ANTIMICROBIAL RESISTANCE IN *SERRATIA MARCESCENS*

by

Danielle Susan Sopovski

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STATEMENT OF COMMITTEE APPROVAL

Dr. Lindsay Gielda, Chair

Department of Biological Sciences

Dr. Scott Bates

Department of Biological Sciences

Dr. Michelle Spaulding

Department of Biological Sciences

Approved by:

Dr. W.T. Evert Ting

Head of the Graduate Program

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ABSTRACT

Author: Sopovski, Danielle, S. MS
Institution: Purdue University
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Title: Antimicrobial Resistance in *Serratia marcescens*
Committee Chair: Lindsay Gielda

With the increase of antibiotic resistant bacteria strains, the need to determine the mechanisms of antimicrobial resistance is similarly rising. *Serratia marcescens*, a ubiquitous, Gram-negative opportunistic pathogen is known to have strong, natural resistance to diverse antimicrobial agents including antibiotics and antimicrobial peptides. Recently, we identified *S. marcescens* as one of the few bacteria resistant to antimicrobial compounds produced by *Stemphylium vesicarium*, an isolated fungal spinach endophyte. To identify the mechanism of antimicrobial resistance to the unknown *Stemphylium* antimicrobial compounds, we designed a transposon mutant screen identifying mutants sensitive to antimicrobial inhibition of bacterial growth. A transposon mutant library was constructed using the Tn5 EZ-Transposome (Epicentre) system and contains 1,824 individual mutants with 127 being identified as having a decreased resistance to the *Stemphylium* antimicrobial compounds. The transposon growth inhibition screen initially evaluates the mutants for reduced growth in the presence of 25% fungal metabolite over 24 hours. The growth phenotype is then confirmed in triplicate in a 12-hour time course growth experiment. Identification of the genomic insertion site of the Tn5 transposon utilized a multi-step modified nested-PCR protocol, termed TAIL-PCR. Following PCR purification, nanodrop spectroscopy and gel electrophoresis were performed to ensure the amplification purity of the extracted DNA and was subsequently sequenced via WideSeq analysis. BLAST identified insertions in genes necessary for membrane biogenesis, drug transport, pili formation, and iron metabolism. Future work is aimed at confirming these results and understanding the role of iron sequestration. Not only will this research contribute to our understanding of *S. marcescens* antimicrobial resistance mechanisms, but it aids in our understanding of the mechanisms of antimicrobial resistance development in other human pathogens.

INTRODUCTION

Serratia marcescens is a rod-shaped Gram-negative, facultative anaerobic bacterium from the family *Enterobacteriaceae* (Grimont & Grimont, 2015). Discovered in 1819 by Bartolomeo Bizio in Padua, Italy, *S. marcescens* was first thought to be a harmless bacterium that could be a useful marker for tracing bacterial transmission due to its distinctive red pigmentation (Yu, 1979). *S. marcescens* was used in this way by the United States government until it was discovered that it could cause severe infections in the 1950s during project "Operation Sea Spray" where the U.S. Navy secretly released *S. marcescens* off the coast of the San Francisco Bay Area in California (Yu, 1979). During this time, 11 people contracted rare urinary tract infections, stemming from *S. marcescens*, after undergoing a medical procedure in a local hospital, with one of those eleven succumbing to the infection (Yu, 1979). Though there are 14 species in the genus *Serratia*, the most prevalent human pathogenic species is *S. marcescens*, which has a high prevalence of multidrug-resistance strains (Iguchi et al., 2014). *S. marcescens* is now regarded as a ubiquitous opportunistic pathogen responsible for a high incidence of nosocomial infections; with novel, multiple multidrug-resistant strains appearing that could pose a major public health threat worldwide (Campos-Cortés et al., 2018). The mechanisms of resistance development seen in this particular *Serratia* species has been an ongoing area of research for decades.

S. marcescens has been isolated from diverse environmental niches and it demonstrates considerable growth plasticity. For example, *S. marcescens* has been isolated from animals, humans, insects, mushrooms, soil, and water (Hejazi & Falkiner, 1997), and growth has been reported under a wide range of temperature (10–36°C), pH (5–9 units), and osmotic pressure (0–4% NaCl; Grimont and Grimont, 2015). The adaptability demonstrated by *S. marcescens* to these diverse conditions also extends to its ability to evolve resistance to antimicrobial compounds. Studies have suggested bacteria capable of surviving in diverse microbial communities, including the human microbiome, develop resistance due to exposure and interaction with a wide range of antibiotic resistant bacteria and resistance-related genes (Vaz-Moreira 2014). To date, approximately 4,000 genes have been characterized in *Serratia* (Iguchi et al., 2014), and roughly 70% of these genes are shared across all species, with a majority of these being involved in basic cellular function (Li et al., 2015). There are, however, nearly 400 genes primarily thought to be

related to pathogenicity that are unique to *S. marcescens*, including virulence factors and antimicrobial resistance mechanisms (Iguchi et al., 2014). Analysis of two different *S. marcescens* strains, human clinical (SM39) and insect pathogenic (Db11) isolates, showed a high degree of colinearity despite low nucleotide similarity between coding regions of the two genomes; while the prevalence of isolate-specific sequences was thought to reflect genetic adaptation to the distinct niches these strains occupied (Iguchi et al., 2014).

It is estimated that there are 10^{30} bacteria currently inhabiting the planet (Schloss & Handelsmann 2004), and only a small portion of these are antibiotic producers (Alonso et al., 2001). Origination of antibiotic resistance genes is thought to occur in antibiotic producers. For example, tetracycline resistance genes present in some *Mycobacteria* strains originated in the bacterium *Streptomyces rimosus*, which is a tetracycline producer (Alonso et al., 2001). However, there are numerous antibiotic resistance genes that do not appear to have homologs among antibiotic producers (Alonso et al., 2001). A growing body of bioinformatic evidence suggests multiple origins of antibiotic resistance genes (Alonso et al., 2001). All bacterial genomes, including those of small size, have genetic information dedicated to the production of molecules for nutrient procurement, environmental sensing, and protection. These include genes coding for receptors, transport and efflux proteins, immune system elements, and catabolic enzymes (Wright, 2010). Microbial genes such as these can be adapted via natural selection to respond to the exposure of antimicrobial compounds in the environment, highlighting the role selective pressure plays in the evolution of antibiotic resistance genes (Wright 2010). For instance, *S. marcescens* may have obtained one resistance mechanism in this manner, as aminoglycoside-modifying enzymes present in some *S. marcescens* isolates perform metabolic roles in addition to their aminoglycoside antibiotic inactivation activity, indicating that these enzymes likely evolved from sugar kinases or acyltransferases (Alonso et al., 2001). Given the various roles the environment plays in the evolution of known antibiotic resistance genes, its potential to give rise to novel forms, and its capacity to contribute to microbial resistance gains, ubiquitous and plastic organisms, such as *S. marcescens*, hold great potential for providing insights into the dynamics of bacterial antibiotic resistance.

Further demonstrating a remarkable capacity for adaptation, *S. marcescens* has been shown to be capable of growing in disinfectants, antiseptics, and even double-distilled water (Szewzyk et al., 1993), which likely contributes to *S. marcescens* being one of the most common causes of nosocomial infections. *S. marcescens* is considered a true opportunistic pathogen, with only a few rare cases of community-acquired, rather than nosocomial infections, being reported (Hagiya et al., 2016). A recent study showed in the U.S. and Europe that *S. marcescens* is responsible, on average, for 6.5% of all Gram-negative infections in Intensive Care Units, ranking 5th place among Gram-negative organisms present in ICUs (Sader et al., 2014). Further, *S. marcescens* have a predilection for infecting the respiratory and urinary tracts, as well as the bloodstream (Hagiya et al., 2016). In an analysis of records spanning from 1966 to 2015, 16 cases of necrotizing fasciitis caused by *S. marcescens* have been reported, with 75% of these cases ultimately leading to death (Majumdar & Crum-Cianflone, 2016). Virulence of *S. marcescens* may be directly related to its capacity for growth and survival outside of the human host. While initially it was thought that the evolution of virulence traits was highly dependent on the efficiency of host-to-host transmission (Mikoranta et al., 2015), the current theory suggests conflicting selection pressures of inside vs. outside-host have more of a role to play in shaping the evolution of virulence. In a study of *S. marcescens* grown both in the host (*Drosophila melanogaster*) and outside-host environment, bacterial adaptation to each condition was marked by rapid evolutionary changes (Mikoranta et al., 2015). Significantly, the *S. marcescens* cells grown within *D. melanogaster* showed reduced virulence as compared to outside-host colonies that maintained the same level of virulence (Mikoranta et al., 2015). This suggests *S. marcescens* may gain a competitive advantage in infecting hosts over other bacteria with a lesser capacity of survival in the outside-host environment.

The evolutionary history of *Serratia* has not been estimated; however, its ubiquitous nature suggests an early divergence for the genus within the Enterobacteriacieae (Burke, 2011; Iguchi, 2014). Production of antibiotic compounds is also likely an ancient condition within *Serratia* species, as β-lactamases are known to have evolved over 2 billion years ago before the split between Gram-positive and Gram-negative bacteria (Hall & Barlow, 2004). It is reasonable to assume, therefore, that mechanisms of resistance co-evolved within *Serratia* as they diverged from the Enterobacteriacieae. With the rising threat of antibiotic resistance, the need to understand the

evolution, development, and acquisition of resistance by pathogens is essential for combating emerging pathogens as well as emergent drug-resistant bacterial strains. *S. marcescens,* with its ubiquitous and competitive nature, broad resistance to antimicrobial compounds, and importance to human health, represents an ideal model organism for study. Here, we review the known virulence factors contributing to the transmission and pathogenicity of *S. marcescens,* highlighting the mechanisms of antimicrobial resistance that demonstrate its adaptability due to genomic plasticity.

