*. This particular deletion is important as the conserved receiver domain within these proteins are involved in the phosphotransfer signaling between the sensor kinase and response regulator to produce a change in expression of genes regulated by these systems (4, 18). These regions contain a conserved histidine and aspartate residue within the sensor kinase and response regulator, respectively (4, 18, 36). qRT-PCR transcript analysis of deletion mutants resulted in generally consistent findings to our preliminary studies, with the exception of the sensor kinase deletion mutant (Δ1405) in *P. aeruginosa*. Reduced expression of *acsA* was seen in full gene deletions within the encoded sensor kinase and response regulator of *P. aeruginosa*, as well as in receiver domain portion of the response regulator. *P. entomophila* showed significant decrease in expression of *acsA* only in the response regulator and receiver domain portion of the response regulator deletion mutants, but not the sensor kinase deletion mutant. We were surprised to see the mutant strains with a deletion in the receiver domain of the sensor kinase encoded in either *Pseudomonas spp*. did not show a decrease in expression of *acsA.* This could

result because the conserved histidine residue of this region may not have been successfully deleted. As there are multiple histidine residues within this region and the prediction of this region utilized nucleotides encoding this receiver domain rather than the amino acid sequence, the prediction program may not have identified the entire receiver domain. In order to investigate this possibility, deletion mutants could be created where a larger region of the receiver domain is deleted or deletions could be constructed utilizing the amino acid rather than nucleotide sequence for this region.

The regulation of *acsA* expression was confirmed through the introduction of a plasmid complemented with the gene encoding the response regulator of either *P. aeruginosa* or *P. entomophila* into deletion mutant strains. Unfortunately, a complementation plasmid containing the gene encoding the sensor kinase of either *P. aeruginosa* or *P. entomophila* could not be created, though multiple attempts were conducted. One reasoning for this could be due to the size and complexity of this particular gene. The studied sensor kinase in *P. aeruginosa* and *P. entomophila* has a gene length of 3480 and 3477 nucleotides, respectively. The GC content for each of these genes is about 68%. Because of this size and GC content, amplification of the gene is difficult through PCR.

Final experiments in this study utilized solid and liquid minimal media to elucidate the effect this novel TCS plays on *P. aeruginosa* and *P. entomophila* in regulating acetate metabolism. In these experiments, M63 minimal media was supplemented with only 5 mM acetate or 5 mM acetate and 5mM glucose as carbon sources. Minimal media qualitative plate experiments allowed for the observation of a growth defect when this

TCS is deleted in either *P. aeruginosa* or *P. entomophila* when acetate was the only carbon source that could be metabolized. No difference in growth of wild-type and deletion mutants was seen when glucose was added as a second carbon source in the minimal media. Growth assays with liquid minimal media allowed us to quantify the growth defect in TCS deletion mutant strains compared to wild-type strains. Results of these assays confirmed there was a significant decrease in growth of deletion mutant strains of either *Pseduomonas spp*. compared to wild-type when grown in media supplemented solely with acetate as the carbon source. Again, these strains had growth similar to wild-type strains when grown in a medium where multiple carbon sources are present. These results suggest that this novel TCS plays a role in the regulation of acetate metabolism in both *P. aeruginosa* and *P. entomophila.*

One anomaly seen in these growth experiments was a delayed growth in Δ*acsA* deletion mutants in *P. entomophila*. An exponential increase in growth was seen in qualitative growth assays around 12 hours post inoculation of M63 minimal media containing acetate as the sole carbon source. This latent increase in cells could be due to suppressor mutations, a phenomenon where random mutations occur during cell starvation to suppress a negative growth phenotype, such as a previous mutation, to allow survival in nutrient limiting environments (2). These types of mutations accumulate due to the strong selective pressure (such as those present in a nutrient limiting environment). One explanation for this mutation occurring could be that this allows these strains to survive in the minimal media where acetate is the sole carbon source. One possible reason for this phenotypic rescue could be that such suppressor

mutations might occur in the promoter region of other acetate metabolism genes. Such

mutations could result in increased expression of other acetyl-CoA synthetases

annotated as *acsB* and *acs-2* in *P. aeruginosa* and *P. entomophila*, respectively, which

could allow for the rescue of *acsA* mutations.

