Serratia marcescens: Outer Membrane Porins and Comparative Genomics

By

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Abstract

The Gram negative bacterial species *Serratia marcescens* is an increasingly prevalent and dangerous cause of health care acquired infections. Its high inherent resistance to first line antimicrobials, paired with a very plastic genome and diverse plasmid compatibility make multi drug resistance a common challenge to treatment of *S. marcescens* infections. One such genetic change that can lead to drug resistance in this species is the loss of certain Outer Membrane Porins (OMP) that normally facilitate diffusion of antimicrobials, particularly β-lactam antibiotics. Previous research into other enterobacteriaceae species has indicated that these strains often exhibit altered virulence in host models. We used an existing library of *S. marcescens* Db11 OMP mutants to evaluate virulence in *Caenorhabditis elegans* and *Galleria mellonella* host models. We observed significant but not biologically relevant increases in virulence of the Δ*ompC*Δ*ompF* and Δ*ompC*Δ*ompF*Δ*phoE* strains. Other porin genes were evaluated for expression in the mutant strain backgrounds in order to observe changes resulting from loss of *ompC*, *ompF* and *phoE*. A significant reduction of the *lamB* gene in all strains, and significantly increased expression of *ompA* and *ompX* in both double and triple deletion strains. This is the first evidence of *ompA* overexpression due to loss of *ompC* and *ompF* function. This study also sought to investigate the genomic properties of two clinical isolates, in order to establish their compatibility with existing genetic tools for the manipulation of OMP genes, for the purpose of studying the effects in different genetic backgrounds. Two strains of *S. marcescens* isolated from platelet concentrate, CBS11 and CBS12, had their genomes sequences prepared and analysed. The two strains were also evaluated for virulence in *C. elegans*, revealing CBS11 as significantly hyper virulent as compared to both CBS12 and Db11. The high homology of OMP genes in these strains and Db11 makes them excellent candidates for manipulation with existing tools designed for a Db11 background.

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1. Literature Review:

1.1. *Serratia marcescens*

Overview: The organism *Serratia marcescens* has recently become a growing concern among public health observers. The ubiquity of *S. marcescens* in the environment has made it robust and adaptable to many ecological niches (Iguchi *et al.* 2014; Mahlen 2011). Such flexibility has made it particularity receptive to horizontal transfer of antimicrobial or antiseptic resistance genes. Furthermore, the propensity to form biofilms makes *S. marcescens* a frequent causative agent of healthcare associated infections; particularly those introduced by contaminated transplants, prosthetics or transfusions (Mahlen 2011; Marrie and Costerton 1981). Compared to closely related enterobacteriaceae such as *Escherichia coli* or *Yersinia pestis*, *S. marcescens* has been largely under investigated. Only within the last century has *S. marcescens* been recognized as a pathogen at all. It was thought so harmless that it was in fact sprayed on civilians as a type of marker to assess the spread of biological contamination (Mahlen 2011). It has since been shown that the genus *Serratia* contains pathogens of plants and animals, both aquatic and terrestrial. This literature review will cover the historical background, taxonomy and species classification of *Serratia* genus, and the current state of knowledge pertaining to pathogenesis and antimicrobial resistance associated with *S. marcescens*.

1.1.1. Species

History of Discovery: The organism *Serratia marcescens* was originally identified in the year 1819 (Bizio 1823; Mahlen 2011). In the rural Italian town of Legnaro, the unusually high humidity and temperature led to a strange and alarming phenomenon. The village's polenta stores began to develop a vibrant red color, closely resembling that of blood. Although a priest was first asked to investigate, the task was eventually given to the University of Padua. The lead investigator was Bartolomeo Bizio; a professor of pharmacy at the university. Using simple microbiological techniques, he was able to isolate brightly red pigmented colonies. Upon observation with a primitive light microscope, he believed that the organism resembled the cap of a mucoid mushroom lacking a stem. Whilst Dr. Bizio named the organism *Serratia marcescens*, after the contemporary physicist Serafino Serrati, his colleague Dr. Vincenzo Sette had named the same isolated organism *Zoagalactina imetrofa* following the conclusion of his own experiments. This independent identification became a recurring theme in the history of *S. marcescens*. In 1848, it was again characterized by a German researcher, this time as *Monas progigiosa*. This pattern of independent discovery, isolation and renaming continued until the modern name *Serratia marcescens* was adopted in 1980, with the first release of the "Approved List of Bacterial Names" (Mahlen, 2011; Skerman *et al.* 1980). Now working under the umbrella of a common name for the organism, interest in its role as a pathogen and environmental organism has expanded significantly.