Mechanisms of Virulence and Pathogenesis

Like other opportunistic pathogens, *S. marcescens* has low intrinsic pathogenicity. Many patients become infected with *S. marcescens* through invasive instrumentation, such as catheterization or intubation (Maki et al., 1973). Many strains carry several plasmid-borne antibiotic resistance factors, and even among patients receiving heavy doses of broad-spectrum antibiotics, exposure to *S. marcescens* quickly causes infection. *S. marcescens* infection often leads to sepsis due to its capacity for effective colonization, which includes its ability to survive aerobic and anaerobic conditions, and the presence of specific virulence factors, such as hemolysins, the lipopolysaccharide membrane, quorum sensing, and the ability to produce biofilms (Campos-Cortés et al., 2018). Virulence factors of *S. marcescens* are complex and interconnected and likely reflect ancient acquisition and subsequent co-evolution of several factors.

Serratamolide

Serratamolide, a cyclic and aliphatic aminolipid, is a hemolytic factor produced by *S. marcescens* that contributes to virulence (Shanks et al., 2012). Hemolysins are important virulence factors for *S. marcescens* due to their diverse cytotoxicity potential, including their ability to destroy red blood cells, fibroblasts, and epithelial cells, and the resultant release of histamine and generation of leukotriene. These events increase vascular permeability, granulocyte accumulation, and edema formation, which contributes to further spread and progression of the infection (Campos-Cortés et al., 2018). Production of serratamolide is part of the RssAB-FIhDC-ShIBA pathway that regulates the switch between biofilm production and swarming ability and is therefore responsible for regulating key virulence traits. At 37°C (temperature of the average human body) the RssA-RssB sensor kinase and response regulator system represses flagellar and hemolysin related genes,

decreases swarming, and eventually leads to the production of a mature biofilm. When the RssA-RssB system is non-functional due to mutations, the *S. marcescens* can become hypervirulent due to increased hemolysis and swarming (Lin et al., 2010).

Motility and Quorum Sensing

S. marcescens strains are capable of several types of motility including swarming and swimming motility, as well as flagellar-independent sliding. Flagella of *S. marcescens* serve for both movement and sensory purposes, as they can help propel a cell through various linings of tissue while sensing and mediating adaptation to their surroundings. Regulation of flagella synthesis is dependent on several factors, mediated by the aforementioned RssAB-FIhDC-ShIBA regulatory pathway. In addition, biosynthesis conditions play a key part in regulating flagella. PhlA phospholipase is secreted by the flagellar apparatus and simultaneously takes part in regulation. It was recently discovered that PhlA can be inhibited by the assembly of enterobacterial common antigen (ECA), a glycolipid that can be found in the outer membrane of Gram-negative bacteria (Castelli et al., 2008). The role of ECA was not clearly understood until a study looked at a cluster of flagellar assembly mutants. In ECA defective strains, the transcriptional process was completely turned off for flagellar assembly due to down regulation of expression of *flhDC* (Castelli et al., 2008). Temperature is another key determinant of swarming behavior. At 37°C the RssA-RssB component system signals and directly inhibits *flhDC* promoter activity which is directly responsible for synthesis of flagella (Soo et al., 2008). Swimming behavior in *Serratia* is also regulated by physiochemical conditions, like other Enterobacteriacia species. (Darton 2010). Bacteria grown in liquid media were short rods with either one or two flagella that showed classical swimming behavior. When these bacteria were transferred to a solid surface, cells elongated and expressed anywhere from ten to one hundred flagella, switching to a swarming locomotion method. (Alberti & Harshey, 1990). These dynamics enable *S. marcescens* to move in a variety of environmental conditions and contribute to pathogenesis in humans as shown in the hypervirulent strains mentioned above that had no regulation of swarming (Lin et al., 2010).

Quorum sensing, or the ability of bacteria to detect one-another and communicate within a population, is a common strategy of bacteria living in various environments, and it can also lead to the regulation of virulence traits in several species (Antunes, 2010). For example, quorum

sensing by *Serratia* species has been shown to regulate several activities including motility, cell differentiation, as well as prodiogiosin and biofilm production (Antunes, 2010). Like other Gram-negative bacteria, *S. marcescens* produces N-acylhomoserine lactone (AHL) as the main quorum-signaling molecule (Van Houdt et al., 2007). In low population densities, AHLs are synthesized and secreted from the cell; however, as populations grow, extracellular AHL levels increase, leading to an intracellular influx of AHL. At this point, AHL binds into a complex with transcriptional regulatory R protein, resulting in the expression of downstream target genes in multiple cells in the population (Van Houdt et al., 2007). AHL regulates a variety of phenotypic changes including biofilm formation, prodigiosin production, and motility (Van Houdt et al., 2007). Quorum sensing inhibitors (QSI) have also been shown to have a variety of effects on *S. marcescens* colonies. For example, cells that interacted with QSIs demonstrated up to a 93% reduction in prodigiosin production, a 93% reduction of protease production, a 47% reduction of hemolysin production, and showed a visible reduction in biofilm formation (Annapoorani et al., 2012). Petroselinic acid (PSA), a fatty acid produced by humans, was also shown to significantly affect quorum sensing through down regulation of QS-controlled virulence genes, which slowed motility and swarming and resulted in the inhibition of prodigiosin production (by 75%), protease production (by 59%), and biofilm formation (by 72%; Ramanathan et al., 2018).

Biofilms

Biofilms are a self-secreted complex of bacterial cells and biomolecules like polysaccharides, nucleic acids, lipids, and proteins. Biofilm formation by *S. marcescens* mediate the attachment and growth of cells on a variety of surfaces including contact lenses and epithelial cells and has been implicated in the development of nosocomial infections due to adherence to medical devices. Biofilms contribute to the pathogenicity of up to 80% of all human bacterial infections (Jamal et al., 2018), even with the advancement of engineered materials and discovery of substances designed to inhibit bacterial biofilm formation. Within the body, biofilm formation is so adaptive bacteria can utilize the cells of the host's immune system to benefit in the formation. For example, in cases of colonization on contact lenses the biofilm formation is strengthened in the presence of dying neutrophils and their excretions (Patel et al., 2018). While biofilm formation represents a threat in terms of human health and bioindustrial settings, bacteria embedded within an

extracellular matrix can develop resistance to environmental stresses necessary for environmental survival and competition, which represents an adaptive strategy by *S. marcescens*.

While adherence to diverse substrates is known, the mechanism of *S. marcescens* attachment to biotic and abiotic surfaces remains unclear (Kalivoda 2013). Abiotic surface adhesion is AHL regulated, but biotic surface adhesion is not (Labbate 2007). However, type I fimbriae, widely expressed bacterial surface pili implicated in the virulence of several pathogens including *S. marcescens*, have been shown to be necessary for *S. marcescens* biotic surface adhesion. While quorum sensing has been suggested to be responsible for several stages of biofilm formation, including attachment, swarming, and detachment (Rice et al., 2005) (Labatte 2007), alternative studies suggest that genes necessary for nutrient utilization regulate biofilm production (Kalivoda 2013). Under reduced carbon or nitrogen conditions, *S. marcescens* forms a classic microcolony biofilm. The biofilm type is dependent on media nutrient composition though. *S. marcescens* can switch between a microcolony and filamentous biofilm in response to media nutrient composition changes even after establishment of the biofilm (Rice et al., 2005). This is due to the catabolite repression system (CRS) that regulates the utilization of less preferred carbon sources through modulation of cyclic AMP (cAMP) levels and influences *S. marcescens* biofilm formation (Kalivoda 2013). In a study assessing the contribution of cAMP in regulating motility, type I fimbriae (encoded by the *fimABCD* operon) and biofilm formation were decreased in response to cAMP increase due to a deletion of cAMP-phosphodiesterase. (Kalivoda 2013). These results suggest that nutrient cues play an important role in biofilm synthesis.

In *S. marcescens*, cAMP-CRP dependent pathways not only regulate biofilm formation, but control motility, prodigiosin production, hemolysis, and the production of flagella, demonstrating complex regulatory pathways that are not well understood. A homolog of the PigP transcription factor has been shown to positively regulate secondary metabolite biosynthesis, including prodigiosin through serratamolide production. PigP works in conjunction with CRP and HexS, transcription factors that negatively regulate serratamolide, within the cAMP-CRP pathway (Shanks et al. 2013). Mutations of the *pigP* gene resulted in a loss of swarming motility, hemolysis, and had drastic reductions in both prodigiosin and serratamolide synthesis indicating the large role PigP has in regulating several key virulence factors of *S. marcescens* (Shanks et al. 2013). In addition,

the IgaA/UmoB family proteins are found in members of the *Enterobacteriaceae* family and play a large role in regulating secondary metabolites as well. These are inner membrane proteins that report extracellular stress to intracellular signaling pathways that respond to environmental change (like the cAMP-CRP pathway). The *gumB* gene was a novel discovery that when mutated conferred a severe loss of prodigiosin and serratamolide, but it is not essential for growth like other IgaA/UmoB family members. The *igaA* gene from *Salmonella enterica, yrfF* gene from *Escherichia coli,* and uncharacterized predicted ortholog from *Klebsiella pneumoniae* complemented the *gumB* mutant defects suggesting a highly conserved and selected function (Stella et al. 2018).