Full genome sequencing of any possible suppressor mutants could be used to ascertain

which mutations are responsible for rescuing the growth defect seen in *acsA* mutants.

Further studies of *acsB* and *acs-2* could be conducted to elucidate any possible

relationship to this novel TCS and any role these genes may play in acetate metabolism.

These additional studies could identify if these genes are regulated in a similar fashion

to *acsA* in these organisms. One such experiment could include the creation of a double

deletion mutant in either of the model *Pseudomonas spp.* where both genes encoding

acetyl-CoA synthetases were deleted. This double deletion mutant could then be

utilized in growth assays, such as those performed in this study, to determine if the

additional deletion of either *acsB* or *acs-2* has an effect on the growth of either *P.

aeruginosa* or *P. entomophila*, respectively, in the presence of acetate.

 A Δ*acsA* deletion mutant was not constructed in *P. aeruginosa* in this study to allow for

comparison of this phenomenon in both *Pseudomonas spp*. Previous studies in *P.

aeruginosa* have shown that a deletion in the gene *acsA* will result in the decrease of

cell growth on both minimal media supplemented with acetate or ethanol which can be

utilized in other cellular processes, such as the tricarboxylic acid cycle (19, 20, 47).

Growth on ethanol is important for *P. aeruginosa* as it can be utilized as a carbon source

where acetate is produced through the oxidation of ethanol within this organism (19).

With this knowledge, further studies could be conducted to elucidate if this novel TCS

plays a role in the regulation of ethanol metabolism, along with acetate metabolism in

both *P. aeruginosa* and *P. entomophila.*

Conclusion

In summary, the purpose of this study was to investigate the role of a novel two-component system in the regulation of acetate metabolism and virulence within two Pseudomonads, *P. aeruginosa* and *P. entomophila*, and compare its functionality to a recently identified homologous system, CrbRS in *V. cholerae*. We have shown that this novel TCS system is involved in acetate metabolism of *P. aeruginosa* through transcription regulation of *acsA*, but has no involvement in regulation of virulence of this organism. Varied results in *acsA* expression through qRT-PCR analysis in *P. entomophila* indicate that further analysis of this TCS in this organism is needed before confidently determining that this TCS play a role in regulating *acsA* expression. Fly survival assays indicate that this TCS does not regulate virulence in *P. entomophila*. Growth assay of both Pseudomonads show inhibited growth when deletion mutant strains, other than the receiver domain region of the sensor kinase, on media where acetate is the sole carbon source.

These results suggest that virulence is regulated independently of this two-component system in our model *Pseudomonas sp.*, but plays a role in acetate metabolism of *P. aeruginosa* and possibly in *P. entomophila.* These results were unlike what was shown in the investigation of CrbRS in *V. cholerae,* where this system was also involved in virulence and acetate metabolism. As TCS are widely utilized among bacteria to regulate many cellular processes, knowledge gained from study of this novel system is not

limited solely to Pseudomonads or *Vibrio spp.* Further studies of this novel, homolgous

TCS could identify additional genes regulated by this system.

RNAseq experiments comparing the transcriptome of wild-type *P. aeruginosa* or *P. entomophila* to the transcriptomes of TCS mutants could elucidate all the genes regulated by this TCS in these species. Knowledge gained from increased understanding of gene regulation pathways in bacteria has many important uses. As bacterial resistance to currently available antibiotics is increasing, novel treatments are needed. By understanding gene regulation in these organisms, novel treatment methods for combating pathogenic organisms could be developed. Furthermore, manipulating gene regulation pathways in bacteria could be use in the development of useful products such as unique insecticidal toxins that could be utilized in the food/agriculture industry. Overall, TCS are important gene regulatory mechanisms found among most bacterial families. Studying these TCS could allow for many industrial and health applications.

