CHARACTERIZATION OF A PHYLOGENETICALLY CONVERGENT NITROGEN-DEPENDENT ANTIMICROBIAL MECHANISM AGAINST *SERRATIA MARCESCENS* UTILIZING A *D. MELANOGASTER* INFECTION MODEL

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Nathan James Poling

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STATEMENT OF COMMITTEE APPROVAL

Dr. Lindsay Gielda, Chair

Department of Biological Sciences

Dr. Michelle Spaulding

Department of Biological Sciences

Dr. Michael Zimmer

Department of Biological Sciences

Approved by:

Dr. Barbara Mania-Farnell

Dedicated to Everyone Who Supported Me

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ABSTRACT

Host-pathogen interactions are the result of long term evolutionary processes due to the conflicting goals of the host and the infections pathogens in their quest for survival, creating an interplay of co-evolution as various adaptation are acquired by one and then in turned adapted to by the other. Selection of the host's antimicrobial strategies and the resultant adaptations of infectious microorganisms leads to the development of complex and dynamic relationships ranging from symbiotic to commensal to pathogenic. In an effort to understand the selective process and identify unique mechanisms of antimicrobial defense, sera from 18 species (7 invertebrate, 11 vertebrate) were tested for antimicrobial potential against 20 Gram-negative and 11 Gram-positive bacteria. *Alligator mississippiensis* sera exhibited the strongest inhibitory potential. A transposon mutagenesis screen performed on the resistant bacterium *Serratia marcescens* identified several genes, including *glnL*, as necessary for defense. The *glnL* gene encodes for the sensory histidine kinase/phosphatase NtrB, controlling the expression of regulatory genes in response to nitrogen limitation. Attenuated growth of the Tn::*glnL* mutant in the presence of alligator serum and minimal media was rescued with nitrogen supplementation, suggesting the existence of a mechanism for nitrogen limitation as an antimicrobial strategy in alligator sera. Utilization of a *Drosophila melanogaster* oral model of infection showed that *glnL* is required for *S. marcescens* virulence, and nitrogen supplementation rescued the phenotype, as measured by fly mortality and bacterial cfu recovery. *S. marcescens*, an environmentally ubiquitous Gram-negative bacterium, is an opportunistic pathogen in several species, including alligators and *Drosophila*. Subsequent *in vitro* testing of the antimicrobial potential of invertebrate hemolymph utilizing the Tn::*glnL* mutant showed a nitrogen-dependent growth inhibition of species in the order Dipteria. Combined, these results support a model of evolutionary convergence of nitrogen limitation as an antimicrobial mechanism. This work not only identifies a novel antimicrobial strategy that could be used in the development of therapeutics, and a novel virulence factor in *S. marcescens*, but has broad implications for bacterial management and can provide insight into the evolutionary history of host-pathogen interactions.

CHAPTER 1. INTRODUCTION

Serratia marcescens is a rod shaped, Gram-negative ubiquitous bacterium commonly found in soil, sediments, water, and plant roots (Chen *et al*., 2017). Once regarded as generally harmless, starting in the 1950's, *S. marcescens* is now classified as an opportunistic pathogen with a broad host range including humans (Pittman *et al*., 2015; Yu, 1979). Differing strains are able to survive in environments ranging from the nutrient rich environments of soil, to the minimally rich and harsh clinical environments including saline, and some antiseptics *(*Pittman *et al*., 2015). This ubiquitous nature of *S. marcescens* is due, in some part, to its robust intrinsic and acquired resistance to antimicrobial compounds, as well its production of antimicrobial secondary metabolites (Iguchi *et al*., 2014).

A defining characteristic of *S. marcescens* is its production of prodigiosin, a bright red tripyrrole pigment known to have antimicrobial properties hypothesized to allow propagation of *S. marcescens* within highly competitive bacterial environments (Hage-Hülsmann *et al*., 2018; Li *et al*., 2015). Expression of prodigiosin is variable between strains, with a lower frequency of pigmentation in clinical isolates compared to environmental isolates (Kurz *et al*., 2003). A variety of transcription factors and environmental stimuli influence prodigiosin biosynthesis, including thermoregulation where production is inhibited at $37°C$, while synthesis occurs at temperatures below $32°C$ (Williamson *et al*., 2006). In addition to prodigiosin, *S. marcescens* produces other antibiotic secondary metabolites such as the biosurfactant serratamolide serrawettin W1 (Hage-Hülsmann *et al*., 2018). Serrawettin W1, produced by most pigmented *S. marcescens* strains, is a nonionic biosurfactant composed of serine, and beta-hydroxyl fatty acids with anti-cancer and antimicrobial activity predominantly against Gram-positive bacteria (Kadouri & Shanks, 2013). The biosurfactant's surface-tension reducing properties not only influence *Serratia's* motility, but also enhances prodigiosin biosynthesis (Eberl *et al*., 1999). Thus the combinatorial effects from production of serrawettin W1 and prodigiosin together yields higher antimicrobial activity (Hage-Hülsmann *et al*., 2018). Another secondary metabolite produced is the peptide althiomycin which is a broad spectrum antibiotic pentapeptide that blocks the action of the peptidyl transferase activity on ribosomes, resulting in the inhibition of protein biosynthesis in targeted bacteria (Gerc *et al*., 2012). *S. marcescens*, like other environmental microbes develop competitive mechanisms to interfere with microbial competitors and harvest nutrients (Ghoul & Mitri, 2016). Research to

date has primarily focused on *Serratia's* production of secondary metabolites with antimicrobial potential as a competitive mechanism due to their potential in therapeutic development. It is clear that within the diverse and competitive environment of soil niches, *S. marcescens* has adapted to produce complementary metabolites that work synergistically to reduce the growth of competing bacteria to create a colonizing advantage (Hage-Hülsmann *et al*., 2018).

Highly competitive environmental niches not only influence development of offensive antimicrobial strategies in microbes, but defense mechanisms as well. Ubiquitous bacteria are under selection pressure to develop resistances to antimicrobials, however *S. marcesens* due to its large repertoire of chromosomally encoded resistances, has defense strategies against antimicrobial molecules in environmental and clinical settings. This resistance is conserved between large numbers of *S. marcescens* strains, and includes resistances to antimicrobials including aminoglycosides, quinolones, macrolides, and beta-lactams (Iguchi *et al*., 2014). Antimicrobial resistance along with virulence factors can be acquired through lateral gene transfer, compensatory mutations, and adaptive mutations.

Antimicrobial resistance acquisition, though mutation or mobile genetic elements, can partly be attributed to pharmaceutical runoff within natural environments from the leakage and over use of current antimicrobials (Hiltunen *et al*., 2017). The variable exposure of antimicrobial agents into natural environments in lower than minimal growth inhibitory concentrations, allows for bacteria to adapt and resist these antimicrobials. chromosomal mutations conferring resistance to antimicrobial agents are often found to be deleterious to fitness without the presence of the antimicrobial agent (Andersson & Hughes, 2010; Hiltunen *et al*., 2017). However, this newly acquired antimicrobial resistance adaptation is then able to transfer from one bacterial strain to another through horizontal gene transfer and increase new bacterial strain fitness within the presence of the antimicrobial agent (Hiltunen *et al*., 2017). Horizontal gene transfer, enables the transfer of acquired antimicrobial resistances of a primary pathogen to an opportunistic pathogen such as *S. marcescens* (Huddleston, 2014). An example of this is the *alb* biosynthetic gene cluster, which encodes for althiomycin production in *S. marcescens* isolates, was discovered in 1957 and demonstrated that horizontal gene transfer had to occur for this acquired antimicrobial metabolite production due to phylogenetic divergence of these species (Gerc *et al*., 2012). It's prevalence as a ubiquitous microbe, and subsequent genomic plasticity enabling the acquisition of antimicrobial

biosynthetic pathways and competitive defensive strategies, has led to *S. marcescens* emergence as a multidrug resistant pathogen in both the clinical, and environmental settings.

S. marcescens is a known entomopathogen, and has been isolated from flies *(Drosophila),* mosquitos *(Anopheles),* and nematodes (*Caenorhabdbitis*) (Chen *et al*., 2017; Lancaster *et al*., 2012; Nehme *et al*., 2007). *Drosophila* have a wide array of food sources from fruits, and other plant materials to rotting flesh, and commonly acquire *S. marcescens* through ingestion of contaminated food (Markow, 2015). When infected by *S. marcescens*, or any Gram-negative bacterium, the *immune deficiency* (*imd*) pathway in *Drosophila* is activated, mediating an immune response (Nehme *et al*., 2007). The *imd* response activates production and secretion of AMPs along with transcription of greater than 200 or more genes, some of which are unknown in function (Hetru *et al*., 2003). While certain strains of *S. marcescens* are sensitive to the *imd* response, *S. marcescens* strains including Db11 (the sequenced reference strain) are resistant and can infect flies through the intestinal epithelial cells, and can propagate a systemic infection in the fly's hemolymph. Bacterial proliferation is normally kept in check by the phagocytic hemocytes in the hemolymph, however systemic infection by *S. marcescens* has resulted in a significant reduction of hemocytes in insects, enabling bacterial proliferation and subsequent death of the host (Burritt *et al*., 2016). Additionally, infection of intestinal epithelia cells leads to degradation of the cells via protease production by *S. marcescens*, contributing to the mortality of the fly (Nehme *et al*., 2007).

Drosophila host immune defenses are able to inhibit the proliferation of pathogens via multiple mechanisms. Due to *Drosophila*'s lifestyle as an ecological generalist microbial interactions encountered during feeding, have allowed for the development of immune strategies to prevent infection from orally ingesting bacteria, including a cellular response and a systemic humoral response (Markow, 2015; Nehme *et al*., 2007). Within the body cavity that contains hemolymph, called the hemocoel, the cellular arm of the innate immunity controls proliferation of pathogens. In the digestive tract, the *imd* pathway induces the production of large quantities of anti-microbial peptides (AMP) from the fat body (Nehme *et al*., 2007). Anti-microbial peptides produced by the fat body, similar to the mammalian liver, include metchnikowin, diptericin and drosomycin (Mylonakis *et al*., 2016; Nehme *et al*., 2007). Expression of these AMPs allow for partial protection from entomopathogenic bacteria while also complimenting another part of innate immunity, namely the production of reactive oxygen species. Orally ingested bacteria present in

the digestive tract induce the *imd* pathway locally in the cardia of the fly, as well as systemically (Nehme *et al*., 2007). Outside of the digestive tract and within the hemolymph, hemocytes phagocytize foreign pathogens (Lemaitre & Hoffmann, 2007). All these antimicrobial strategies employed by *Drosophila* were adapted over time to defend against infection due to its constant interactions with microbial life. However, microbial adaptation to selective pressures such as environmental conditions or immunologic factors in the host can lead to virulence strategies that enable microbes to become pathogenic due to its constant interactions with the host.

Like *Drosophila*, alligators (*Alligator mississippiensis*) live in microbial diverse and laden environments such as swamps. However, even in these bacterial abundant environments and injury prone activities, alligators do not often acquire infections (Barksdale *et al*., 2017). It is hypothesized that the lack of infections is due to the potency of the innate immune system. Alligators are evolutionarily ancient organisms, the order Crocodilia diverged from their closest living relative the birds over 250 million years ago (Benton & Clark, 1988). Within their semi-aquatic environments alligators are phenotypically the apex predator, however exposure to a high quantity and diversity of microbial species means one of their main biological threats is infection from pathogens. The long evolutionary time, with modern alligators being roughly 83 million years old, and selective pressure from a wide array of diverse pathogens has led to adaptation of their innate immune system to provide protection from ecologically diverse pathogens and has resulted in robust and diverse defense strategies (Bishop *et al*., 2015; van Hoek, 2014). Alligator's innate immune system potency is due in part to AMPs, and cationic antimicrobial peptides (CAMP), Including lysozyme, defensin, hepcidin, and hemocidin (van Hoek, 2014). A cathelicidin found in alligator sera was found to have strong activity against gram negative bacteria, along with multidrug resistant (MDR) bacterial strains that also have resistance to carbapenem (Barksdale *et al*., 2017). Hemocidin are fragments of hemoglobin that have been found to have broad spectrum antimicrobial properties. In comparison with other avians alligators express hepcidins while birds do not, expressing critical differences between innate immune system expressions. This highly potent innate immune system could be attributed to the evolutionary age of alligators and the adaptation to the microbial flora within the brackish, harsh environment that they reside in (van Hoek, 2014). Even with such a potent innate immune system, alligators are still susceptible to septicemia via infection from gram negative bacteria such as *S. marcescens* (Novak & Seigel, 1986).