Prodigiosin

The most identifying factor of *S. marcescens* is its red pigment, prodigiosin, produced at temperatures below 30°C (Tanaka et al. 2004). Prodiogiosin is categorized as a secondary metabolite that belongs to a class of tripyrrole compounds and has a range of colors from light pink to blood red. It is produced not only by *Serratia*, but by *Pseudomonas*, *Vibrio*, and *Hahell* as well (Kimyon et al. 2016). Prodigiosin's biological activities have been the focus of several recent studies that show its ability to aid in *Serratia* competition within the environment by inhibiting other species. Acting as a hydrophobic stressor with the use of redox active metabolites it can cause membrane damage in a variety of bacteria (Surywanshi 2017). For example, *Pseudomona* biofilm formation is inhibited in the presence of these metabolites (Kimyon 2016). When applied to food stuff bioactive prodigiosin scavenges free radicals inhibiting bacterial growth (Arivizhivendhan 2018). Not only is prodigiosin efficient at eliminating competitors, but it also has benefits to metabolic functions. Prodigiosin has been found to have a key role in *S. marcescens* fitness, with pigmented cells accumulating ATP more rapidly and multiplying quicker leading to an overall increase in biomass (Haddix & Shanks 2018). Prodigiosin has also been discovered to play a role against cancer cells. When treated with prodigiosin, high-levels of survivin expression in human hepatocellular carcinoma cells were reduced to levels found in normal cells. It also changed the morphology of these cells to apoptotic forms and disrupted cell connections by increasing growth inhibition rates and decreasing metabolic activity (Yenkejeh & Esmaeillou 2017). In two different lines of human oral squamous carcinoma cells, prodigiosin was shown to effectively cause cell death and cell-cycle arrest (Chen et al. 2017). While *S. marcescens* is

ubiquitous, it more than likely does not encounter cancer cells very frequently in its environment. This shows how *S. marcescens* has evolutionary adaptations that can serve multiple purposes that are beneficial to the organisms, even if that wasn't the original function of the adaptation.

Interestingly, even though prodigiosin enables *S. marcescens* to eliminate competition and improve its own growth; its role in virulence is not very well understood. While there are some instances of *S. marcescens* colonization causing breast milk to turn pink and the babies that feed on this milk to have pink stool (Quinn et al. 2018), most strains associated with hospital outbreaks are of the non-pigmented variety (Hejazi & Falkiner, 1997). In the 8 case studies mentioned above in the colonization of breast milk, good clinical outcomes were reported even in cases where antibiotics were not used whereas the non-pigmented strains can often prove fatal. In a study done on silkworms, while the two different pigmented strains caused different symptoms (red coloration in silkworms with the pigmented strain), there were very similar LD50 values between them indicating that prodigiosin did not play a key part in virulence here (Zhou et al. 2016).

Lipopolysaccharide Membrane

The virulence factor common to all Gram-negative bacteria is the lipopolysaccharide (LPS) membrane. An LPS consists of a hydrophobic lipid section, lipid A, which is the main cause of the toxic properties of the membrane, a core oligosaccharide, and either an O-specific polysaccharide or O antigen (Coderch et al. 2004). While the hydrophobic/hydrophilic nature of the LPS membrane protects *S. marcescens* from a variety of factors, lipopolysaccharides are also recognized as stable endotoxins that induce a strong immune response in mammalian cells (Makimura et al. 2009). The LPS endotoxin lipid A resides in the innermost of the LPS and anchors it to the outer membrane of the cell (Raetz & Whitfield, 2002). Studies have shown that due to the penta-acylated structure of the lipid A in *S. marcescens* it has high levels of both endotoxin and immunobiological activities with severe lethality that makes it a major virulence factor as a TLR4/MD-2 agonist (Makimura et al. 2009). Not only does the LPS enable *S. marcescens* to invade, but it also protects the bacterial cell. The LPS layer has been shown to protect the cell from highly hydrophobic quinolones partially. Quinolones act specifically against Gram-negative bacteria targeting intracellular enzymes for inhibition. In a study utilizing strains of *S. marcescens* with mutations that rendered them porin deficient, there was less accumulation of quinolones

demonstrating this method of resistance was more than likely born from a simple mutation (Berlanga et al. 2000). An additional function of the LPS is as a mediator for cell-to-cell communication that could potentially result in the acquisition of new resistance. Some bacteriocins produced bind the core oligosaccharide to the outer membrane proteins of other bacteria enabling them to confer bacteriocin-resistant phenotypes when encountering alterations of the usual outer membrane molecules (Coderch et al. 2004). This key virulence factor triples in function as it allows bacteria to infect cells, protect itself, and confer and gain resistance.

Plasmids

Plasmids are the main vector of gene transference for bacteria across the world. Plasmids that specifically code for antimicrobial resistance are often coined as resistance transfer factors (R-factors). These R-factors can not only confer one type of resistance, but often times multiple different types. *S. marcescens* has the pSMC1 plasmid that confirms resistance to metallo-β-lactamase and other drug resistance. This plasmid is closely related to plasmids that circulate in *Pseudomonas* species (Iguchi, 2014). Another study demonstrated the pBBR1MCS plasmid from *Klebsiella* that contains cefotaxime, cefamandole and cefuroxime resistance can also co-transfer cefoxitin resistance after multiple rounds of transfer between *Escherichia coli* K-12, *S. marcescens*, *Proteus mirabilis*, and *Salmonella typhimurium*. This study showed that even though a plasmid may originally contain resistance to a certain type of cephalosporin, a broader range from the whole class could be transmissible through nosocomial *Enterobacteriaceae* (Knothe et al. 1983). This can be applied to other antibiotic classes as well, possibly giving a hint as to how *S. marcescens* developed such a wide diversity to so many antibiotics through these plasmid-located resistance factors.

Virulence factors have been defined as factors that allow a pathogen “to replicate and disseminate within a host in part by subverting or eluding host defenses” (Cross 2008). All the aforementioned structures of *S. marcescens* help accomplish that with their functions. Studies using knockout mutations of the genes responsible for these virulence factors have been key in determining which factors are key to virulence. When in vivo, if pathogens have a decreased virulence rate with a knockout mutation of something hypothesized to be a virulence factor, it can be assumed the function of that knocked-out gene is key to virulence (Shanks et al., 2007; Posadas et al., 2007;

Swenson et al., 1996). While these virulence factors aid in the pathogenesis of *Serratia*, antimicrobial resistance of *Serratia* is the forefront concern due to the high rate of nosocomial infections and our era of robust emergence of antimicrobial resistance.

Antimicrobial Resistance

β-Lactams

An important characteristic of *S. marcescens* is its ability to produce a β-lactamase. β-lactamases confer resistance to the large spectrum of bacteriocidial β-lactam antibiotics including penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems (Khanna et al. 2013). β-lactam antibiotics inhibit proper cell wall synthesis by binding to the bacterial transpeptidase penicillin binding proteins (PBP), inhibiting peptidoglycan synthesis. Peptidoglycan is a necessary component of cell walls, especially in Gram-positive bacteria, and β-lactam thereby inhibit bacterial replication. β-lactamases are enzymes that work by breaking down the antimicrobial molecule. It targets the commonly found four-ring atom β-lactam found in β-lactams and breaks the ring structure via hydrolysis, inactivating the antibiotics antibacterial properties (English et al., 1978).

In a study done by the Departments of Medicine and Laboratory Medicine at the Medical University of South Carolina disk sensitivity was used to measure the resistance capabilities of 102 clinical isolates of *Serratia* across the span of three different hospitals. Two types of β-lactam antibiotics were used (ampicillin and cephalothin) along with 10 other different antibiotics of varying classes. 93% of the strains were shown to have complete resistant to ampicillin and 100% of the strains were completely resistant to cephalothin indicating how broadly this resistance his spread through the species. (Cooksey et al. 1975). In this study it was determined that in 21 of the 22 multidrug resistant strains (resistant to five or more of the total antibiotics applied in this study) there was transferable resistance traced to R factors. All of the 22 multidrug resistant strains included both cephalothin and ampicillin resistance, indicating that *S. marcescens* may have an innate resistance to these drugs, then in combination with R factors additional degrees of resistance can be conferred. β-lactams compounds are commonly found in nature originating in filamentous fungi, actinomycetes, and gram-negative bacteria, so a ubiquitous bacterium such as *S. marcescens*

would have a high chance of encountering the properties in its environment enabling them to potentially gain resistance before they began being broadly produced as antibiotics (Liras & Martin, 2006).

Topoisomerase Inhibitors

Topoisomerase inhibitors, including the quinolones nalidixic acid and ciprofloxacin, inhibit the enzyme topoisomerase, which is necessary for controlling DNA structure for replication. While these drugs are known as potent anti-cancer drugs, they also have antibacterial properties. It has been shown in studies that the prodigiosin pigment produced by *S. marcescens* has similar activities, inhibiting topoisomerase through DNA intercalation (Montaner et al. 2005). *S. marcescens* possesses membrane porin channels and a unique LPS structure that enable resistance to the hydrophobic quinolones (as reviewed above). Speculatively, the evolutionary history of *S. marcescens* included a chromosomal mutation that led to porin loss or downregulation that resulted in substantially less diffusion rate of quinolones and other topoisomerase inhibitors due to prodigiosin production. This reduced influx acts in conjunction with an increased efflux pumps to create the highly resistant phenotype seen in many strains (Correia et al. 2017).

Aminoglycosides

Similar to the β-lactam resistance studies, aminoglycoside studies have shown that in 90% of isolates there was resistance to the aminoglycoside gentamicin (García et al. 1995). Aminoglycosides are bacteriocidal compounds that inhibit protein synthesis by binding to ribosomes. Originally derived from *Streptomyces*, this class of antibiotics is specific against Gram-negative bacteria and includes streptomycin, kanamycin, gentamycin, neomycin, and tobramycin. Resistance to aminoglycosides is typically mediated by the expression of modification enzymes, including transferases, phosphotransferases and nucleotidyltransferases. *S. marcescens* resistance is due to the expression of acetyltransferases such as the enzyme acetyltransferase AAC(3)-I that inactivates gentamicin (Platt & Sommerville, 1981). Other enzymes like AAC(3)-V, AAC(6')-I, and APH(3')-I also conferred resistance to tobramycin, netilmicin, and amikacin giving *S. marcescens* another wide range of resistance to the aminoglycoside antibiotic class (García et al. 1995). Expression of these modification enzymes is commonly controlled by

plasmids, which have shown to be highly transferable, resulting in the ubiquitous resistance seen in S. *marcescens* strains (Platt & Sommerville, 1981).