Taxonomy: The genus *Serratia* is part of the family enterobacteriaceae, which contains a large number of Gram negative bacilli species. It is part of the larger class gammaproteobacteria, which itself falls into the phylum proteobacteria. The Enterobacteriaceae is home to many widely

known human and animal pathogens, such as those of the genus *Yersinia*, *Escherichia*, *Shigella*, *Salmonella* and *Enterobacter*. Plant pathogens are also present in this family, such as the genus *Erwinia*. The phylogenetic trees of this diverse family indicate that the genus *Serratia* maps closely to the genus *Yersinia* and the genus *Erwinia*. The genus *Serratia* itself contains 14 species, and the species *S. marcescens* has two subspecies. The first, *S. marcescens* subsp. *marcescens* is the most common human disease agent in this genus. The second subspecies, *S. marcescens* subsp. *sakuensis* was isolated from activated sludge in a Japanese water treatment plant in 1998. It does not have any reported pathogenic ability, and is physiologically distinct from subspecies *marcescens* (Ajithkumar *et al.* 2003).

Environmentally isolated *S. marcescens* is characteristically identified by its bright red pigmentation, as was observed by Dr. Bizio in 1823. This striking coloration is due to the synthesis and secretion of prodigiosin, a tripyrrole pigment (Bennett and Bentley 2000). Many *S. marcescens* strains produce this molecule, but most clinically isolated strains do not. Production of prodigiosin is increased in response to cold temperatures; thus, in a warm environment such as the human body the ability to produce prodigiosin is either an impediment or is simply too resource intensive (Shanks *et al.* 2013; Williams *et al.* 1971).

On rich or complex growth media, *Serratia marcescens* form opaque, slightly mucoid rounded colonies with smooth edges. Isolates are known to produce a musty, ammonia like odor. Without the production of red pigment, *Serratia* can be difficult to differentiate from other enterobacteriaceae on the basis of colony features. Biochemical identification serves as the mainstay of *S. marcescens* confirmation, shown in Table 1, with ornithine decarboxylase or DNAse presence differentiating it from organisms such as *E. coli* of *Salmonella enterica* serovar

Typhimurium. The inability to ferment L- arabinose seen in *S. marcescens* allows differentiating from other *Serratia* species such as *S. rubidaea* (Wilfert *et al.* 1970).

Table 1: Biochemical test results characteristic of *S. marcescens* isolates (Zimbro *et al.* 2009).

Basic Characteristics	Test Result for *Serratia marcescens*
Catalase	(+)
Gelatinase	(+)
Oxidase	(-)
DNAse	(+)
Indole	(-)
Citrate	(+)
Ornithine decarboxylase	(+)
L-arabinose Fermentation	(-)

Species of the genus *Serratia* produce flagella, and are capable of rapid swarming motility. This motility is temperature sensitive, and appears most active between 30^{o}C and 37^{o}C (Shanks *et al.* 2013). The cell's motility is aided by secreted surfactants, called serrawettin 1, 2 and 3. These three molecules are cyclodepsipeptides that capture water molecules to form a slippery gel. Bound to the cell's surface, serrawettin molecules enhance flagellum independent expansion of bacterial populations (Pradel *et al.* 2007).

Role in Bioterror Models: Due to its characteristic red pigmentation and simple biochemical identification, *S. marcescens* has a long history of use as a marker organism in public health research. While controversial, those early forays into epidemiology were informative. The first recorded use of *S. marcescens* as a tracer was by James Cumming in 1920, shortly after the end of the first world war (Mahlen 2011). Two groups of troops were gathered; one to have their

eating utensils sprayed with red pigmented *S. marcescens*, and the other to use the first group's utensils after washing in warm water. Both groups had their lips, teeth and tonsils swabbed to evaluate the spread from the first group to the second. Bright red colonies were isolated from the mouths of both groups, serving as proof of oral transmission of bacteria.

Early military studies using *S. marcescens* were small in scale, but later studies were much larger and far more controversial. A massive aerosolized release of *S. marcescens* off the coast of San Francisco, labelled Operation Sea-Spray, aimed to measure the wind's ability to spread biological weapons. Inland collecting stations capture airborne bacteria and cultured them for identification (Mahlen 2011; The New York Times 1981).

As part of congressional hearings on the release of pathogens onto non-military populations, the Centers for Disease Control (CDC) bio typed *S. marcescens* prepared for the release over San Francisco (Mahlen 2011; Rubin *et al.* 1976). It was categorized as biotype A6, serotype O8:H3, phage type 678. The CDC then serotyped over 2000 *S. marcescens* cultures from the continental United States. Of those 2000, only 7 matched the Operation Sea-Spray strain type. The conclusion of the CDC was that the military strain did not contribute to a significant increase in outbreaks, or represent an abnormally pathogenic strain.