One of the driving principles of the theory of evolution via natural selection is that competition for survival between two or multiple organisms leads to a cycle of adaptations in an evolutionary arms race. Red Queen dynamics is a hypothesis concerning the coevolution of organisms, stating that an organism must adapt and proliferate against and in response to an equally ever evolving species (Papkou *et al*., 2019). Simply put, the dynamics between a pathogen and host competing for nutrients will drive adaptation, and evolutionary change. Once a pathogen evolves virulence factors to infect and cause detriment to the host, then the host evolves to kill or stop the proliferation of the pathogen via immunological mechanisms, and vice versa. Microbes require certain nutrients to survive and proliferate in a given environment (Bruhn & Spellberg, 2015). Pathogens acquire or "steal" these nutrients from a host, leading to stress on the host cells which ultimately results in an immune response in order to regain fitness. An inability of a pathogen to acquire said nutrients in contrast, would inhibit efficient replication and decrease the bacteria's fitness. Therefore, host-pathogen dynamics are often adaptations of each organism to acquire specific required nutrients in order to maintain fitness.

Nutritional immunity is the ability to control nutrient concentrations by the host in order to reduce the proliferation of pathogens (Hesse *et al*., 2019). Vertebrates have evolved to exploit this need of nutrients by pathogens via nutrient sequestration. Examples of nutrient sequestration that produce antimicrobial effects is the limiting of zinc during the innate immune response to bacteria (Hesse *et al*., 2019). Further nutrient sequestration mechanisms include reduction of the amount of iron available to the pathogen by the host via the transferrin family of iron sequestering proteins (Bruhn & Spellberg, 2015). In order for pathogens to overcome host mediated nutrient sequestration, pathogens develop scavenging pathways. These pathways can be protein receptors and trans-membrane transporters that have an extremely high affinity for sequestered nutrients (Hesse *et al*., 2019). Transferrin is a glycoprotein that transports Iron in the blood. Bacteria such as *Staphyloccocus aureus* have transferrin binding proteins that scavenge the necessary iron required for virulence within mammals (Li *et al*., 2015). The area of nutrient-competition is not as well explored as direct mechanisms of microbial competition (such as through antimicrobial products), and therefore there are potentially undiscovered mechanisms with which host-microbe interactions are indicated.

This study is aimed at the characterization of a novel virulence factor of the opportunistic pathogen *S. marcescens* that enables proliferation and survival in host niches. Due to the ability of

S. marcescens to survive in harsh clinical environments, and cause nosocomial infections we aim to identify further antimicrobial mechanisms that may be used to exploit required key virulence factors as new means of control. Through targeted therapeutics, such as the against the *glnL* sensory pathway, possibilities to inhibit virulence in MDR *S. marcescens* strains may open up. Further insights into the evolutionary relationship between host, and pathogen survival interactions can be discovered as well. We tested the ability of *S. marcescens* to resist diverse animal sera to determine whether there is an evolutionary relationship between bacterial and host exposure. We identified an evolutionary convergent mechanism of antimicrobial activity found in alligator and fly sera against *S. marcescens* that depends on available nitrogen. *S. marcescens* requires a functional *glnL* gene to survive and remain resistant to alligator and fly sera

CHAPTER 2. MATERIALS AND METHODS

2.1 Preparation and Growth of Bacterial Samples

Bacterial growth for stock samples was quadrant streaked on TSB agar or LB agar petri plates for bacteria colony formation. Stock plates were incubated at their respective temperatures for each bacteria species. Bacterial samples are from -80°C stocks of bacteria. Isolates of single colonies are grown overnight in TSB at either 37°C or 23°C depending on the assay to be performed.

2.2 Kirby-Bauer

Bacterial species listed in Table 1 were sub cultured out into 2.5mL TSB broth tubes overnight at 37C in an incubator. After overnight incubation 1mL of bacteria culture was then pipetted onto agar petri plates of the designated nutrient type for each bacteria. Full lawn inoculation was achieved by using autoclaved glass 3mm beads to spread the inoculum over the whole plate. Upon inoculation the petri plates were set aside for drying. Whatman® filter paper was hole punched by an office type hole punch then autoclaved to sterilize. The Whatman® discs were then saturated by micro pipetting various animal sera directly onto the sterile discs. Upon full saturation the discs were then placed onto a nutrient media plate containing a single species of bacteria. The discs were gently pressed onto the agar to ensure the adhesion to prevent dropping during incubation. All plates were then incubated agar side up overnight in incubators with respective optimum bacterial temperatures listed in table 1. After incubation overnight, zones of growth inhibition was then measured in millimeters by a rule and recorded.

2.3 Transposon Mutagenesis

An EZ- Tn5 plasmid with a kanamycin resistance cassette from Lucigen® was utilized to perform a transposon mutagenesis screen. Protocol was followed to manufactures specifications. After culturing *S. marcescens* overnight it was pipetted into an electroporation cuvette along with 1μl of the EZ-Tn5 plasmid just before electroporation. Immediately after electroporation SOC media was added to the cuvette. Next the contents of the cuvette was transferred into a test tube

containing sterile 37C TSB. After 30 minutes the newly electroporated culture was then lawn plated on TSA plates with antibiotic kanamycin to select for only newly mutated cultures. Plates were incubated at 37C overnight for isolate colony formation. Post overnight incubation isolate colonies were then collected in two 96 well plates containing TSB broth. All colonies were then incubated at 37C to proliferate

2.4 Mutant Selection

S. marcescens transposon mutants collected in the two 96 well plates were then used to inoculate two separate 96 well plates containing TSB with 2.5% alligator serum by volume. A negative control of wild type *S. marcescens* was used as baseline to compare growth inhibition by the alligator serum in the mutants. The plates were then incubated over 12 hours at 37C while recording optical density at 600nm (OD^{600}) every hour. After the 12 hour cycle a single Transposon mutant was selected by having a lower OD^{600}, showing increased sensitivity to the alligator serum, while still allowing significant growth.

2.5 Serum Collection

Serum and hemolymph that was not donated was extracted within the lab. Animal sera samples were graciously donated to Purdue University Northwest from Westchester Animal Clinic in Chesterton IN. Hemolymph, and serum samples that were not donated were collected in lab using several methods. Hemolymph extraction from all flies was achieved by piercing the thorax using a 29 gauge insulin needle. Once pierced 20-30 flies were collected in to a 1mL centrifuge tube with five 20 gauge needle holes in the bottom to allow hemolymph to flow out during centrifuge. The 1mL tubes were then placed in 1.5mL centrifuge tubes and cold spun at 14k RPM at 4C for five minutes (Musselman, 2013). Cricket hemolymph was extracted via removing the hind legs and centrifuge in same procedure as the flies. All centrifuge extraction methods had 50uL of heparin added to prevent degradation of the samples. Tarantula hemolymph was collected from live specimens via fine needle blood draw 4-6mm posterior of the pedicel. Snail blood was collected via fine needle aspiration from live specimens. Sea urchin blood was collected via fine needle aspiration. All hemolymph and sera samples had heparin added and kept in storage at -80C until used.

2.6 Sensitivity Screen

The Tn::*glnL* mutant and wild type *S. marcescens* was subcultured in sterile TSB overnight. After incubation the active cultures was then diluted and calibrated to 0.1 OD^{600} in subsequent TSB suspensions. The standard 0.1 OD cultures were then transferred into 96 well plates by 200uL in each well. The experimental plate contained the Tn::*glnL* cultures while the negative control plate contained *S. marcescens* cultures. Serum that was collected from multiple vertebrate and invertebrate organisms was then was then added to wells on both plates to a final well concentration of 2.5% serum by volume. After addition of the serum a 12 hour growth curve was performed using the ThermoFisher MultiSkan plate reader to incubate at 37C and record OD^{600} every hour for the 12 hours. All serum tested was performed in triplicate to ensure accuracy of test results.

2.7 Mutant Genome Sequencing

DNA extraction from the mutant was done utilizing a Qiagen DNA extraction kit using the standardized procedure as per the manufactures specifications. Steps include lysing bacteria cells via lysis buffer, and proteinase K. following steps were the isolation of the bacterial DNA, and the purification of the DNA via ethanol, and filter spin columns.

2.8 Nitrogen Complementation

A 24 hour incubation growth experiment was utilized using a 96 well sterile plate with sterile TSB as a growth medium. The plate was inoculated in all 12 rows into six groups. Each group contained the wild type *S. marcescens,* and the Tn::*glnL* mutant in triplicate. Each group consisted of two rows with one row being the wild type, and the second row being the Tn::*glnL* mutant. The first group was left as experimental controls and grown in TSB. The second group was grown in TSB with the inclusion of 10% by volume of alligator serum. The third and fourth group was grown similarly as the previous group, however group three had the inclusion of 0.1mM of urea. Group four was the same as group three except for a tenfold lower concentration of urea was added (.01mM). The final groups five and six were controls which included growth in TSB with inclusion of 0.1mM urea, and group six grown with the inclusion of 0.01mM urea. The plate was measured for OD^{600}, and incubated using ThermoFisher MultiSkan plate reader over the

course of 24 hours. Aggregate data was compiled to show growth fold change compared to the first group of controls.

2.9 Total Nitrogen Cell Test

A Spectroquant® total nitrogen test was utilized to determine total nitrogen levels for each sera type. All reagents and procedures were provided and followed to manufactures' specifications. For each test 1ml of sample was loaded along with 9ml of distilled water, 1 cap microspoon of reagent N-1K, and 6 drops of reagent N-2K. The sample was then heated cooled after digestion 1ml of the sample was then added into a test cell along with 1ml of reagent N-3K. After the final step samples were then transferred into a cuvette and recorded via spectrophotometry. A single test was used for each sera type separately.

2.10 Heat Kill Sensitivity Assay

Subcultured Tn::*glnL* mutant and wild type *S. marcescens* strains are aliquoted into separate wells of a sterile 96 well plate in triplicate. Alligator serum used in a four hour growth assay to determine if the antimicrobial killing was caused by a proteinacous or chemical compound by growing *S. marcescens* and the mutant in sterile TSB in separate wells of the 96 well plate. Alligator serum was heated at 65C for 20 minutes to denature any possible antimicrobial peptides or proteins. After the alligator serum was cooled to safe levels then a 10% by volume concentration was made in one third of the wells used in the 96 well plate. OD^{600} was measured by the plate reader every hour. Data was collected and compiled.

2.11 Phylogenetic Tree Construction

Construction of synthetic phylogenetic trees was done using Mesquite version 3.61 (Maddison & Maddison, 2019). Relationships were based off of already existing database within the National Center for Biotechnology information (NCBI) as well as other primary literature sources. NCBI databases were used for phylogenetic taxonomy relationships (Kjer *et al*., 2016; Schoch *et al*., 2020).

2.12 *D. melanogaster* Oral Infection Model

Conical fly tubes with foam caps were autoclaved with 20 Whatman® paper punched discs at the bottom for bacterial growth. Sub cultures of bacterial species were grown 24 hours prior to infection. Bacterial cultures were standardized to 0.1OD at 600nm into filter sterilized 50uMol sucrose. 2ml of each bacterial sucrose solution was then micropipetted into the bottom of the sterile paper bottomed fly tubes. The tubes were then left aside to absorb the 2mL prior to addition of flies. Flies were anesthetized via CO_2 gas being introduced into the live fly holding tubes. Flies were then transferred out onto a gas diffusion pad with a constant supply of CO_2 to ensure easy counting and constant anesthetization. Upon counting the specific number of flies, the flies were transferred into the infection tubes, and air was wafted into the tubes to reduce the time taken for the flies to return to normal. All fly tubes; infection, breeding, and holding vials were stored in an incubator held at 23C. Infection flies were fed every 24 hours by micro pipetting 50ul of 50uM sucrose into the infection tubes. Changing sterile tips after every tube was to reduce potential contamination. Counts of dead flies were recorded at time of feeding.