Antimicrobial Peptides

There are over 2600 natural antimicrobial properties in the world that offer protection against bacterial, viral, fungal, and protozoan infections (Andersson et al., 2016). Antimicrobial peptides (AMPs) are a large and differential group of small peptides with a variable amino acid compositions and chain lengths. When a pathogen infects a host, they are recognized by a variety of pattern recognition receptors that promote AMP synthesis via activation of the toll and immune deficiency pathways (Romoli et al., 2017). One model organism that exhibits the use of AMPs is *Bombyx mori*, the domesticated silkworm. There are at least 23 AMPs utilized by this species in addition to 13 AMP-like genes (Romoli et al., 2017). In a study done utilizing one silkworm from each of four geographical regions (Japan, China, Europe, and Tropical) defense mechanisms were successful only when humoral immune response was prompt and the AMP cocktail produced had a wide birth of antimicrobial activity (Romoli et al., 2017). This indicates that while *S. marcescens* can withstand individual AMPs, when a cocktail mixture is introduced with ample amounts of a variety of antimicrobial functions it can be susceptible despite its various resistance mechanisms. Because bacteria encounter AMPs of different kinds frequently in their natural environments they have evolved mechanisms to resist their action. One such mechanism comes from *S. marcescens* having inherently more positively charged lipid A (mentioned above) that reduced AMP interaction. Another mechanism of resistance against AMPs is bacteria will incorporate positively charged molecules into their cell surface in order to reduce interaction and binding of AMPs (Andersson et al. 2016). In addition to holding resistance mechanisms to AMPs, *Serratia marcescens* has exhibited production of antimicrobial secondary metabolites. Recent studies show a strain of *S. marcescens* (Db10 a model insect pathogen) produces three secondary metabolites; the antibiotic althiomycin, the biosurfactant serrawettin W2, and a siderophore that all have shown antimicrobial effects against *Staphylococcus aureus* (Gerc et al., 2014). So not only is *S. marcescens* able to resist AMPs, but it can also utilize them to its advantage against specific bacteria. These results highlight the ability of *S. marcescens* to not only resist AMPs but utilize them for a competitive advantage as well.

Conclusions

With a variety of complex and interconnected virulence factors and a multitude of resistance for antimicrobial compounds, and the prevalence of *Serratia marcescens* within the medical community globally the possibilities that could emerge from a greater understanding of *S. marcescens* are of clear importance. With the ubiquitous nature of *S. marcescens* it is plausible to believe that long before antimicrobial compounds were discovered and synthesized for use in antibiotics, *S. marcescens* was already encountering these in its environment. With several innate virulence mechanisms and resistance mechanisms that have origins as other metabolic functions, it can be hypothesized that *S. marcescens* had some type of evolutionary adaptation already in place that doubled in terms of providing resistance to these antimicrobial compounds. When antibiotics became widely manufactured and hospitals became a main environment for *S. marcescens*, interactions with other bacteria that held other resistance on plasmids happened and *S. marcescens* began proliferating strains that had multidrug resistance. In this study we aim to examine the genome of *S. marcescens* to determine key genes responsible for these plasmid and chromosomal encoded antimicrobial resistance and their function within the cell. This will be done using a transposon mutant screen to identify specific genomic sites that correspond to resistance factors. Previous studies determined that *S. marcescens* has robust resistance to natural antimicrobial compounds produced by the fungal isolate *Stemphylium* PNW-2016-03. We expect to find mutations in sites corresponding to the virulence factors listed above, including membrane biogenesis and multi-drug efflux pumps, and hypothesize that novel mechanisms of resistance will be identified.

MATERIALS AND METHODS

Transposon Mutant Library Construction

Serratia marcescens strain UMH8 was used in this study and propagated in tryptic soy (TS) broth and agar. Tn5 mutant strain construction utilized the EZ-Tn5 Tranposome Kit (Epicentere), according to manufacturer's instructions. Briefly, the EZ-Tn5 Transposome is a stable complex formed between the EZ-Tn5 transposase enzyme and the EZ-Tn5 transposon, which contains the Tn903 kanamycin resistance gene. The Ez-Tn5 Transposome was electroporated into electrocompetent cells where the EZ-Tn5 Transposase is activated by Mg2+ in the cellular environment. Electroporated cells were plated on TSA containing 50µg/ml kanamycin. Individual colonies were inoculated into TSB+Kan in 96 well plates and grown overnight at 37°C. Glycerol to 50% v/v was added to each culture and the strains stored at -80°C.

Fungal Metabolite Isolation

Stemphylium strain PNW-2016-03 was isolated from commercially available spinach through endophytic selective process. Briefly, spinach leaves were surface sterilized by 10% bleach solution, with 3 subsequent sterile water washes. Leaves were cut, and plated on potato dextrose (PD) agar containing 30µg/ml ampicillin, and incubated at room temperature for 1 week. *Stemphylium* was grown in a biphasic media, composed of PD agar, topped with PD broth in a tissue culture flask for 7 days. The broth was collected, filter sterilized using at 2µm filter (Millipore), and stored at 7°C.

Screen Conditions

Mutants were first tested in a 24hr growth screen. 96-well plates were pulled from the -80°C deep freeze storage and warmed to 37°C. 5µl of the original culture was transferred to an overnight growth 96-well plate consisting of 195µl of TSB+Kan. This was grown for 24hrs at 37°C. 5µl of this fresh culture was transferred to a control 96-well plate of 195µl of TSB+Kan and to an experimental 96-well plate of 195µl of TSB, 25% of fungal metabolite isolate 3, and kanamycin. Cultures were grown at 37°C for 24hrs then read with the Multiskan FC Microplate Photometer from ThermoFisher at 620nm. Strains that showed decreased growth were selected for a triplicate

12hr growth screen, done in a similar manner to the 24hr growth screens. The 96-well plate was left in the photometer for 12hrs and a reading was done at every hour at 620nm.

DNA Extractions

DNA was extracted from 1ml cultures of Serratia marcescens grown overnight in TSB+Kan. The Promega Wizard Genomic DNA Purification Kit was used and manufacturers protocol was followed. The quantity and quality of DNA were assessed utilizing a Nanodrop spectrophotometer. Samples were stored at -20°C until needed for PCR.

PCR and Purification

Amplification of the Tn5 insertion site utilized a modified thermal asymmetric interlaced polymerase chain reaction TAIL-PCR protocol (Jia et al., 2017). Primers F389, LAD1, LAD3, F536, and LAC1 were used. The first step was a linear reaction with 20 cycles of a 20ul sample mixture of primeSTAR Max DNA polymerase, primer F389 (100uM), genomic DNA, and diH2O. The second step was an exponential reaction for 19 cycles of a 20ul sample mixture of primeSTAR Max DNA polymerase, primer F389 (10uM), primer LAD1 (10uM), primer LAD3 (10uM), template from Step 1, and diH2O. A 1% agarose gel was run to ensure sample purity. The final 3rd step is another exponential reaction with 7 cycles of a 20ul sample mixture of primeSTAR Max DNA polymerase, primer F536 (10uM), primer LAC1 (10uM), template from Step 2, and diH2O. Samples were purified using the Promega Wizard SV Gel and PCR Clean-Up Kit. Manufacturer's protocol was followed except for the addition of the nuclease-free water. Instead of adding 50ul and incubating for 1min then centrifuging at 160,000rpm for 1 minute, the nuclease-free water was added twice at a total of 25ul each then incubated for a minute each time and centrifuged each time to increase the total DNA yield. The quantity and quality of DNA were assessed utilized a Nanodrop spectrophotometer, and samples were stored at -20°C.

Sequence Identification

DNA samples were sequenced via Next Generation WideSeq at Purdue University Genomics Core Facility. Assembled sequences were Blasted using the NCBI database for sequence identification (https://www.ncbi.nlm.nih.gov/).

RESULTS

Transposon Mutant Library Screen

A transposon mutant library screen was designed to identify individual mutants that had at least 40% reduction in growth over 24 hours in the presence of 25% fungal metabolites in TSB, compared to TSB alone. Preliminary testing showed that 25% of the fungal metabolite in the presence of TSB actually provided optimal growth for *Serratia marcescens.* So any mutants that showed a decreased growth in this media were then being negatively affected by the metabolite indicating a loss in resistance.