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Table 5: Nucleotide sequences of forward and reverse primers utilized during colony PCR to confirm proper deletion of listed genes in either *P. aeruginosa* or *P. entomophila*.

Primers Utilized in Confirmation of Correct Deletions in Mutant Strains

Organism	Target Gene	Forward primer	Reverse Primer
Pseudomonas aeruginosa	PA3271	GCTCAAGCATGCGTGCGACC	CTGCTCTATCGAGACGGAGC
Pseudomonas aeruginosa	PA3604	GACGAACGCGGTGTACGAGG	CGTTGAGGATACGCTTGAGG
Pseudomonas aeruginosa	PA3271 Receiver Domain	CGGACAAGCAGAAAGTCATCT	GCAACCGCCTGCTCTATC
Pseudomonas aeruginosa	PA3604 Receiver Domain	GGTGATCAGCATCAGGACATC	GGGAACCGTCTCCATTTGTT
Pseudomonas entomophila	PSEEN3888	GACAGAATCTGTAACAATC	CACAAGCGGTGACAGCCATG
Pseudomonas entomophila	PSEEN1405	CTCCTACAGGATGTGCCATG	GTTCGTGCTGTTCATCCAGG
Pseudomonas entomophila	PSEEN4122	GCTTCAGGCATGACGAAGCTG	CAGTACATTGAGCAGCACCA
Pseudomonas entomophila	PSEEN1405 Receiver Domain	GGTGATCTTCCAGGAGTTCAA	AAGTTCGTGCTGTTCATCCA
Pseudomonas entomophila	PSEEN4122 Receiver Domain	GGCGTTTGCATACCTTCCTA	TCATAGGCGATCTGCTTGTTC

Table 6: Nucleotide sequences of forward and reverse primers utilized during qualitative real-time PCR transcript analysis of listed genes in either *P. aeruginosa* or *P. entomophila*. This study did not look at the transcript analysis of receiver domain mutations within either the sensor kinase or response regulator encoded in *P. aeruginosa* or *P.*

Primers Utilized in Transcript Analysis of Target Gene Expression

Organism	Target Gene	Forward primer	Reverse Primer
Pseudomonas aeruginosa	PA3271	TCAGCAAGCAGGAGAACATC	GAGGACGATGCCTTTCAGTT
Pseudomonas aeruginosa	PA3604	AGATCGCCTACGACCTGAA	GGGAACCGTCTCCATTTGTT
Pseudomonas aeruginosa	PA3271 Receiver Domain	-	-
Pseudomonas aeruginosa	PA3604 Receiver Domain	-	-
Pseudomonas entomophila	PSEEN3888	AAAGTGGTGATCACTGCCGACGAA	ATGATCTTCTGCACGCTGCTGGTT
Pseudomonas entomophila	PSEEN1405	CGAGCTGGGCAAGATTCA	CTTGGCTTCAGCCGATACA
Pseudomonas entomophila	PSEEN4122	ATGAGCTTCACCGACATCAC	TGGTTGAGTTGCGACAGTT
Pseudomonas entomophila	PSEEN1405 Receiver Domain	-	-
Pseudomonas entomophila	PSEEN4122 Receiver Domain	-	-