2.13 CFU Recovery Collection

Flies were infected using the oral infection method described in 2.10. 110 total flies were incubated for 5 days total during infection. There were 5 total groups, 10 control flies, 25 wild type at 0.1 OD^{600}, 25 wild type 0.05 OD^{600}, 25 mutant at 0.1 OD^{600}, and 25 mutant at 0.05 OD^{600}. However every 24 hours 5 flies from each of the 4 experimental condition tubes, and 2 from the control tube was CO_2 anesthetized and transferred 1 flies into 1 screw cap top bead beater tubes. Each of the Bead beater tubes contained 1 mL of Autoclave sterilized PBS, and 5 sterile 3mm glass beads. Upon all flies collected into tubes all samples were homogenized via 1 minute in a bead beater. 200uL of supernatant was collected in the top row of 96 well plates. The remaining wells contained 180uL of TSB. The collected supernatant samples were serial diluted in a 1:10 fashion to 10^{-7} dilution. All wells were plated via micro pipetting 10uL dots onto Agar plates with Kanamycin for the Tn::*glnL* mutant, and Tetracycline for the WT samples. Plates were left to dry then incubated at 37C for 18 hours. After incubation colonies were counted and bacterial load was determined for each fly.

2.14 Bacterial Heat Kill Oral Infection

An oral infection of flies was set up using procedure described in **2.12**. However, additional separate mutant and WT bacterial samples were heated at 65C for 20 minutes to kill all active bacteria. All tubes including the additional tubes were set up in the same manner as traditional infection. Dead flies were recorded at time of feeding. Survivability was determined at end of 14 days.

2.15 Nitrogen Complementation Oral Infection

Oral infection procedure used in **2.12** was used for initial infection. Post infection by 4-6 hours flies were fed with 100uL of 50mMol sucrose solution containing 200mMol urea to compliment the Tn::*glnL* mutant. Flies were fed every 24 hours with the same 100uL of the sucrose containing additional 200 mMol urea solution. Fly death counts were recorded at time of feeding

CHAPTER 3. RESULTS

3.1 Identifying the Antimicrobial Potential of Animal Sera

We hypothesized that a phylogenetic relationship existed in regard to the potency of the innate immune response of organisms. To test this, the antimicrobial potential of animal sera was tested against 31 bacterial (Gram-negative and Gram-positive) strains. Similar to a Kirby-Bauer disc diffusion assay, 20ul of sera taken from several animal donors (Table 1) were added to sterile Whatman® discs placed onto bacterial lawns, and incubated overnight at temperatures specific for each bacterial strain. Zones of growth inhibition were measured in millimeters (mm) and are reported in Table 1. Alligator serum was significantly more potent in terms of inhibiting the growth of more bacterial species (16/31) as well as the size of the zone of inhibition compared to the other sera tested (average, 6.4 bacteria inhibited/31 strains (Table 1)). These results are consistent with previous reports that alligator sera has potent antimicrobial properties due, in part, to diverse antimicrobial peptides (M. E. Merchant *et al*., 2006).

Table 1: Kirby-Bauer like disc diffusion method was utilized to test bacterial susceptibility to animal sera. Growth Media types are: (NA): Nutrient Agar, (TSA): Tryptic soy Agar, and (LB): Luira-Bertani Agar. Temperatures listed are in degrees of centigrade. Numbers indicate millimeters of clearing of bacterial lawn. Dashes indicate no zones of clearing or bacterial death. Totals are numbers of bacterial species that each animal sera that produced zones of inhibition. Alligator was proven to be most potent, and able to kill 16 different bacteria, while the next highest was the cat at 9. However the alligator was susceptible to *S. marcescens* while all other sera was able to kill *S. Marcescens*.

Bacteria Species	Media	Temp	*Alligator mississippiensis*	*Canis lupus*	*Felis catus*	*Anas platyrhynchos*	*Didelphis virginiana*	*Boa constrictor*
Enterococcus faecalis	NA	37	4mm	-	-	-	-	-
Mycobacterium smegmatis	NA	37	5mm	-	-	-	-	-
Alcaligenes faecalis	NA	37	-	0.5mm	0.5mm	-	-	-
Enterobacter aerogenes	NA	37	1mm	-	1mm	-	1mm	1mm
Staphylococcus saprophyticus	NA	37	-	5mm	-	-	-	-
Lactobacillus plantarum	TSA	37	2mm	-	-	-	-	-
Streptococcus faecalis	TSA	37	5mm	-	-	-	1.5mm	-

Table 1 continued

Lactococcus lactis	TSA	37	4mm	1mm	-	-	-	-
Staphylococcus epidermidis	TSA	37	1mm	-	-	-	-	-
Salmonella typhi	TSA	37	3mm	1mm	1mm	1mm	1mm	-
Vibrio parahaemolyticus	TSA	37	5mm	-	-	-	1mm	-
Staphylococcus aureus	TSA	37	1mm	-	-	-	-	-
Escherichia coli K12	TSA	37	-	-	1mm	-	-	-
Klebsiella pneumoniae	TSA	37	-	-	1mm	-	-	-
Citrobacter freundii	TSA	37	-	1mm	1mm	1mm	-	-
Psuedomonas aeruginosa	TSA	37	-	-	1	-	-	-
Proteus vulgaris	TSA	30	1mm	1	-	-	-	1mm
Hafnia alvei	TSA	30	-	-	-	2mm	-	-
Serratia liqufaciens	TSA	30	-	1mm	1mm	-	1mm	-
Bacillus cerues	TSA	30	-	-	-	-	-	-
Micrococcus luteus	TSA	30	-	-	-	-	-	-
Bacillus megaterium	TSA	30	-	-	-	-	-	-
Pseudomonas fluorescens	TSA	30	-	-	-	-	-	0.5mm
Aeromonas hydrophila	TSA	25	-	-	-	-	-	-
Micrococcus roseus	TSA	30	1mm	-	-	-	-	2mm
Serratia marcescens	TSA	25	-	1mm	1mm	1mm	1mm	1mm
Streptococcus thermophilus	TSA	45	-	-	-	-	-	-
E. coli MG1655	LB	37	1mm	-	-	-	-	-
E. coli DH5a	LB	37	1mm	-	-	-	-	-
E. coli EDL933	LB	37	1mm	-	-	-	-	-
E. coli BL21	LB	37	1mm	-	-	-	-	-
Total			16	8	9	4	6	5

S. marcescens, a Gram-negative ubiquitous bacterium was susceptible to all sera tested in this assay, but was resistant to the alligator serum (Table 1). Due to this finding, *S. marcescens* was chosen to be used as a model organism to not only identify microbial required resistance factors and the associated mechanism of antimicrobial activity observed in alligator sera. *S. marcescens* has diverse antimicrobial resistance factors and we hypothesized that identifying the

specific bacterial genetic factor that mediated resistance to the alligator sera would aid in our understanding of the mechanisms of alligator antimicrobial action.

3.2 Transposon Mutagenesis Screen Identifies *glnL* as Necessary for *S. marcescens* Resistance to Alligator Serum

To identify genes necessary for resistance in the presence if alligator serums, a transposon mutant library was constructed using the EZ-Tn5 Transposase system (Lucigen). Individual mutants in a 96-well format were tested for increased susceptibility in the presence of 5% alligator serum in broth (TSB), compared to broth only controls. Of the 153 mutants tested, one (AB5) had over 2-fold difference in growth over 24 hours in the presence of alligator serum. These results were confirmed by a time-course analysis of growth of the AB5 mutant compared to wild type in increasing concentrations of alligator serum (1%, 2.5%, 5%) in TSB broth (Figure 1). The results showed a dose-dependent growth inhibition of AB5 compared to wild-type and broth controls (Figure 1)

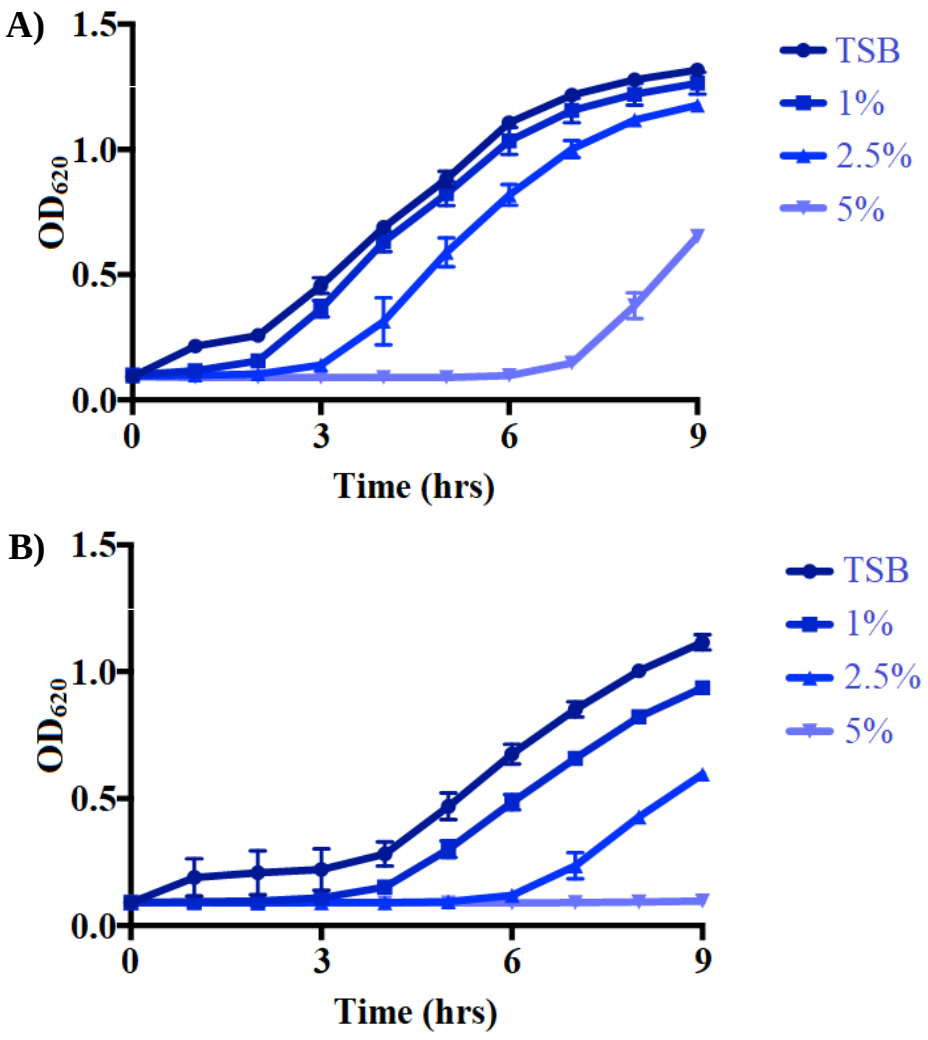

Figure 1. A) 9 hour growth curve showing the growth of *S. marcescens* in differing percentages of alligator serum by volume 1%, 2.5%, and 5% in comparison to standard TSB. B) 9 hour growth curve showing the growth of Tn::*glnL* mutant in differing percentages of alligator serum by volume 1%, 2.5%, and 5% in comparison to standard TSB. Comparing the 5% Tn::*glnL* mutant to the *S. marcescens* there is attenuation in the resistance factors of the mutant. Without the *glnL* gene the Tn::*glnL* mutant has increased sensitivity to the alligator serum while the wild type *S. marcescens* remains resistant to the alligator serum.