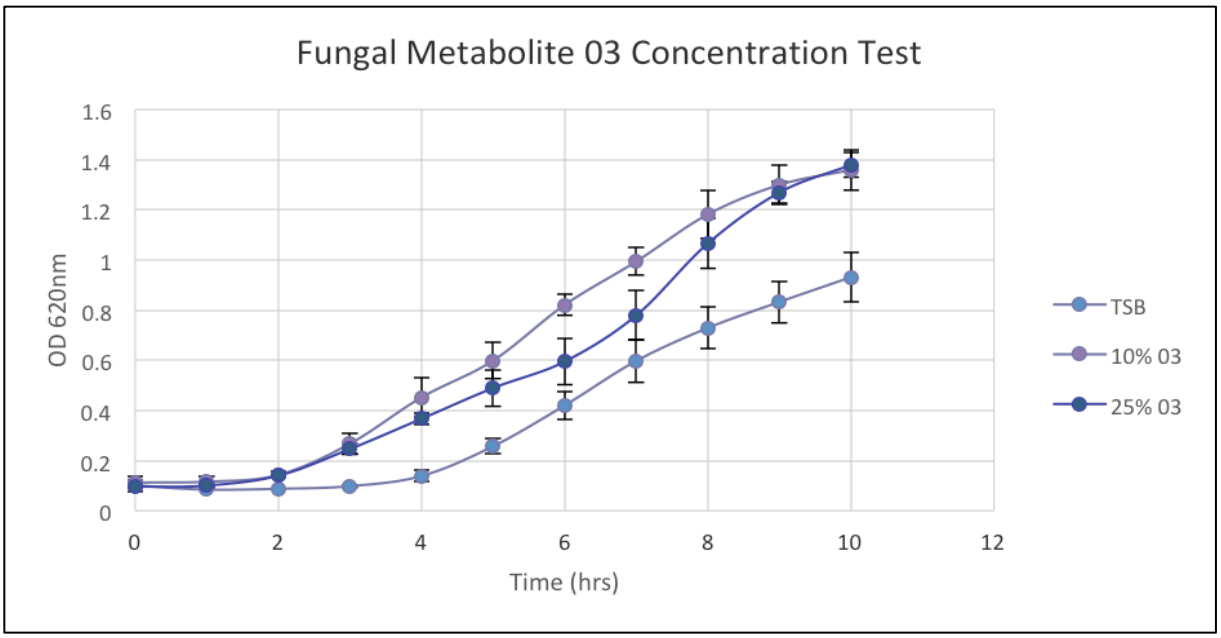

Figure 1: Fungal Metabolite 03 Concentration Determination

Following the initial screening of mutants after 24 hours, selected mutants that demonstrated altered growth were confirmed in triplicate in 12-hour trials where growth was observed at every hour time point. This was done to determine if the selected strains were in fact not growing at all, or if growth was occurring up to a certain point with a crash following. Any mutants that exhibited the later type of growth were not chosen for this specific study, as we were more interested in strains that showed an overall decrease in growth, not just a segmented decreased in growth.

Out of 1,824 of the mutant strains screened 127 were identified as having a decreased resistance to the fungal metabolite isolate 03. Mutants selected as having a decreased resistance were those that showed an optical density of less than 1.069 (a 40% decrease from the wild type growth peak at 24hrs of 1.782). Of the 127 mutants identified 64 (shown below in Table 1) were sent for sequencing of the area of the genome affected by the transposon insertion.

Table 1: WideSeq Sample Data

Mutant	**DNA Concentration (ng/µl)**	**260/280 Ratio**
4A1	35.6	1.59
4B2	35.7	1.62
5B3	44	1.60
5B5	26.3	1.56
5B8	34.2	1.52
5B9	35.0	1.57
5C8	30.6	1.50
5D8	27.4	1.60
5D11	49.4	1.72
5D12	47.1	1.76
5E11	36.3	1.60
5E12	52.3	1.70
5F11	50.8	1.67
5G10	50.0	1.58
5G11	52.3	1.71
5G12	37.4	1.62
6C8	56.5	1.65
6D9	51.3	1.66
6F7	60.3	1.59
6G7	53.1	1.71
7A11	48.4	1.68
7B9	48.4	1.68
7B12	52.1	1.63

Table 1 continued

7C3	48.4	1.72
7D2	51.4	1.56
7D7	45.1	1.56
7D9	43.7	1.60
7D10	51.1	1.68
7E4	45.7	1.62
7E7	53.5	1.59
7E12	51.7	1.58
7F8	56.5	1.62
7F10	39.4	1.62
7F12	52.5	1.67
8D12	50.8	1.70
8E10	49.5	1.68
8G7	55.5	1.74
8G11	46.1	1.66
8G12	48.5	1.71
8H3	54.7	1.74
8H5	52.3	1.72
9A1	43.1	1.63
10B2	46.5	1.66
10B5	48.0	1.70
10B9	48.5	1.72
10F11	44.6	1.63
10G6	13.1	1.91
10G8	11.7	1.85
12C9	38.2	1.46
12C12	46.8	1.47
12E12	47.4	1.57
12H4	48.6	1.55

Table 1 continued

12H7	47.1	1.74
12H8	41.4	1.55
13C9	37.4	1.57
13C10	38.8	1.59
14F10	39.8	1.57
14H9	38.6	1.53
15A11	34.2	1.61
15B11	30.0	1.46
15C9	33.7	1.45
15E1	32.6	1.42
15E7	37.1	1.38
15H3	29.4	1.50

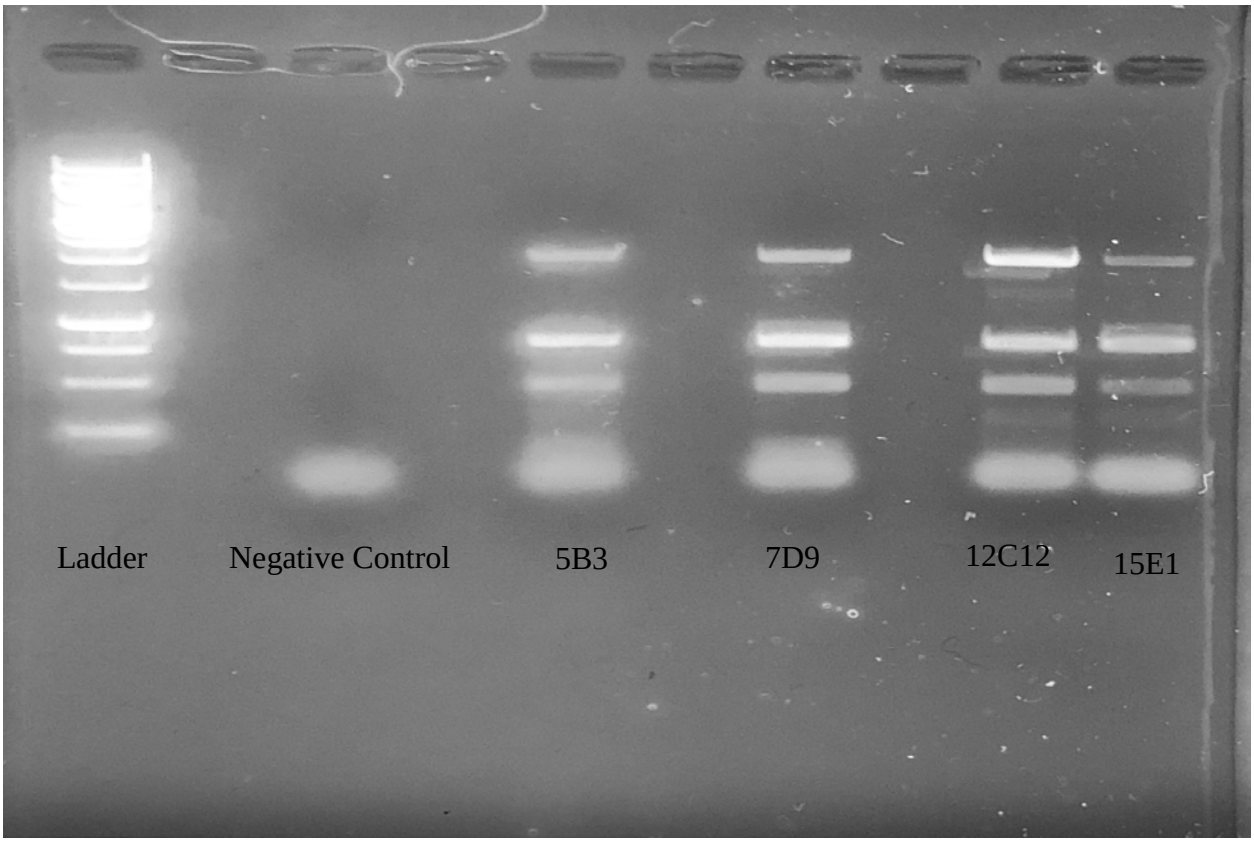

Figure 2: Agarose Gel of Randomly Selected *S. marcescens* Mutants

With the transposon inserting randomly into the genome it is expected that strains would appear that host the same gene mutation. Once a point in the study was reached where sequencing came back that showed multiple strains having a mutation in the same gene, sequencing was halted. 56 strains were successfully sequenced with results shown below.

Table 2: Sequenced Mutant Strains

Mutant	**24hr Growth (OD 620nm)**	**Colony Size (cm)**	**BLAST Gene**
4A1	0.8697	n.d	*fecR*
4B2	0.7188	n.d	*aphA1b*
5B3	0.0389	0.136	*fecR*
5B5	1.0013	0.184	*ygbN*
5B8	0.8203	0.192	*mrfE1*
5B9	0.0146	0.124	[ferredoxin]
5D8	0.1129	0.150	[alkaline shock protein]
5D11	0.6667	0.072	*acrAB*
5D12	1.0332	0.152	*aphA1b*
5E11	0.9895	n.d	*aphA1b*
5E12	0.9601	0.146	*pagP*
5F11	0.9134	0.174	*cpxA*
5G10	1.0032	0.098	hypothetical
5G11	0.0122	0.120	*mtdI*
5G12	0.0909	0.136	*iptD*
6C8	0.6672	0.100	*hasA*
6F7	0.1395	0.162	*traC*
6G7	0.6741	0.130	*fecB*
7B9	0.6098	0.106	*fecR*
7B12	0.6980	0.062	hypothetical
7C3	0.3610	0.102	[alkaline shock protein]
7D2	0.6604	0.108	*fecR*

Table 2 continued

7D7	0.4738	0.108	[ferredoxin]
7D9	0.4682	0.056	*fecR*
7D10	0.6578	0.098	[ferredoxin]
7E4	0.3528	0.086	*tamB*
7E7	0.5960	0.094	*wzi*
7E12	0.5616	0.124	*mrfG*
7F8	0.6752	0.134	*ilvB*
7F10	0.6013	0.084	*aroP*
7F12	0.5674	0.128	hypothetical
8D12	0.5478	0.118	*yeiG*
8E10	0.5833	0.088	*tamB*
8G7	0.5463	0.108	*sdeC*
8G11	0.4021	0.078	*aphA1b*
8H3	0.4621	0.132	*marR*
8H5	0.5656	0.094	*robA*
10B2	0.5598	0.102	*smbD*
10B9	0.4580	0.094	hypothetical
10F11	0.5882	0.086	*smbD*
10G8	0.5401	0.170	*lptG*
12C9	0.5855	n.d	*wzi*
12C12	0.5244	0.102	*traC*
12E12	0.5596	0.102	*gyrA*
12H4	0.5511	n.d	[SAM dependent methyltransferase]
12H7	0.5023	n.d	hypothetical
12H8	0.5285	n.d	hypothetical
13C9	0.4788	n.d	hypothetical
13C10	0.5160	n.d	[SAM dependent methyltransferase]