qRT-PCR Transcript Analysis of *acsA* in Various Deletion Mutants of *P. aeruginosa*
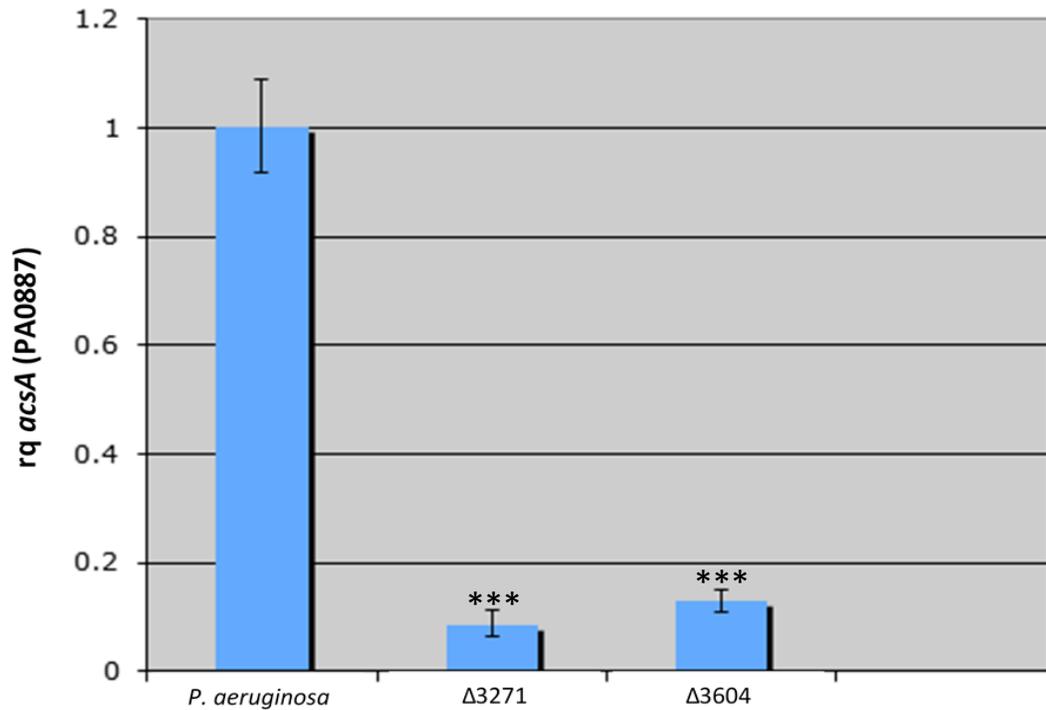
Figure 23: qRT-PCR transcript analysis of *acsA* in a sensor kinase (PA3234) and response regulator (PA3604) deletion mutant in *Pseudomonas aeruginosa*. A decrease in expression of *acsA* was seen in both deletion mutants of *P. aeruginosa*. Statistical significance (P-value) was determined comparing results of each mutant strain to the wild-type strain. One asterisk (*) denotes a P-value less than 0.05, two asterisks (**) denotes a P-value less than 0.01, and three asterisk (***) denotes a P-value less than 0.001.