Identification of the transposon insertion site via PCR amplification and subsequent sequence analysis using internal transposon primers identified the transposon insertion site as in the 5' end of the *glnL* gene (Figure 2). The *glnL* gene is a sensory pathway that encodes for an NtrB kinase which activates a nitrogen scavenging uptake pathway which alleviates nitrogen stress imparted on the bacterial cell. We then hypothesized that the loss of this sensory pathway in *S.*

marcescens would limit growth in nitrogen-limiting environments and could be complimented via excess available nitrogen.

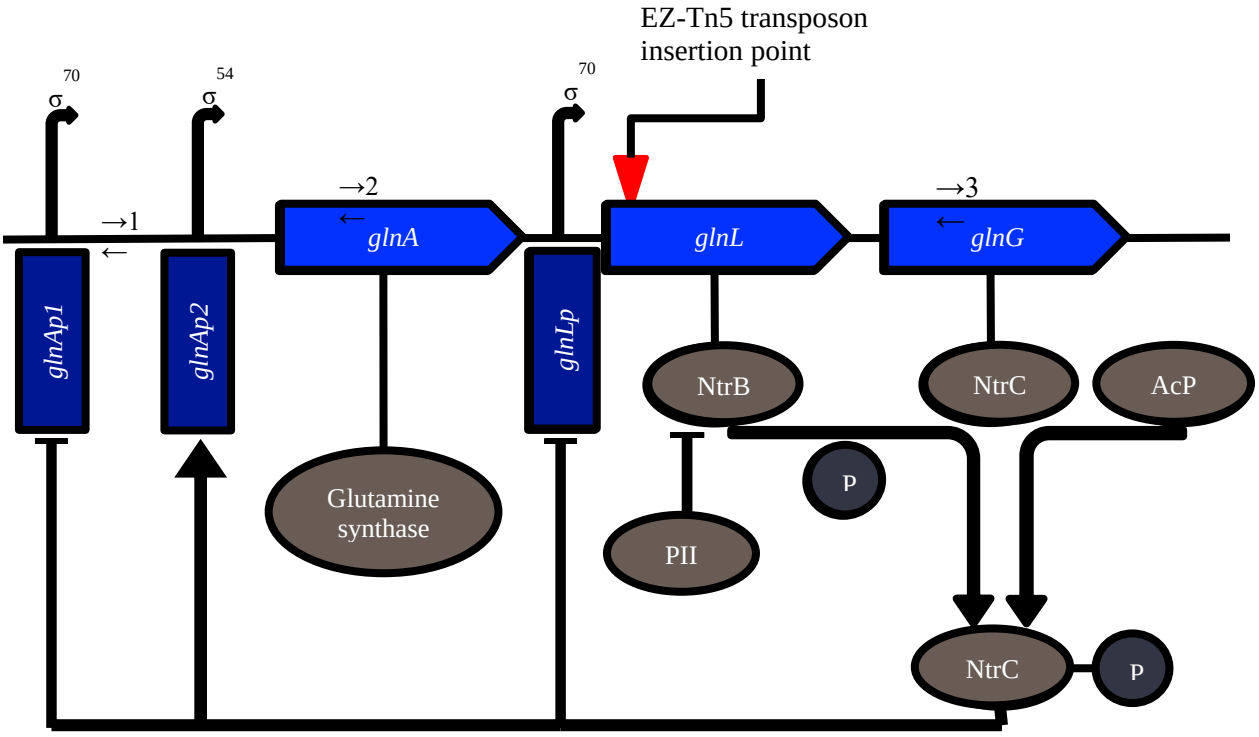

Figure 2. Upon genetic sequencing of the Tn::*glnL* mutant the transposon had attenuated the *glnL* which is a sensory pathway that encodes for an NtrB kinase that activates a nitrogen uptake system to alleviate nitrogen stress imparted on the bacterial cell.

3.3 Nitrogen Limitation is Linked to Antimicrobial Potential of Alligator Sera

To test the effects of nitrogen levels on the growth of the Tn::*glnL* mutant, growth of wild type *S. marcescens* and the mutant in minimal media was tested with increasing concentrations of urea over 12 hours. An altered M9 Minimal Media lacking ammonium chloride was mixed and filer sterilized. Increasing concentrations of urea (0.1mM to 19mM) were added to provide variable nitrogen levels. Wild-type and Tn::*glnL* strain were inoculated into each medium in triplicate in a 96 well format. After 12 hours at 37℃, bacteria were serially diluted and plated on TSB for cfu/ml recovery. Fold change of growth of each condition compared to complete M9 media change of growth demonstrates an attenuation of growth by the Tn::*glnL* mutant in media with lower concentrations of urea than 19mM (Figure 3).

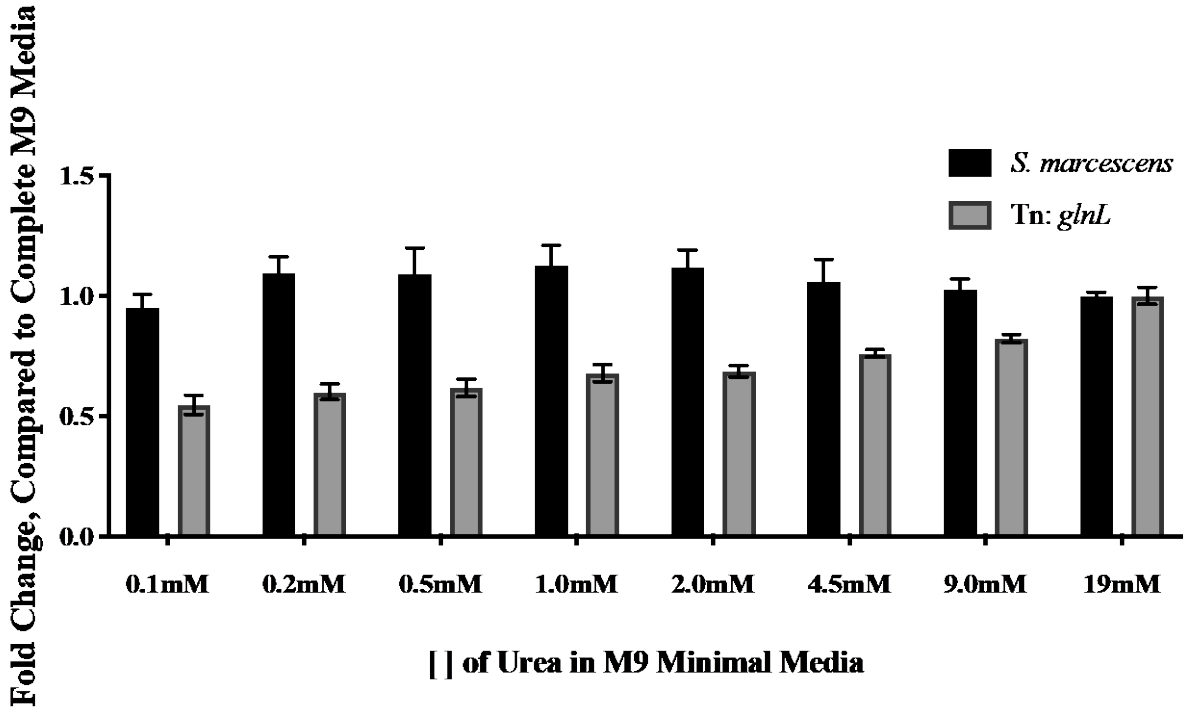

Figure 3: to test the effects of nitrogen levels on the Tn::*glnL* mutant growth compared to WT in nitrogen minimal media. The attenuation of the *glnL* gene shows at minimal nitrogen level there is significant growth attenuation.

Together, the Tn::*glnL* mutant growth attenuation in the presence of alligator serum (Figure 1), growth attenuation in low nitrogen media (Figure 3), and the role of *glnL* in regulating nitrogen levels within bacteria, led to hypothesis that growth inhibition of the Tn::*glnL* mutant in the presence of alligator serum is due to low nitrogen levels. To test this hypothesis, we measured the levels of free nitrogen in collected animal sera (alligator, cat, dog, lizard, and turtle) using a Spectroquant total cell nitrogen test. All samples tested were quantified in triplicate and used multiple animals when possible. The alligator sera (n=2) had significantly less total nitrogen at 15 mg/dL compared to the other samples (Figure 4). Our data was similar to published averages for the indicated species of blood urea nitrogen (BUN). Nitrogenous waste products are present in sera due to protein and nucleic acid metabolism in all animals, and include ammonia, urea, or uric acid. Most animals process and excrete nitrogenous waste as urea. Uric acid is the main form of nitrogenous waste for alligators, and therefore there is no known standard BUN concentration expected in these animals (Figure 4).

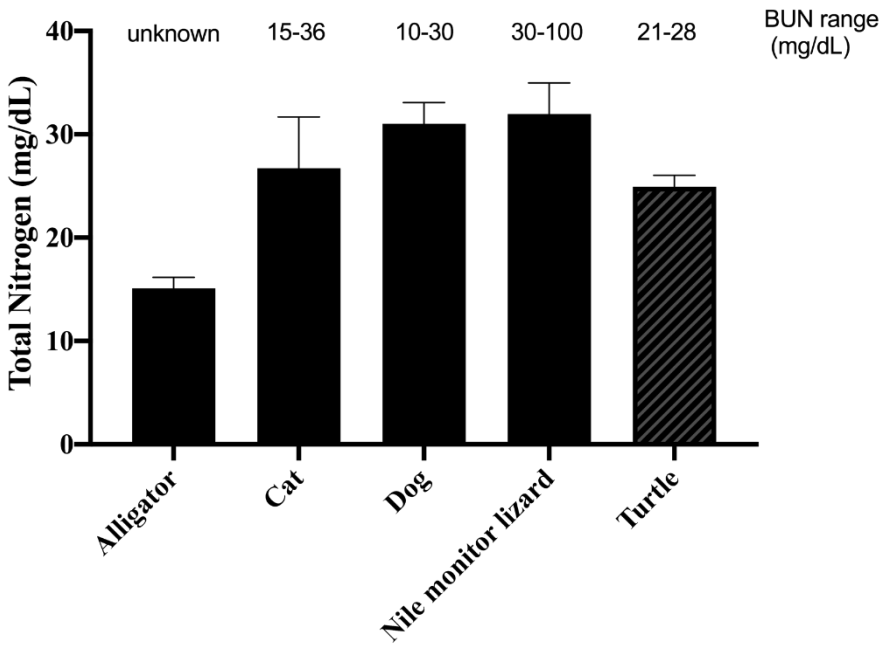

Figure 4. Utilizing a spectroquant total cell nitrogen test, animal sera was tested to determine total available cellular nitrogen. Results are reported in mg/dL. These results show that the alligator has the lowest available nitrogen thus explaining why the *glnL* nitrogen scavenging gene is required for successful resistance in the presence of alligator serum. Alligator (n=2), Cat (n=3), Dog (n=3), Nile monitor lizard (n =1), turtle (n=1)

If the presence of alligator sera is leading to a decrease in available nitrogen in the TSB broth and subsequent attenuation of growth of the *glnL* mutant, then nitrogen supplementation would rescue the growth phenotype. To test this, urea (0.1mM and 1.0mM) was added to broth containing 10% alligator serum and growth and grown overnight at 37℃. Bacteria in each condition were serially diluted and plated on TSB agar to determine cfu/ml and were compared to broth only controls. Urea supplementation of 1.0mM rescued the Tn::*glnL* growth phenotype in the presence of alligator serum, suggesting that nitrogen limitation could be the mechanism of antimicrobial activity (Figure 5).