Table 2 continued

14F10	0.2902	0.122	*mrfE1*
14H9	0.4612	0.132	[aspartate aminotransferase]/[oxidoreductase]
15A11	0.3387	0.108	*aphA1b*
15B11	0.4637	0.112	hypothetical
15C9	0.3865	0.042	*traC*
15E1	0.4771	0.104	[N-acetyletransferase]
15E7	0.4474	0.046	*csiE*

There were 6 distinct types of mutations observed within this group, the first being mutations affecting the cell's ability to regulate iron. This could mean mechanisms that intake iron into the cell, transport iron through the cell, or detect iron outside of the cell. The second was mutations affecting drug transport. This refers mainly to the drug efflux pumps located on the membrane of the cell that expels a variety of toxins from within the cell. The third group was mutations with regard to membrane biogenesis. This includes the formation of the membrane and regulation of porins found on the surface. The fourth was stress mechanisms. This includes any means of regulation the cell has in response to changes of pH, osmotic pressure, temperature, or any other factor that brings the cell out of homeostasis. The fifth was other mutations. This refers to mutations that didn't occur frequently enough to be categorized in their own group. Mutations here include pilus assembly, fimbriae, various amino acid pathways, and adhesion secretion. The last category of mutations was the hypotheticals. These include genes and proteins that are predicted to exist, but lack enough experimental evidence for in vivo expression.

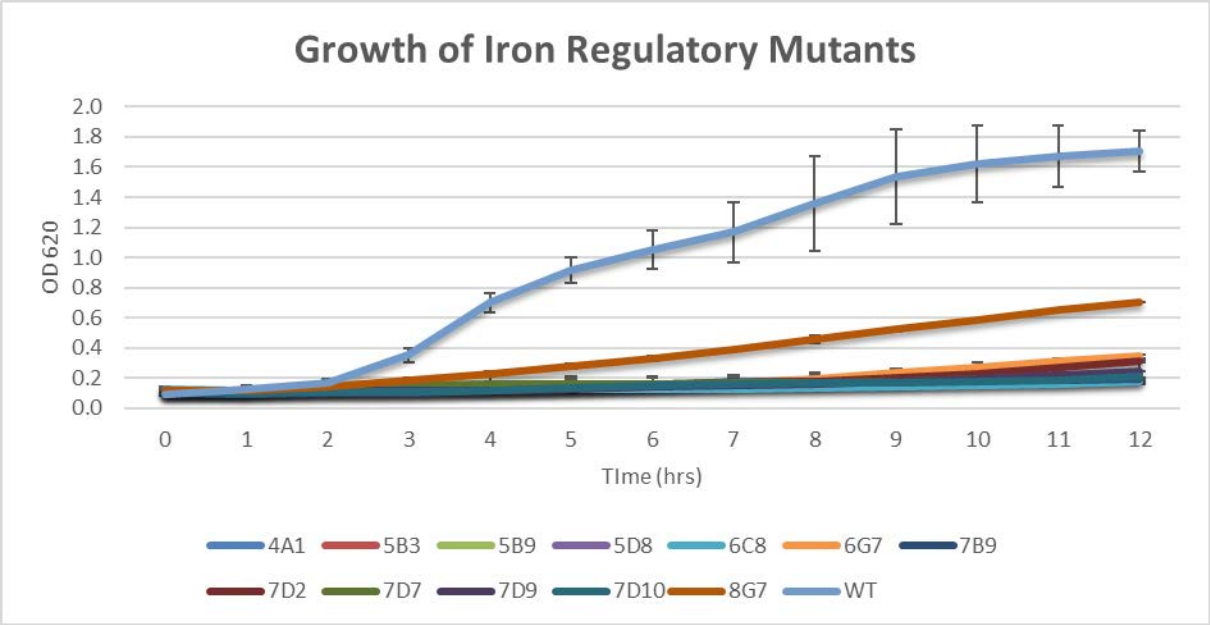

Figure 3: 12hr Growth of Iron Regulatory Mutants

Iron Regulatory Mutants

Out of the 56 mutants 12 had an irregularity in iron regulation. There was a range of optical densities at the 24hr time point from 0.02-0.87 with the mean being 0.48 and a standard deviation of 0.27. Over the course of the 12hr growth experiment no strains reached an optical density of greater than 0.60 with 11 of the 12 strains not going above 0.40, a stark difference than the wild type at an optical density of 1.70. The size colonies vary from 0.06-0.14cm with the mean being 0.11 and a standard deviation of 0.02.

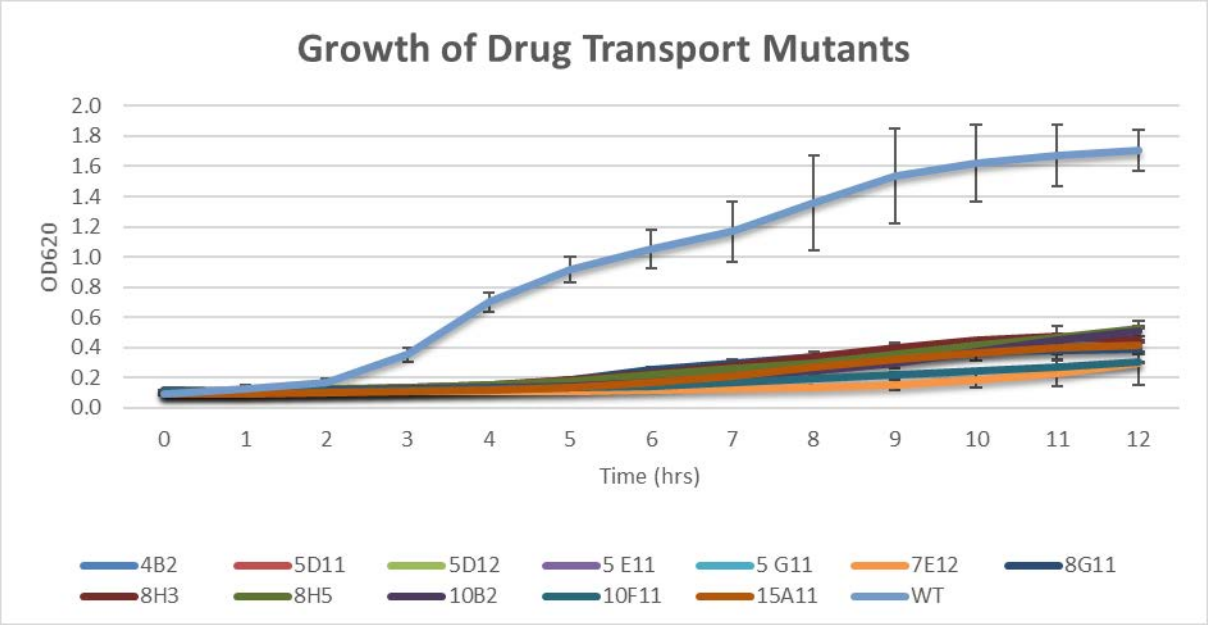

Figure 4: 12hr Growth of Drug Transport Mutants

Drug Transport Mutants

Out of the 56 mutants 12 had an irregularity in drug transport. There was a range of optical densities at the 24hr time point from 0.01-1.03 with the mean being 0.57 and a standard deviation of 0.28. Over the course of the 12hr growth experiment no strains reached an optical density of greater than 0.60, a stark difference than the wild type at an optical density of 1.7. The size colonies vary from 0.07-0.15cm with the mean being 0.11 and a standard deviation of 0.03.

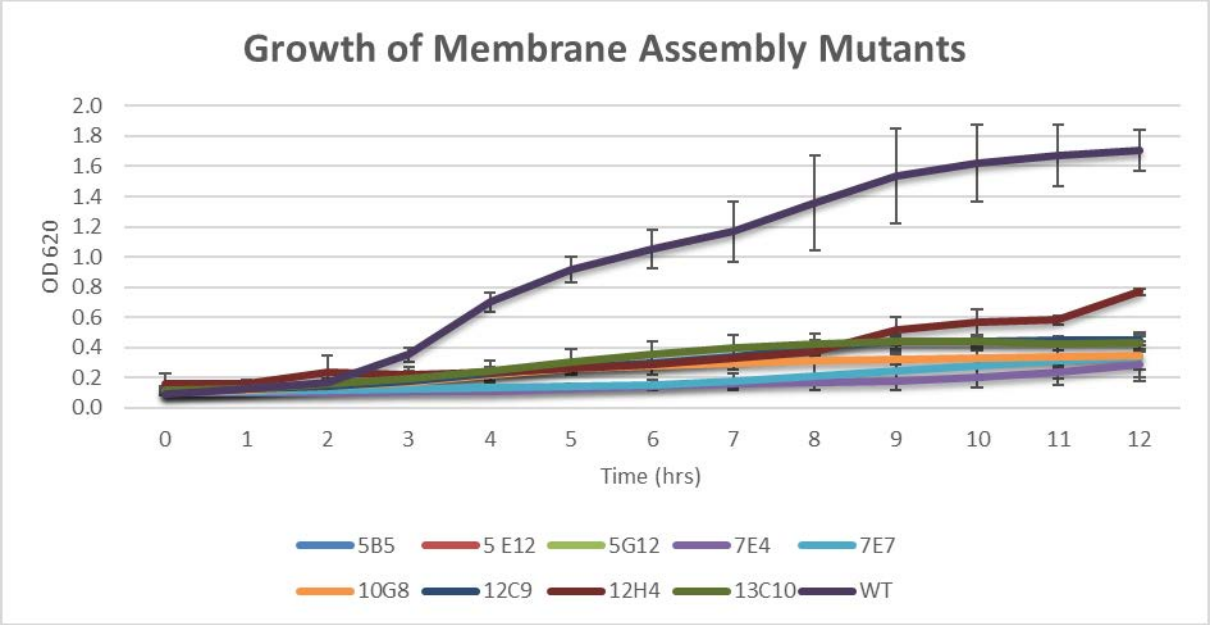

Figure 5: 12hr Growth Curve of Membrane Assembly Mutants

Membrane Assembly Mutants

Out of the 56 mutants 9 had an irregularity in membrane assembly. There was a range of optical densities at the 24hr time point from 0.09-1.00 with the mean being 0.58 and a standard deviation of 0.28. Over the course of the 12hr growth experiment no strains reached an optical density of greater than 0.80 with 8 of the 9 strains not going above 0.50, a stark difference than the wild type at an optical density of 1.70. The size colonies vary from 0.09-0.18cm with the mean being 0.14 and a standard deviation of 0.04.