qRT-PCR Transcript Analysis of *acsA* in Various Deletion Mutants of *P. entomophila*
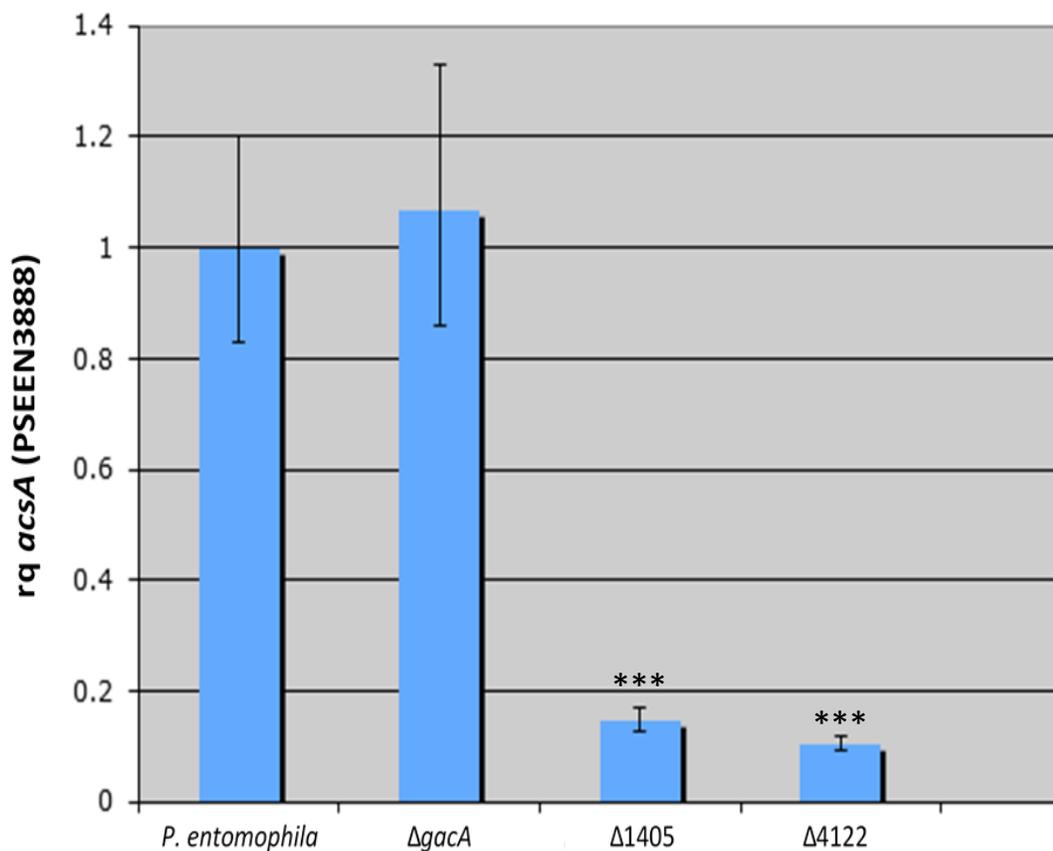
Figure 24: qRT-PCR transcript analysis of *acsA* in a sensor kinase (PSEEN1405) and response regulator (PSEEN4122) deletion mutant in *Pseudomonas aeruginosa*. A Δ*gacA* deletion mutant in *P. entomophila* is utilized as a negative control. A decrease in expression of *acsA* was seen in both deletion mutants of *P. aeruginosa*. Statistical significance (P-value) was determined comparing results of each mutant strain to the wild-type strain. One asterisk (*) denotes a P-value less than 0.05, two asterisks (**) denotes a P-value less than 0.01, and three asterisk (***) denotes a P-value less than 0.001.
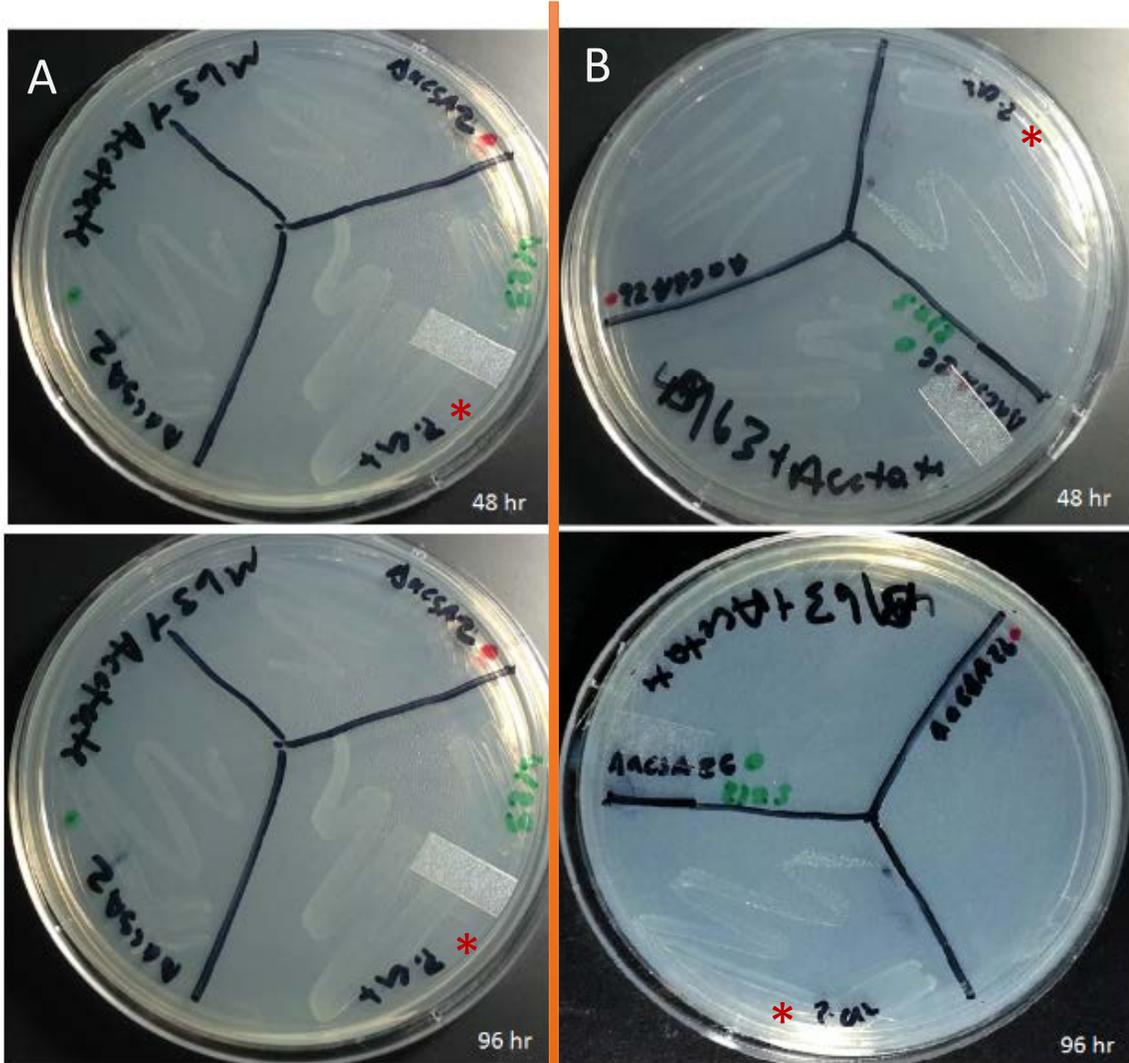
Figure 25: Wild-type *P. entomophila* and Δ*acsA* clone 2 (A) and clone 26 (B) deletion mutants plated on M63 minimal media supplemented with 5mM acetate. Wild-type *P. entomophila* is denoted by a " * ". Two copies of each clone were plated and denoted with either a red dot or green dot. Strains were grown at 30°C for 96 hours, where results were observed at 48 and 96 hours. Growth was seen for all strains, where reduce growth was observed in all Δ*acsA* deletion mutants compared to wild-type strain.

Figure 26: Colony PCR to confirm proper deletion of the gen *acsA* in *P. entomophila*. Lanes are loaded from left to right as follows; 10kb loading ladder (Denville Scientific) , BenchTop 100bp loading ladder (VWR), wild-type *P. entomophila* (P. ent), Δ*acsA* clone 2 red (R), Δ*acsA* clone 2 green (G), Δ*acsA* clone 26 red (R), and Δ*acsA* clone 26 green (G). An "X" indicates a skipped lane. Expected size of wild-type amplicon should be at 2.2kb. If deletion mutants have a correct deletion of *acsA,* then a distinct band should be observed at about 350bp which is not visible in wild-type *P.* entomophila. Wild-type *P. entomophila* shows a distinct band at about 2.2kB, which is absent in all Δ*acsA* deletion mutant strains. A band can be observed at about 350bp in all Δ*acsA* deletion mutants utilized in this assay, indicating correct deletion of the gene *acsA*.