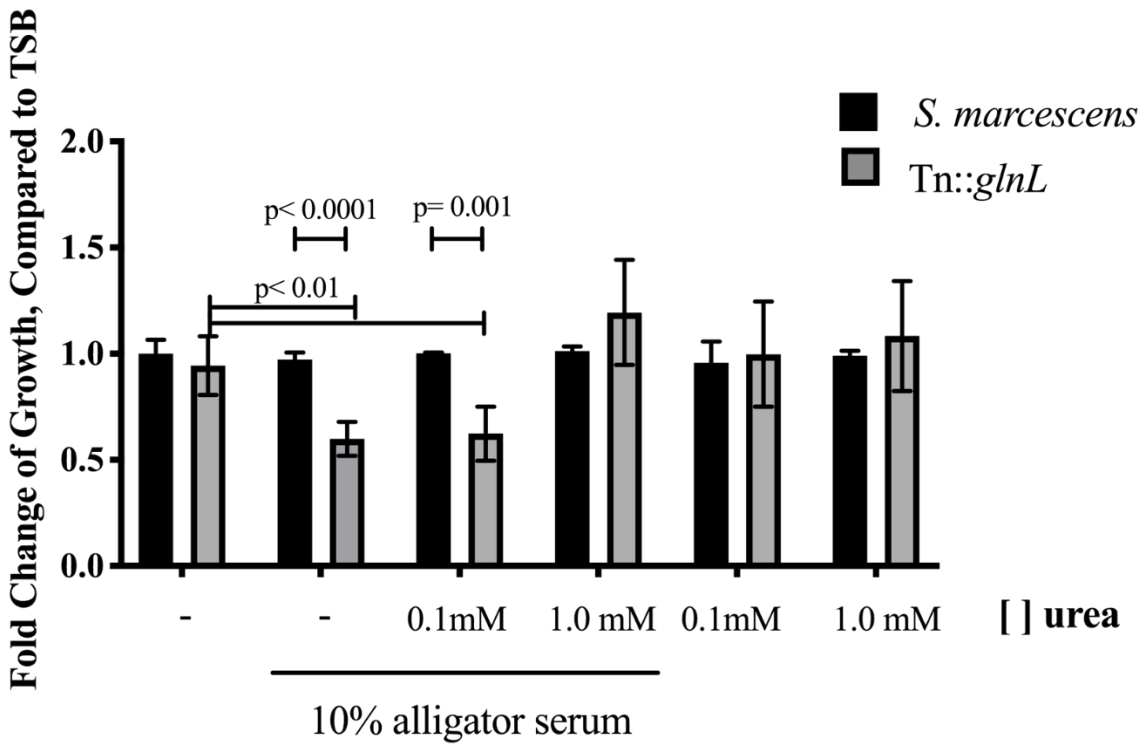

Figure 5. Urea supplementation rescues the growth attenuation of Tn::*glnL* in the presence of alligator sera. Growth of wild-type *S. marcescens* and Tn::*glnL* was assessed after 24 hours at 37ºC in the presence of 10% alligator sera and supplemented with either 0.1mM urea or 1.0mM urea. Growth in TSB only and 0.1mM and 1.0mM urea served as negative controls. Quantification of bacteria in each condition was reported in cfu/ml and performed in triplicate. Growth attenuation of the Tn::*glnL* mutant in the presence of alligator sera was rescued with the addition of 1.0mM urea. Significance was determined by student t-test.

To test whether nitrogen limitation was due to a proteinacous factor in alligator serum pooled alligator sera was heated based on standard conditions (60℃ at 30 minutes) to inactivate proteins. The heat inactivated alligator serum was added to TSB broth, and growth of wild-type *S. marcescens* add the Tn::*glnL* mutants were tested at 2 and 4 hours. Urea supplementation to alligator sera and TSB only conditions were used as controls. All samples were performed in triplicate. Bacteria were serially diluted and plated on TSB agar to quantify viable bacteria (cfu/ml). Both wild-type *S. marcescens* and the Tn::*glnL* mutant grew significantly more in the presence of the heat inactivated sera than compared to the active serum sample, similar to the growth in TSB and urea supplemented conditions (Figure 6) Therefore heat inactivation of the alligator sera reduced or eliminated its antimicrobial efficacy against the Tn::*glnL* mutant. These results suggest that the antimicrobial activity in the alligator serum is caused via a protein or peptide (Figure 6).

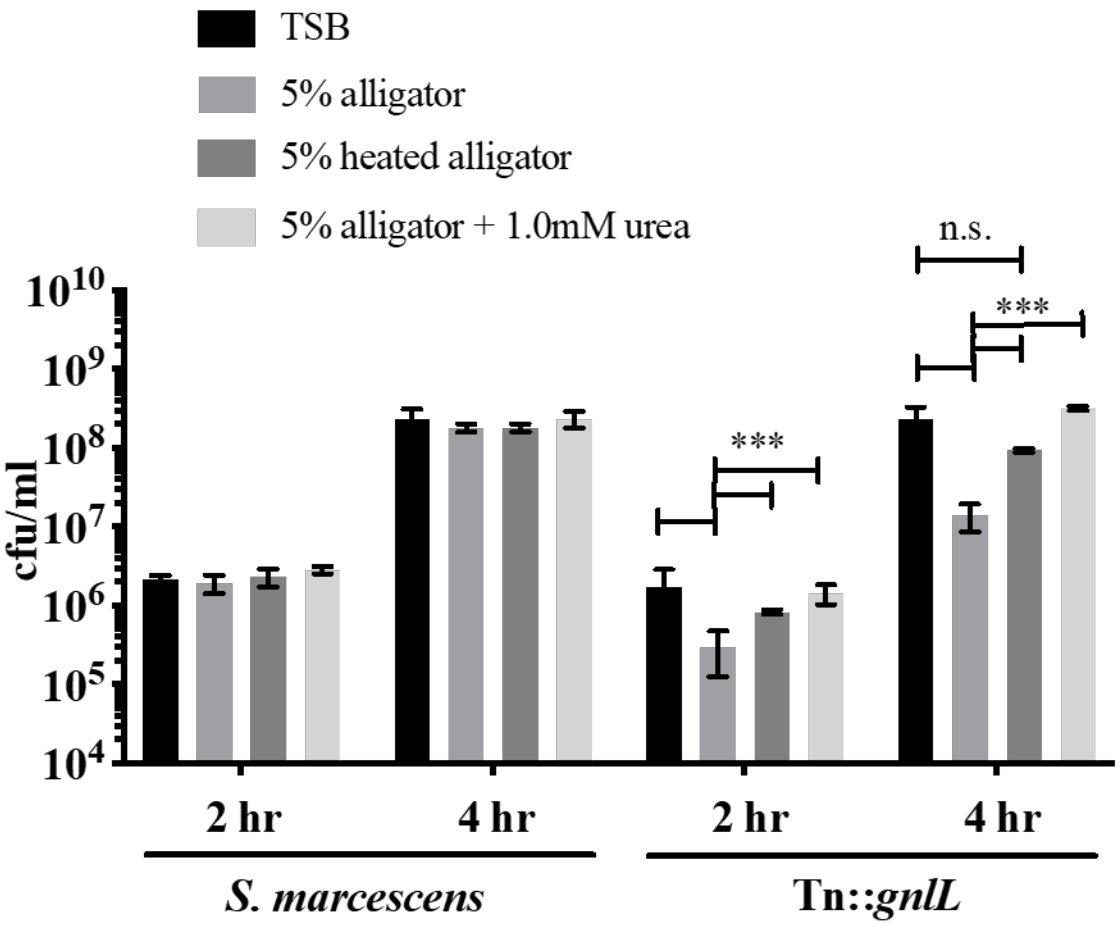

Figure 6. Heat-treatment of alligator sera alleviates growth repression of the Tn::*glnL* mutant. Growth of wild-type *S. marcescens* and the Tn::*glnL* mutant were assessed after 4 hour at 37℃ in the presence of 5% alligator sera, heat-treated alligator sera, and alligator sera with 1.0mM urea supplementation. Recovery of cfu/ml in each condition was performed in triplicate and compared to broth (TSB only controls). Growth attenuation of the Tn::*glnL* mutant in the presence of alligator serum was rescued with heat-treatment of the sera and urea supplementation. Significance is calculated using student t-test. *** $p<0.001$.

3.4 Evolutionary Relationship of Nitrogen Limitation

To better understand the nitrogen limitation as a mechanism of antimicrobial activity, we collected sera from evolutionary diverse species, and tested ability to inhibit the growth of the *glnL* mutant (Figure 7). Growing *S. marcescens* and the Tn::*glnL* mutant in the presence of 10% serum from one of cat, dog, alligator, lobster, fly, lizard, boa constrictor, bearded dragon, snake in a liquid growth broth medium then recording growth fold change compared to a TSB broth media standard (Figure 7). Alligator serum still shows significant growth reduction of Tn::*glnL* mutant compared

to wild type. The only other serum to show significant growth difference between mutant and wild type was the fly. The reduced growth form the Tn::*glnL* mutant in the presence of the fly hemolymph suggests that the fly utilizes a nitrogen limitation as an antimicrobial mechanism similar to the alligator.

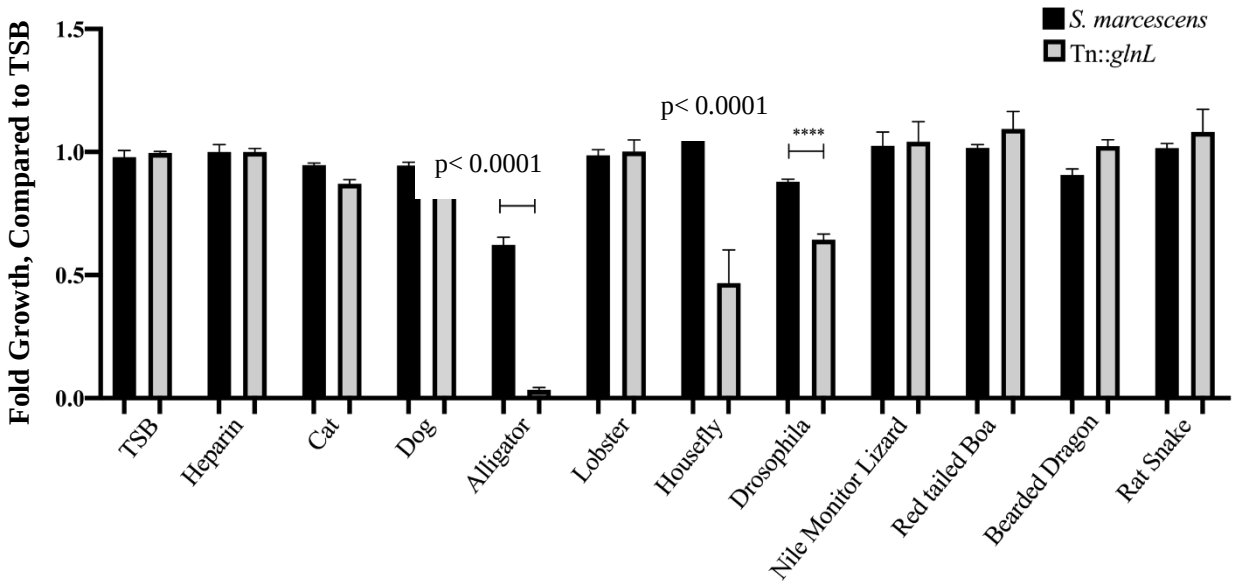

Figure 7. Direct comparison of *S. marcescens* to the Tn::*glnL* mutant in 10%. Black bars represent fold growth change in *S. marcescens* while the gray bars represent fold growth change in Tn::*glnL*. Alligator and fly both exhibit significant growth difference between *S. marcescens* compared to the Tn::*glnL* respectively. Both sera types are utilizing similar antimicrobial properties, and antimicrobial methods based on the significant increased sensitivity by the Tn::*glnL* to both the fly, and alligator

Showing similar results from Tn::*glnL* mutant growth experiments we want to look at a phylogenetic tree to see if this antimicrobial mechanism was conserved, or both species converged to acquire nitrogen limitation proteins or peptides. Based on already existing phylogenetic research, the alligator resides within the phylum of *Chordata* while the fly resides within the *Arthropoda* phylum, thus making them very unrelated to each other (Figure 8). Based on this graphical depiction of phylogenetic relatedness this evolutionary unrelatedness suggests that using nitrogen limitation as an antimicrobial mechanism was acquired independent of each organism and thus making it evolutionarily convergent between the alligator and fly (Figure 8).