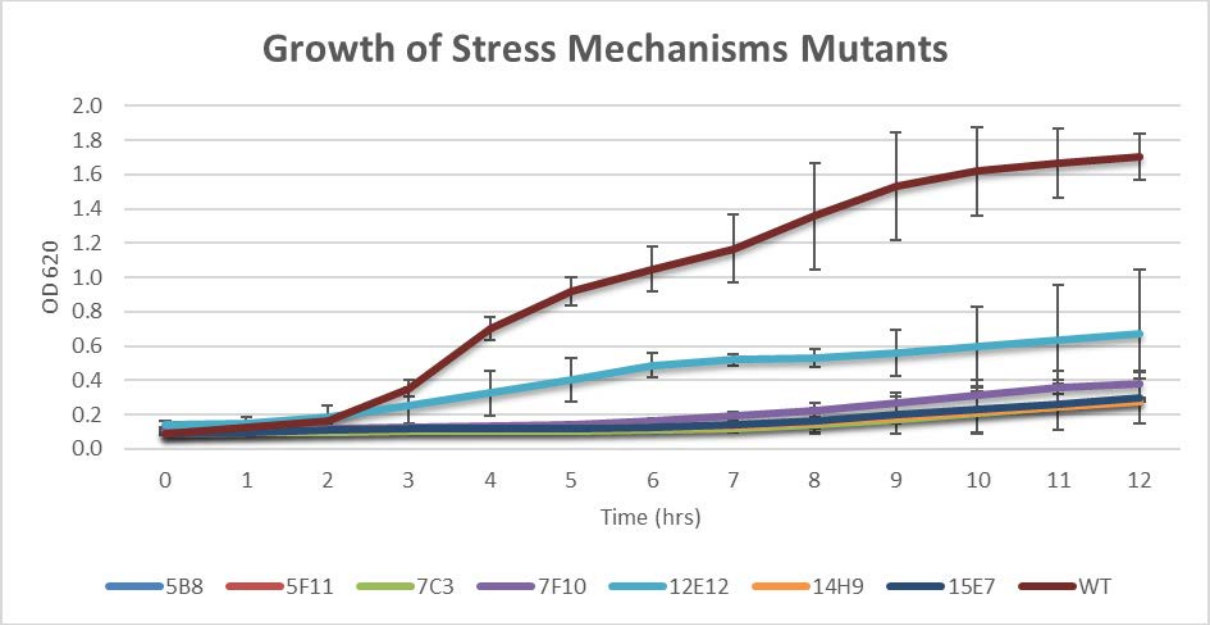

Figure 6: 12hr Growth Curve of Stress Mechanisms Mutants

Stress Mechanism Mutants

Out of the 56 mutants 7 had an irregularity in stress mechanisms. There was a range of optical densities at the 24hr time point from 0.36-0.91 with the mean being 0.59 and a standard deviation of 0.20. Over the course of the 12hr growth experiment no strains reached an optical density of greater than 0.70 with 6 of the 7 strains not going above 0.40, a stark difference than the wild type at an optical density of 1.70. The size colonies vary from 0.05-0.19cm with the mean being 0.12 and a standard deviation of 0.05.

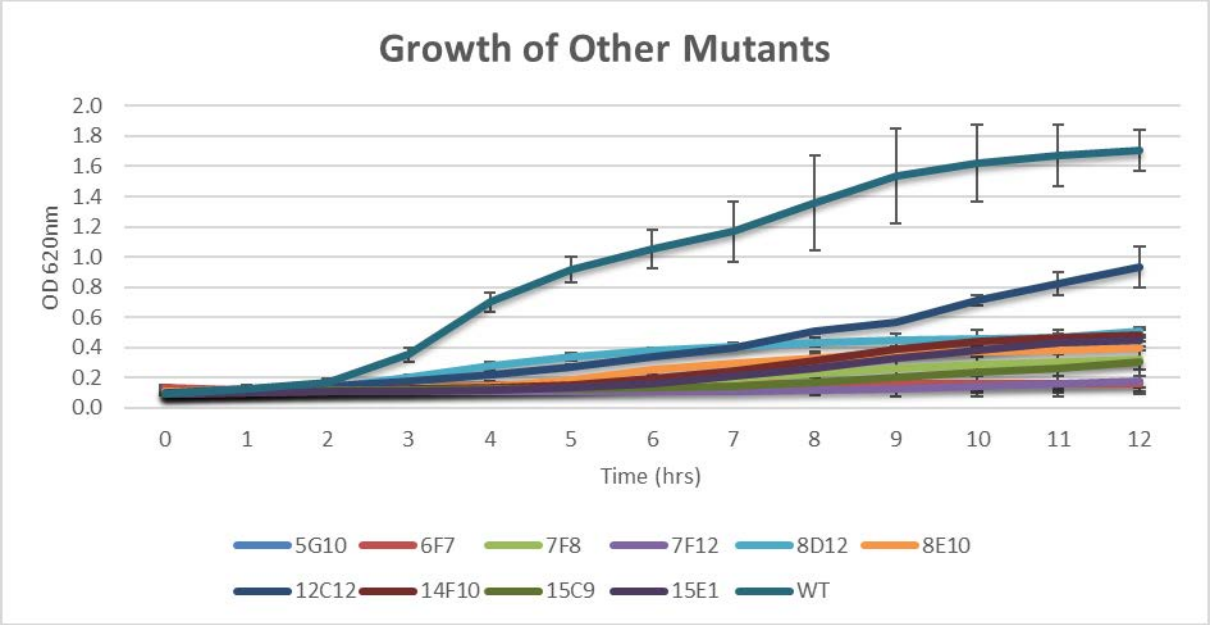

Figure 7: 12hr Growth Curve of Other Mutants

Other Mutants

Out of the 56 mutants 10 had an irregularity in other areas of function (pili formation, adhesion secretion, etc.). There was a range of optical densities at the 24hr time point from 0.14-1.00 with the mean being 0.52 and a standard deviation of 0.23. Over the course of the 12hr growth experiment no strains reached an optical density of greater than 1.00 with 9 of the 10 strains not going above 0.50, a stark difference than the wild type at an optical density of 1.70. The size colonies vary from 0.04-0.16cm with the mean being 0.11 and a standard deviation of 0.03.

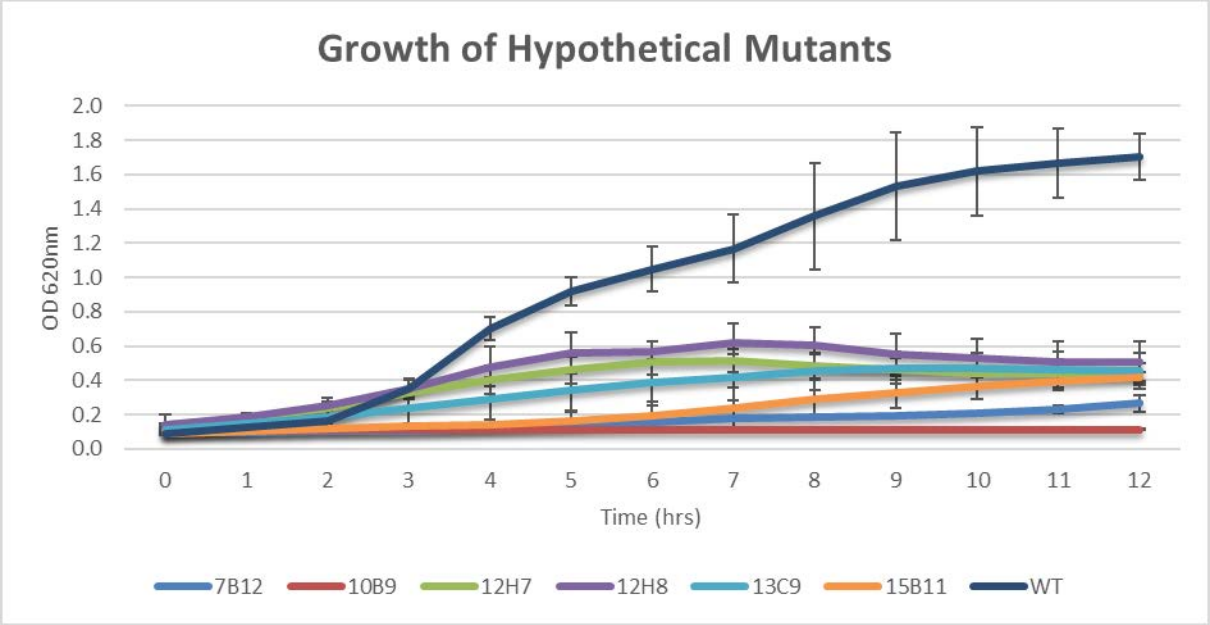

Figure 8: 12hr Growth Curve of Hypothetical Mutants

Hypothetical Mutants

Out of the 56 mutants 6 had an irregularity in hypothetical areas of function. There was a range of optical densities at the 24hr time point from 0.46-0.70 with the mean being 0.52 and a standard deviation of 0.09. Over the course of the 12hr growth experiment no strains reached an optical density of greater than 0.70 with 5 of the 5 strains not going above 0.50, a stark difference than the wild type at an optical density of 1.70. The size colonies vary from 0.06-0.11cm with the mean being 0.09 and a standard deviation of 003.