The phylogenetic mapping shows convergent evolution may have taken place between the alligator and flies suggesting that similar mechanisms arose from each organism due to biological

need for inhibiting the growth of pathogens. The need for such a mechanism could've arose from similar environmental and microbial diverse living conditions. With both organisms able to scavenge for food sources, and both organisms live in brackish, dirty microbial diverse environments.

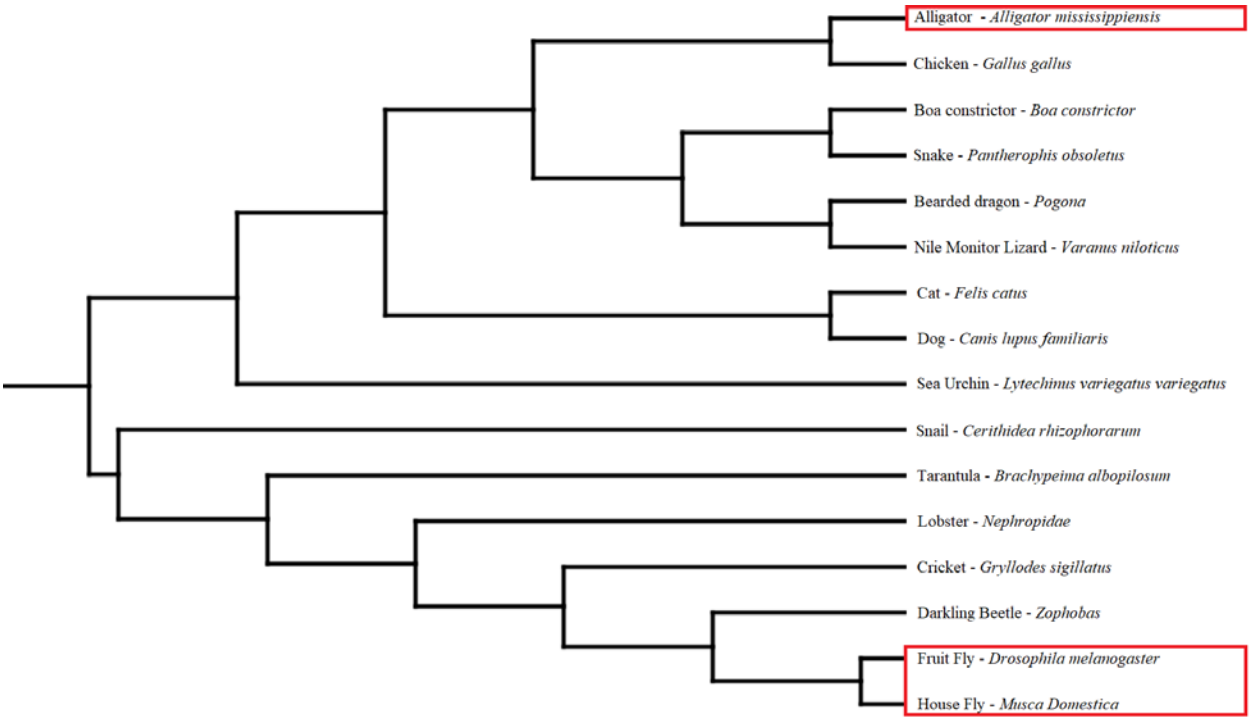

Figure 8. The Alligator and fly, both denoted with red boxes, show an evolutionary convergent antimicrobial mechanism to modulate nitrogen to impart nitrogen stress on the bacterial cell. No other groups showed this unique nitrogen modulation. The ability of two unrelated clades to produce similar antimicrobial effects show that evolutionary convergence has effected these organisms. Figure was made based off of already existing phylogenetic relationships, and is a visual aid for representation of relative relation between alligators and flies.

3.5 *D. melanogaster In vitro* Model of Nitrogen limitation

To test whether the fly hemolymph exhibits the same antimicrobial action as the alligator serum a 12 hour growth curve experiment was done to examine whether the inclusion of excess urea supplements the Tn::*glnL* mutant similar to alligator serum (Figure 9). After 12 hours in the wild type *S. marcescens* grew unimpeded by the addition of the Fly hemolymph (Figure 9A). The Tn::*glnL* mutant with the inclusion of 10% fly hemolymph in a liquid broth showed significantly reduced growth, however in 10% hemolymph and the inclusion of 1mM urea the Tn::*glnL* mutant was restored to control levels of Tn::*glnL* in standard liquid growth media (Figure 9B). These results show the fly hemolymph as limiting nitrogen to cause antimicrobial effects against the Tn::*glnL* mutant in a similar fashion as alligator serum.

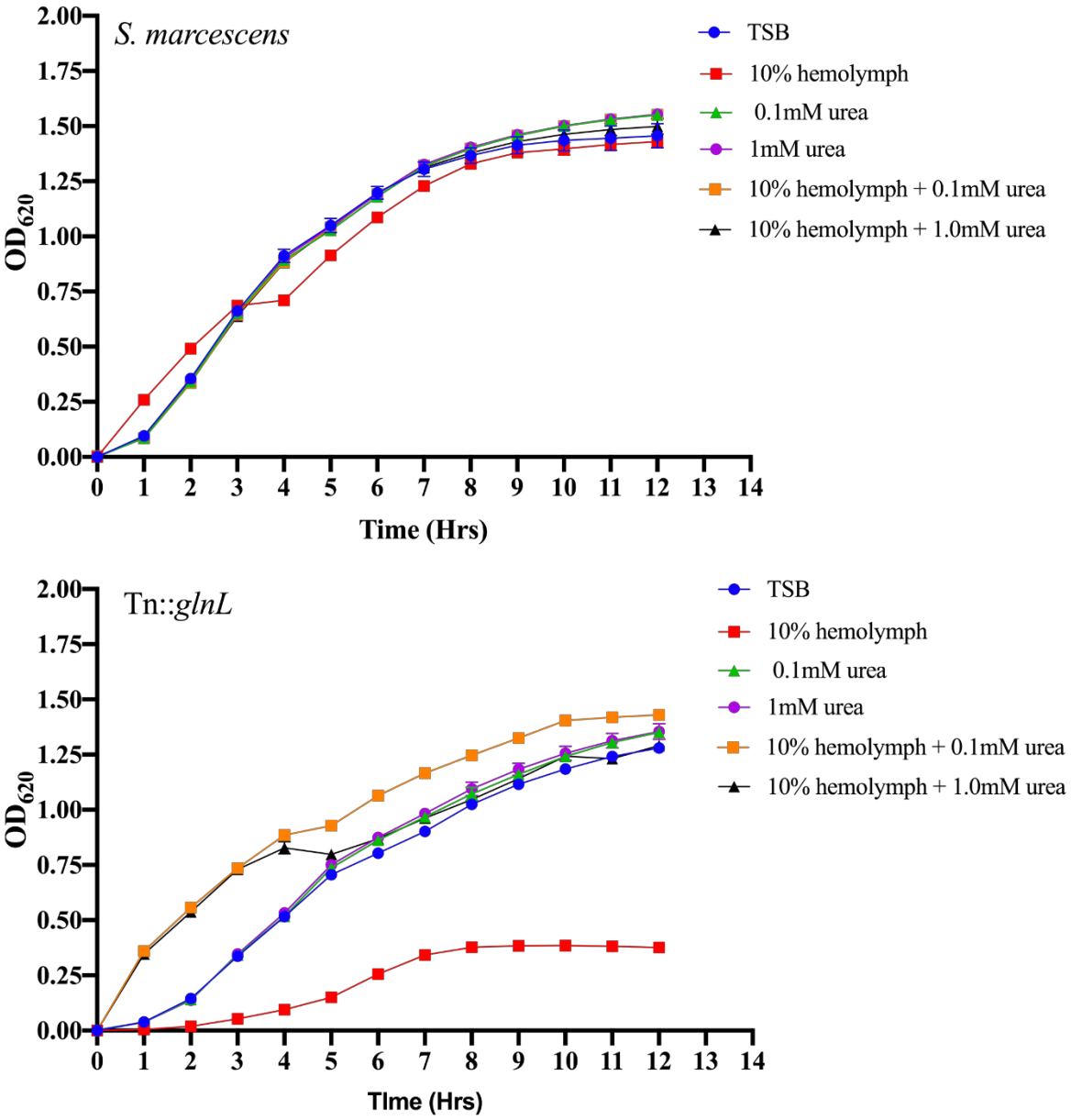

Figure 9. A) *In vitro* hemolymph results against *S. marcescens*. A 12 hour growth curve was done recording *S. marcescens* growing in standard TSB (blue), 10% fly hemolymph (red), 0.1mM urea (green), 1mM urea (purple), 10% fly hemolymph + 0.1mM urea (orange), and 10% hemolymph + 1.0mM urea (black). Wild type *S. marcescens* results to show unimpeded growth in all experimental conditions by increase of optical density (OD) as time progresses. B) *In vitro* hemolymph results against the Tn::*glnL* mutant. A 12 hour growth curve was done recording the Tn::*glnL* mutant growing in standard TSB (blue), 10% fly hemolymph (red), 0.1mM urea (green), 1mM urea (purple), 10% fly hemolymph + 0.1mM urea (orange), and 10% hemolymph + 1.0mM urea (black). Growth of the Tn::*glnL* mutant in 10% hemolymph shows attenuation of growth, while the inclusion of 0.1mM urea or 1.0mM compliments the attenuation of the *glnL* gene and brought growth to similar levels of TSB control.

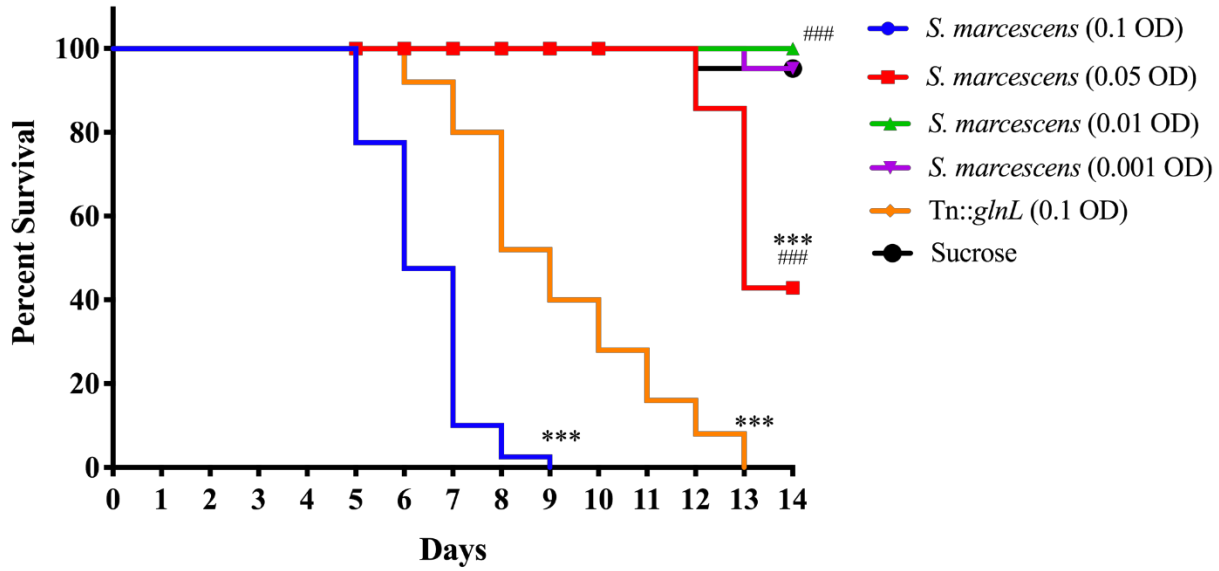

Figure 10. Attenuation of virulence of the Tn::*glnL* mutant in an oral fly model of infection. *D. melanogaster* (n=25 per group) were infected with wild type *S. marcescens* suspended in sucrose at four doses (blue, 0.1 OD; red, 0.05 OD; green, 0.01 OD; purple, 0.001 OD), the Tn::*glnL* mutant (yellow, 0.1 OD), or sucrose alone, and survival monitored daily. Flies inoculated *with S. marcescens* (0.1 OD) had 0% survival by day 9 post inoculation, and 50% of the flies inoculated with *S. marcescens* (0.05 OD) survived after day 14 post-inoculation. Plating of the inoculum for cfu/ml demonstrated that the LD50 for wild type *S. marcescens* is 1-2 x10^7 cfu/ml. Comparatively, flies inoculated with the Tn::*glnL* mutant (0.1 OD) had 0% survival rate by day 13 post infection. This data demonstrates that the Tn::*glnL* mutant is attenuated for virulence in the oral model of infection in *Drosophila*. Statistics were performed by student t-test. ### $p<0.0001$ compared to *S. marcescens* (0.1 OD), *** $p<0.0001$ compared to sucrose control.