DISCUSSION

Our study aimed to examine the various elements of *S. marcescens* that contribute to its wide range of antimicrobial resistance. As expected due to the multitude of virulence and resistance factors *S. marcescens* possesses, we identified multiple genes that contribute to the resistance of this species. These genes were then grouped into broad categories based on functions that did correspond to the known factors of virulence and resistance factors mentioned at the start of this study. All mutation categories had similar means of growth at the 24hr time point (around 0.50 with no standard deviation being higher than 0.30). This indicates that no one part of the *S. marcescens* antimicrobial resistance system is more important than the others. The complex network of factors that correspond to virulence and highlight resistance shows the evolutionary advantage to having multiple systems that ensure fitness.

Approximately 50% of our strains sequenced contained mutations we expected to see, such as membrane assembly, drug transport, and stress mechanisms that are directly responsible for daily cellular function. The cellular membrane is the control mechanisms for what goes in and out of the cell in addition to being necessary for cell adhesion and cell signaling (Takeuchi et al., 2000). Any type of mutation here would leave the cell vulnerable to foreign particles that normally would not be able to enter. This was the main reason we only saw 16% of our mutant strains have a membrane assembly mutation instead of a larger percentage. If a mutation that occurred caused a drastic decrease in membrane function, it's possible the cell wouldn't have been able to grow at all or died off a few hours into the screen. Drastic mutations that affected the overall fitness of the cell were not seen in this screen because we were only targeting decreased growth not complete lack of growth. Our results signify the mutations in membrane assembly were more than likely in regard to things mentioned above that aided in virulence, but not overall growth like lipid A. The positively charged lipid A on the membrane causes reduced antimicrobial interactions (Andersson et al. 2016). Any kind of reduction of lipid A on the surface of the membrane would therefore result in more antimicrobial interactions. In our study, mutants with a membrane assembly gene affected had 8 of the 9 strains not reach an optical density of 0.50 over the course of the 12-hour growth. This was a 70% decrease from the wild type growth at 1.70. These remarkably low growth rates were more than likely due to a combination of the cell not having a barrier against

the metabolite and possible structural failures of the membrane as a whole. 7 different genes were responsible for the mutations viewed indicating the complexity of the membrane biogenesis and the variety of functions the membrane can have for the cell.

Similarly, drug efflux pumps are transport proteins that are responsible for expelling toxic substrates from within cells (Webber &Piddock, 2003). These pumps are also responsible for supporting cell homeostasis and intercellular signal transduction as well. They also play a part in virulence by transporting adhesions, toxins, and proteins involved in infection and colonization (Berlanga et al., 2000). Drug efflux pumps are a good example of an evolutionary adaption that *S. marcescens* possessed that served a secondary function correlating to resistance. Efflux pumps originally are thought to serve a physiological role within the cell of expelling natural toxic substances produced by the cell such as bile, hormones, and host-defense molecules (Piddock, 2006). These efflux pumps could have hypothetically then evolved to transport foreign toxic substances out of the cell as *S. marcescens* encountered them in its environment. *S. marcescens* specifically uses a resistance-nodulation-division (RND) efflux pump typical of Gram-negative bacteria (Chen et al., 2003) that has the highest clinical significance as a proton/antibiotic antiporter (Berlanga et al., 2000). These RND efflux pumps catalyze active expulsion of a wide range of antibacterial compounds, natural and synthetic (Berlanga et al., 2000). So any type of mutation within these drug transport systems would render the cell unable to transport the fungal metabolite out of the cell once it entered. Drug transport mutants were tied for the highest prevalence within our sample group comprising 21% and having 7 different genes responsible for the mutations. This shows their regulation complexity within *S. marcescens* and their importance as a source of variety against a multitude of antimicrobial factors to aid in resistance.

Lastly of the expected mutations, stress mechanisms enable cells to tolerate changes to their environment for a short time that would otherwise damage them. These could include osmotic, salicylate, temperature, and pH stressors (Begic & Worobec, 2006). Stress mechanisms also play a large role in a pathogen's ability to withstand nutritional immunity. Nutritional immunity is when a host organism sequesters trace minerals to stop pathogenicity during infection (Hennigar & McClung, 2016). When infections occur in areas of inflammation there is a dramatic and rapid decrease of circulating concentrations of minerals like iron and zinc that pathogens use in a variety

of metabolic activities (Hennigar & McClung, 2016). So in instances where a stress mechanism was mutated within a cell, that cell would lose the ability to grow as adequately as the wild type in the presence of antimicrobial properties that would not normally cause a decrease in growth. While there is a multitude of resistance factors that *S. marcescens* possess, the presence of the metabolite does ultimately cause overall stress on the cell. 6 of the 7 strains in our study did not reach an optical density of over 0.40 at 12hrs (a 75% decrease) showing just how drastic growth is affected when these stress mechanisms aren't functioning properly. Each of the 7 mutants had a different gene responsible for the mutation indicating the wide variety of stress mechanisms that a part of regulating a cell and how each one can independently affect overall growth in the presence of the fungal metabolite. These expected mutations show that the basics of resistance are the same for *S. marcescens* compared to other species that are highly resistant, but the next aim of our study was to show how *S. marcescens* may differ, and how this difference enables it to be so effective at resistance. Our results show significance in *S. marcescens* ability to defend against nutritional immunity.

A mutation category we were expecting to see, but not at such a high frequency was the iron regulation category. With 21% of total mutations falling in this category, and it having the lowest average for both 24hr growth and 12hr growth, the need to understand more about the role of iron metabolism and transport within *S. marcescens* as an antimicrobial resistance factor becomes clear. Iron is an important nutrient for all forms of life based on its role in cellular processes. Free soluble iron is limiting in most environmental sources at levels required for growth (Skaar, 2010). And as already discussed iron is one of the minerals sequestered as a part of the host's nutritional immunity. *S. marcescens* possesses an extracellular heme-binding protein (HasA) that allows the release of heme from the hemoglobin of hosts, so the cell can have a steady supply of iron (Letoffe et al., 1994). *S. marcescens* also possess siderophores, small molecules used to bind and transport iron as a scavenge mechanism for microorganisms (Angerer et al., 1992). The reason why *S. marcescens* actively search out iron within a host is that iron detection systems have been shown to affect swarming directly and biofilm formation in *Serratia marcescens* using a two-component system (Lin et al. 2016). The RssA-RssB component of the RssAB-FIhDC-ShIBA pathway system that regulates a variety of virulence factors of *Serratia marcescens* also is responsible for directly sensing ferric iron in the environment. When levels of ferric iron are high RssAB signaling

activation is prolonged and swarming initiation is delayed leading to an increase in biofilm formation (Lin et al. 2016). This suggests that iron is a key regulator in the virulence pathway of *S. marcescens* not only for survivability leading up to infection, but prolongation as the infection becomes disease.

As with any study of this nature there were mutations that occurred that could not be group into the main categories that we classified as other. These included this such as include pilus assembly, fimbriae, various amino acid pathways, and adhesion secretion. 9 of the 10 mutants didn't reach an optical density of greater than 0.50 (a 70% decrease from the 1.70 wild type optical density) and they had some of the smaller size colony variants with the mean being 0.09cm and the smallest being 0.04cm. This indicates that while small in number, some of these other mutations might cause more devastating effects on the cell. The various functions listed above are all key in virulence in regards to biofilm. Pili and fimbriae are used for cell-cell adhesion and can also be responsible for transferring DNA between cells (Kohno et al., 1984). While using pili to help attach to cells adhesion is secreted to help adhere to other surfaces in order to help create the biofilm (Hejazi & Falkiner, 1997). A reduced ability to create a biofilm would make the individual cells more susceptible to antimicrobial factors that they would normally have protection against.

Lastly to close out the categories of mutations there were they hypothetical variants. Hypothetical genes or proteins are those that have been experimentally characterized and conserved, but their functions cannot be deduced from simple sequencing alone (Garbom et al., 2004). While at the current moment not much can be deduced from these hypothetical genes, if over time they are found to be conserved across a variety of species that all hold resistance mechanisms they could prove to be a point of further study. With the wealth of information available in databases via large-scale genome sequencing projects these hypothetical genes and proteins could serve as the next route for developing novel antimicrobial agents.

For a further point of study regarding this research though with iron playing a large role in the regulation of the pathway system that controls a variety of virulence factors and being a known limiting factor of nutritional immunity iron sequestration could be the next aspect of *Serratia marcescens* to study. As of current we know *S. marcescens* sequesters iron from its environment

to regulate several key metabolic pathways, one being the RssAB-FIhDC-ShIBA pathway system. What was identified based off of the results of this study is that the fungal metabolite PNW-2016-03 may possibly either be siphoning iron from the environment resulting in a situation where the cells are undergoing stress in a similar manner they would experience when faced with nutritional immunity in a cell or the metabolite is instead directly affecting some area of the iron-regulating pathway leaving the cell unable to use the iron it is taking in. Further studies can use the mutant strains identified within the iron regulation category to see how well they grow in the presence of other antimicrobial peptides, or see if these strains can function in media in the presence of antimicrobials with extra iron supplemented. Another option could be to study the fungal metabolite itself to determine if in the presence of other pathogens known to use iron in a similar manner that *Serratia marcescens* does but have less natural resistance if it has the same effects.

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