3.6 *D. melanogaster in vivo* Model of Nitrogen Limitation

Fly hemolymph shows antimicrobial activity towards the Tn::*glnL* mutant *in vitro* however we want to determine whether these results translate to an *in vivo* model showing virulence phenotype expression. Using live flies held in vials with Whatman® paper as medium for growth by the bacterial samples. Bacterial samples were added via liquid pipetting on the Whatman® paper to allow for oral infection of the flies. The flies were incubated at 23°C which is optimal living temperature for the flies. Fly death and survivability were recorded at feeding time daily. Wild type *S. marcescens* oral infection at an inoculum of 0.1 OD had successfully killed all flies in the vial by day 9. However the Tn::*glnL* mutant at the same inoculum had killed all flies by day

13 showing that the loss of the *glnL* gene may affect virulence in flies (Figure 10). Results also show *in vivo* the Tn::*glnL* mutant has increased sensitivity to fly hemolymph.

To test whether the bacteria are proliferating *in vivo* and causing fly mortality during an infection we want to recover the bacterial samples at differing days of the infection to show proliferation. An oral infection using wild type and Tn::*glnL* mutant both at 0.1 OD and 0.05 OD was performed. The infection lasted 5 days with each day 5 flies taken from each sample to be homogenized then plated to determine cfu/mL from each sample. The wild type *S. marcescens* show as the infection length increases the bacterial load also increases (Figure 11). The Tn::*glnL* mutant at 0.1 OD show as infection length increases the bacterial load increases as well (Figure 11). The increase of bacterial load as infection length increases shows a positive correlation between dose dependence and length of infection as the dose of the inoculum increases the time until fly mortality decreases.

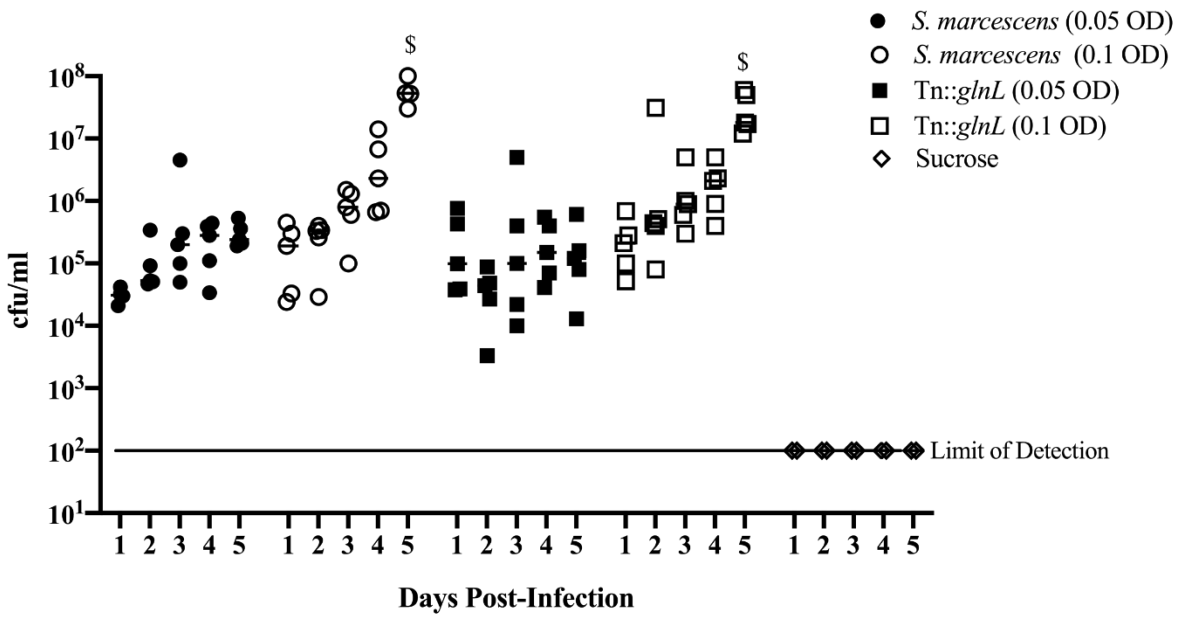

Figure 11. Correlation between fly mortality and bacterial load. Recovery of *S. marcescens* from flies infected with either wild-type *S. marcescens* or Tn::*glnL* over 5 days was quantified via fly homogenization and plating (cfu/ml). Five flies were group were tested at each time point and two doses (0.05 and 0.1 OD) of bacteria were fed to the flies. Bacterial recovery increased in flies infected with wild-type *S. marcescens* and correlated with death of the flies. $, indicating flies were morbid upon collection.

As the bacteria proliferate the immune system of the fly will produce an immunogenic response that could be fatal if there is too much shock on the system, however to determine whether proliferating live bacteria or immunogenic shock is the cause of fly mortality an infection was performed using heat killed bacterial samples. Heat killed bacteria was only heated to kill the bacteria not to denature or destroy bacterial structure. Oral infection using heat killed bacteria was done and fly survivability was recorded daily (Figure 12). Heat killed bacterial samples from both *S. marcescens* and Tn::*glnL* mutant showed no death during the infection, while compared to the control bacterial samples which had 100% mortality respectively (Figure 12). Therefore the results from having no fly mortality from the heat killed samples show that immunogenic shock is not the cause of fly mortality rather by living and proliferating bacterial load.

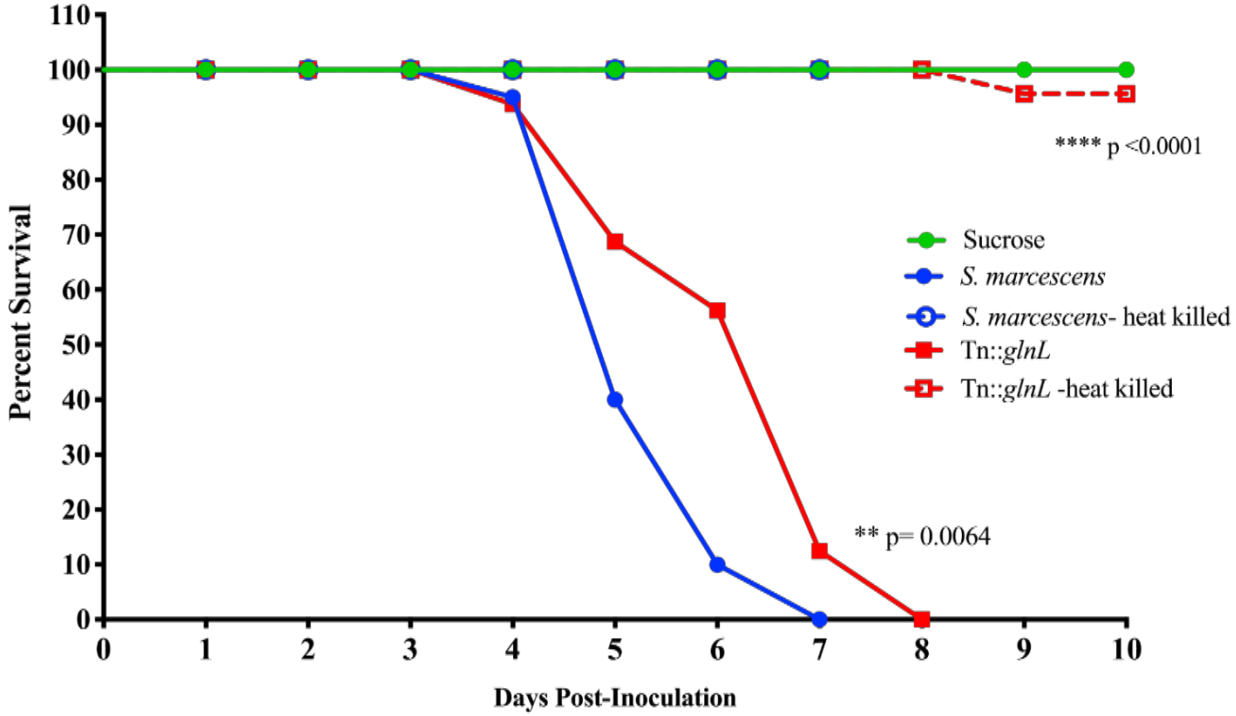

Figure 12. Fly oral infection utilizing live *S. marcescens* and Tn::*glnL* and identical heat killed bacterial samples. Wild type *S. marcescens* (WT) caused 100% mortality by day 7, while the heat killed (WT-heat killed) sample had 0% mortality. The Tn::*glnL* mutant also had 100% fly mortality except later that WT at day 8. Heat killed mutant sample (AB5 -heat killed) shows 0% mortality by the end of the live strain infection (AB5). This data shows that fly mortality is not caused by immunogenic shock rather by live proliferating bacteria. **** denotes p value comparing AB5 -heat killed to WT. ** denotes p value is comparing AB5 mutant to WT. All p values were calculated via student t-test.

To determine whether excess nitrogen can complement the loss of the *glnL* gene *in vivo* we utilized an oral fly infection. To achieve a state of excess nitrogen within the flies. 50uL of a 200mM urea solution was given 4 hours after infection to allow the bacteria to infect the fly, and achieve a high nitrogen state. Further doses of urea was given to the infected flies upon feeding and mortality recording daily. The wild type flies had 100% mortality by day 7. The Tn::*glnL* mutant infected flies with no urea achieved 100% mortality by day 9 which is significant when compared to the wild type infection length (Figure 13). The Tn::*glnL* mutant with the addition of urea achieved 100% mortality by day 7 as similar to the wild type infection length (Figure 13). These results show *in vivo* that providing excess nitrogen levels compliments the Tn::*glnL* mutants' loss of the *glnL* gene thus allowing for 100% mortality at the same rate as wild type *S. marcescens*.

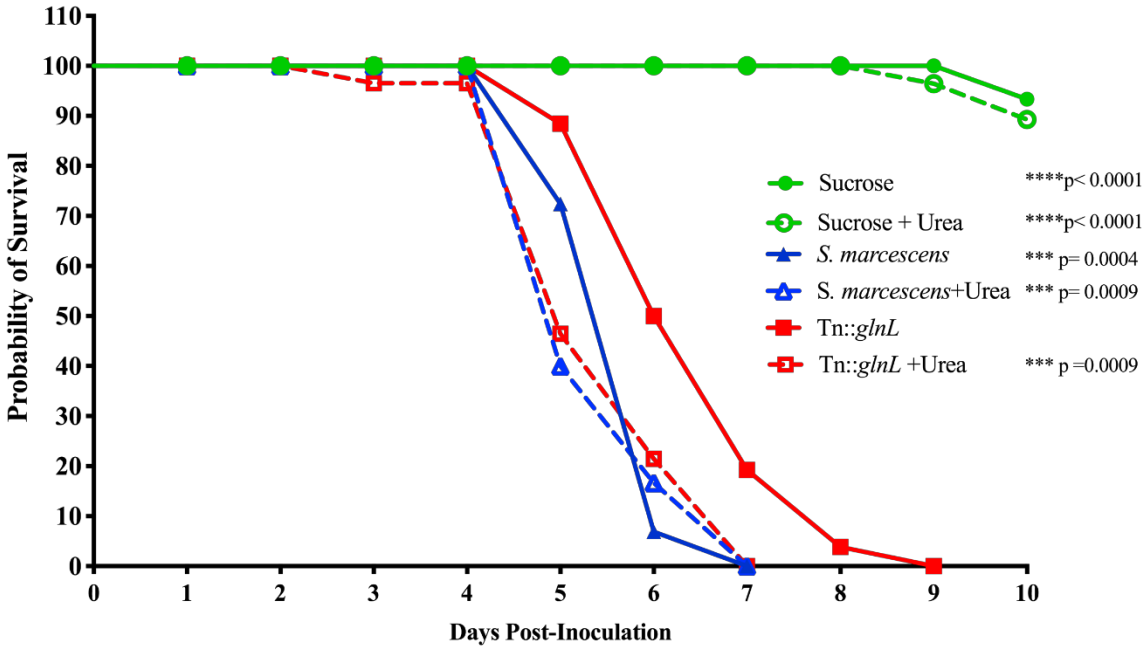

Figure 13. Oral fly infection testing *in vivo* nitrogen complementation of attenuated *glnL* gene. Several samples include an extra nitrogen source to induce complementation for the Tn::*glnL* mutant. The Tn::*glnL* mutant standard infection lasted 9 days, while the Tn::*glnL* + urea was more similar to wild type infection length at 7 days for 0% survivability. The inclusion of urea restores the virulence of the Tn::*glnL* to wild type levels, and thus decreasing the survivability of the flies over a shorter amount of time. These results show that *in vivo* the lack of the *glnL* can be complimented.

CHAPTER 4. DISCUSSION

S. marcescens has evolved many virulence factors in response to direct and indirect competition (e.g. secretion of antimicrobial secondary metabolites, nutrient sequestration, genetic exchange), host mechanisms (e.g. immune response, nutrient sequestration, AMPs), and environmental change (e.g. temperature, salinity, nutrient availability) stressors in order to proliferate and survive in a diverse range of niches and hosts. This is in part acquired through adaptation, mutation, and acquisition of new genetic elements. Opportunistic pathogens are typically characterized as microorganisms that emerge as pathogenic in their host due to a host weakness (immunodeficiency, wound, etc.) from an environmental or commensal source. *S. marcescens* has been recognized as an emerging opportunistic pathogen, and we propose that due to its adaptability to environmental niches and exposure to residing organisms has led to its increased virulence and survival within mammalian hosts. *S. marcescens* requires several virulence genes to remain resistant to immunological defenses. Environmental pressure to evolve antimicrobial mechanisms against a ubiquitous opportunistic pathogen such as *S. marcescens* is not dependent on a single host organism. Due to host-microbe immunological pressure, pathogens caused two unrelated organisms (alligator, and fly) to develop a nitrogen sequestering antimicrobial mechanism against *S. marcescens*, however due to its conserved and acquired resistances is able to overcome this hypothesized convergent antimicrobial mechanism. The evolution of this nitrogen sequestration within alligator and fly hemolymph based off of immunological need due to the constant contact, and ingestion of bacteria

Nutrient acquisition and sequestration may play a more important role in antimicrobial efficiency, and efficacy of antimicrobial peptides than previously thought. Nitrogen is a key important nutrient for proliferation. Comparing a more virulent clinical isolate (SM39) with an insect pathogen (Db11) the clinical isolate carries an operon that encodes for an ATP-binding cassette (ABC) transporter for nitrate and nitrate while the Db11 strain does not. This operon allows for the SM39 clinical isolate to utilize nitrate which is found in urine (Iguchi *et al*., 2014). It could be concluded that the ability of a pathogen to evolve several acquisition mechanisms to be able to acquire host nutrients, such as nitrogen, could lead to a more pathogenic and virulent strain of bacteria. Iron and zinc are important elements to mammals, and bacteria alike. However mammals have evolved systems to sequester iron, through transferrin, and zinc during an infection

to cause antimicrobial effects (Bruhn & Spellberg, 2015; Hesse *et al*., 2019). Alligators are even known to sequester iron as an innate immune response to an infection to produce antimicrobial effects (M. Merchant *et al*., 2007). Even through nutrient sequestration bacteria species have evolved and developed high affinity binding proteins to acquire sequestered iron and zinc (Bruhn & Spellberg, 2015; Hesse *et al*., 2019). This back and forth of nutrient sequestering and nutrient stealing shows that interactions between host and pathogen leads to reciprocal coevolution of antimicrobial and microbial proliferation strategies.

Nutrient sequestration is already known and found in mammals as the sequestering of iron and zinc is known to have antimicrobial effects (Bruhn & Spellberg, 2015; Hesse *et al*., 2019). Due to nitrogen being a vital and abundant nutrient, sequestration of nitrogen to pathogens is a good opportunity for the development of a novel antimicrobial mechanism. This hypothesized sequestering of nitrogen by host mediated immune response is uncharacterized in other research, however as previously stated sequestering of nutrients is not a new phenomenon. Nitrogen sequestration yet remains to be identified or researched. Due to the lack of all peptides and proteins within alligator sera and fly hemolymph being identified and characterized it is possible for an unidentified protein or peptide to bind, and sequester nitrogen from pathogens. Proteomic analysis of all peptides within fly hemolymph and alligator serum could lead to identification of similarly structured peptides reinforcing the hypothesis of this hypothesized evolutionary convergent antimicrobial mechanism. Nitrogen modulation, may be a possible avenue for future therapeutics within the clinical setting. Further modulation of nitrogen may allow for control of microbial populations within environments, or agricultural settings. Targeting of sensory pathways by novel therapeutics may allow for specific antimicrobial clearing or increased sensitivity by targeted pathogens that allow for usage of current therapeutics against previously resistant microbes.

The *glnL* gene is a sensory histadine kinase that gets activated by the nitrogen regulatory protein C (NtrC). This NtrC activated transcription causes the expression of regulatory proteins such as *ntrC* when the bacterial cell is under nitrogen stress. The *glnL* gene is used to ameliorate the slowing of growth when under limiting conditions of nitrogen, or to scavenge nitrogen containing compounds when under nitrogen limiting conditions (Zimmer *et al*., 2000). Enteric *E. coli* detects this low nitrogen state via decrease of internal glutamine. In order to alleviate the low presence of nitrogen the products of *glnL* activation have three main roles. Firstly expressed proteins mediate the assimilation of ammonium into glutamine, and glutamate via transporter

proteins, and glutamine synthetases. Secondly is to produce glutamine from catabolic means such as degradation via production of enzymes. Lastly to relieve nitrogen stress the *glnL* products will spare the requirements for glutamine and glutamate (Zimmer *et al*., 2000). *E. coli* uses the NtrC/*glnL* pathway as a main defense against nitrogen limitation. *S. marcescens* is known to have and utilize the *glnL* gene, however a BLASTN using sequence search reveals that the *E. coli glnL* gene has only 77.81% identity to *S. marcescens* (Zhang *et al*., 2000). The differing of about 22.19% identity between *E. coli*, and *S. marcescens* is a hypothesized cause for the resistance to alligator serum along with fly hemolymph. The *glnL* gene is largely uncharacterized within *Serratia marcescens*. This work shows that the *glnL* gene found in *S. marcescens* is required for virulence *in vitro* and *in vivo* in the presence of alligator sera and fly hemolymph. The *glnL* gene shows to have a downstream effect on the ability of the bacterial cell to defeat antimicrobial mechanisms related to nitrogen sequestration.

Alligators are known to live in swamping, microbial diverse environments. Similarly flies are common insects found eating decaying plant material, fruits, and other organic matter therefore they also live in microbial diverse environments. Fruit flies and alligators are both possible hosts for, *S. marcescens* however the route of infection can differ between organisms. Flies encounter vast arrays of microbes via oral ingestion of food. Alligators are commonly found with wounds on their hide, while also ingesting a large array of microbes through prey as well. Alligators are not often seen to have delirious effects from such open wounds, but *S. marcescens* is a known pathogen that causes septicemia in alligators (Novak & Seigel, 1986). *S marcescens* is a known insect pathogen infecting flies, mosquitos, among others, however it is special in that it is able to escape the digestive tract and cause a systemic infection (Nehme *et al*., 2007). This ability of *S marcescens* to infect hosts such as the evolutionary ancient alligator, and the microbial feasting flies shows the plasticity to adapt to these niches.

Host-pathogen interactions is an ever evolving system similar to the hypothesis of Red Queen dynamics (Papkou *et al*., 2019). Just as pathogens develop new virulence factors to allow for better pathogenicity, hosts will develop stronger, and more effective immune defense mechanisms against those pathogens. Alligators have an extremely potent innate immune system due to being 83 million years old and constantly evolving new antimicrobial mechanisms and peptides to better survive against a vast and diverse range of microbes within their habitat, except for *S. marcescens* (van Hoek, 2014). Selective adaptations is a constantly evolving back and forth

between host and pathogen. Our work has discovered a convergent nitrogen dependent host mediated antimicrobial mechanism from two evolutionary divergent organisms. We were able to discover this mechanism through a transposon mutagenesis screen, and identifying *glnL* as being required for virulence against these hosts.

To further gain a more complete indication of evolutionary convergence more samples from all tested clades would be required. The tested representative species results conclude that evolutionary convergence has taken place to develop similar immune strategies of nitrogen sequestration as an antimicrobial mechanism against, as identified by our *glnL* mutant.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Results from this study indicate that there is an evolutionary convergent nitrogen dependent antimicrobial mechanism against *S. marcescens* via host mediated nitrogen sequestration. The generated Tn::*glnL* mutant shows increased sensitivity to alligator sera, and fly hemolymph through attenuation of the *glnL* gene, however attenuation of this gene did not affect its resistance to other animal sera types tested. Knowing the *glnL* gene encodes for a nitrogen scavenging system to alleviate nitrogen stress, it is hypothesized that both alligator sera, and fly hemolymph is imparting antimicrobial nitrogen stress on the bacterial cell via a nitrogen sequestration mechanism. In contrast the mutant strain can be rescued to wild type phenotype and virulence via addition of an organic nitrogen source in excess. Based on *in vitro* growth assays and *in vivo* infection experiments it was also concluded that the *glnL* gene is required for virulence in *S. marcescens* when in the presence of alligator serum, or fly hemolymph. Phylogenic comparison revealed that flies, and alligators are not phylogenetically related, and no other of tested sera types expressed similar nitrogen dependent antimicrobial results. The hypothesized antimicrobial mechanism of nitrogen sequestration was not evolutionarily conserved among several organisms. This antimicrobial mechanism is hypothesized to be convergent through environmental exposure adaptation to a large array of microbial life. Identification of this novel and previously unidentified antimicrobial mechanism expands on the knowledge of nutrient limitation by host mediated immune responses. This work also emphasizes that evolution of similar antimicrobial immune mechanisms is dependent on habitat factors, and microbe interaction by the host, and not solely on genetic factors.

These results are first to hypothesize an uncharacterized host mediated nitrogen sequestration mechanism as an antimicrobial response to infection by bacteria. Showing two separate unrelated organisms share a similar pathway provides insight into the evolutionary lineage, and expression of the *glnL* gene as a process related to coevolution of hosts and pathogens.

Future outlook regarding this research includes proteomic comparison and isolation between alligators and flies. Genomic mapping of shared antimicrobial peptide sequences between both flies and alligators could provide a more concise indication and identification of evolutionary convergence though similar habitat exposure to diverse microbial species. Fluoresce testing of protein expression during an *in vivo*, and *in vitro* infection will be able to quantify relative protein

expression, and location of expressed proteins. Conversely fluorescence of all active proteins during nitrogen limiting will allow for better understanding of the receptors associated with the *glnL* regulatory pathway.

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