Non-Human Pathogenicity: *Serratia* species can be found in freshwater, marine, and soil environments. These habitats bring it into close contact with both plants and animals. The cause of leafspot disease in the flower *Protea cynaroides* is caused by *S. proteamaculans*. The other species of *Serratia* are commensal with the root surface of plants, such as *S. liquefaciens* and *S. rubidaea*. Species in this genus can also live as symbionts. *Heterorhabditidoides chongmingensis* lives with *Serratia nematophilia* in its intestine, and requires symbiosis for its normal life cycle. *S. marcescens* has been isolated from water, soil, plants and animals. It seems to be a particularity

potent insect pathogen. Honey bees are an economically important victim of *S. marcescens* infections. In a study by Rayman *et al.* 2018, honey bees from 4 different hives across the continental United States had 16s rRNA profiles prepared from gut isolates. Honey bee hives had as low as 20% and as high as 100% of individuals carrying *S. marcescens*, and that subsequent virulence assays using these isolated strains indicated that they were capable opportunistic insect pathogens when introduced orally or via hemolymph injection. It was evidence that *S. marcescens* was not only an opportunistic insect pathogen, but its virulence varied significantly between strains.

1.1.2. Incidence and Mortality

In a 2008 survey by the European Center for Disease Prevention and Control, it was observed that 2.0% of all bloodstream infections were confirmed as *S. marcescens* (Zarb *et al.* 2012). This makes it the 10th most commonly recovered organism from hospital acquired blood stream infections. The same study found that 2.8% of pneumonia cases were also positive for *Serratia*. Data gathered by the CDC in the United States between 2004 and 2008 observed a higher proportion of *Serratia* positive pneumonia cases, at 4.1%. Surveillance of urinary tract infections in Japan found that 6.4% of cases were positive for *S. marcescens*. Several other *S. marcescens* infection routes and outcomes have been observed, such as meningitis and conjunctivitis (Johnson *et al.* 1998; Zingg *et al.* 2017). There does not appear to be a preferred area of colonization, and the most common feature of outbreaks is that they are health care associated. Risk factors for infection are extended hospital stay, gratuitous use of antibiotics, insufficient infection control practices, immune compromise or existing illness. Long term contact with medical devices such as catheters or prosthesis significantly increase probability of infection. The genotype of the *S. marcescens* strain can contribute to mortality as well, with mutations or acquisition of genes that

lead to resistance to treatment options of particular concern. Loss of membrane permeability due to changes in the outer membrane profile, particularly with respect to pore proteins is a frequent reason for heightened resistance (Yang *et al.* 2012).

Mortality among those infected with *S. marcescens* is high. A retrospective study by Kim *et al.* 2015 aimed to determine a 28 day mortality rate for *S. marcescens* bacteremia positive individuals in Seoul, South Korea between 2006 and 2012. Of the 98 individuals considered, 28 day mortality was 22.4%. Significant predictors of mortality were low serum albumin level, elevated Sequential Organ Failure Assessment (SOFA) score and presence of indwelling catheter. For comparison, *Staphylococcus aureus* bacteremia has a 30 day mortality between 10% and 30%, with poorer outcomes significantly linked to infections in developing nations (Hal *et al.* 2012). While *S. marcescens* causes far fewer infections per year, infections are often resistant to health care intervention.

1.1.3. Virulence and Pathogenesis in Humans

The types of infections caused by *S. marcescens* are diverse. Most typical are infections along indwelling medical devices or prosthesis, likely due to contamination in the health care setting. Urinary tract and bladder infections are particularly frequent. Eye infections are also described often, and are typically associated with the wearing of corrective lenses or recent surgery. Very few gastrointestinal infections have been recorded, and they are restricted to pediatric populations. Respiratory infections can often test positive for *S. marcescens*, although to what degree it is a bystander to other species or is in fact the instigator of such infections is unclear (Cristina, Sartini, and Spagnolo 2019). This species is more rare in nasal infections, but a study by Bernard *et al.* (2018) did observe positive *S. marcescens* cultures from patients who had recently undergone reconstructive nasal surgery. Of the 29 patients with nasal samples

positive for *S. marcescens*, 26 had undergone endoscopic sinus surgery prior to becoming culture positive. This further reinforces *S. marcescens* prevalence as a health care acquired pathogen, and raises further questions about its role in the respiratory tract.

1.2. Porins

1.2.1. Background

The term “porin” originated with the researcher Hiroshi Nikaido, who is one of the great cell membrane biochemists of the last half century. In 1971, Dr. Nikaido began investigating the outer membrane composition of *S.* Typhimurium, in efforts to characterise the biochemical properties of lipopolysaccharide (LPS). It is now well known that LPS is a key inflammatory molecule affixed to the outer membrane of Gram positive and negative bacteria. Furthermore, he was first to conclude that LPS served to inhibit the dissolution of the cell membrane by hydrophobic detergents (Ames, Spudich, and Nikaido 1974). In that same study, using an LPS biosynthesis mutant library, Dr. Nikaido observed that substantial reductions in outer membrane protein concentration resulted in reduced permeability of the outer membrane, and profound changes in the phospholipid composition of the lipid bilayer. This led to the theory that transmembrane pores, or “porins”, transverse the cell envelope to facilitate diffusion of water and solutes (Bavoil, Nikaido, and Meyenburg 1977).

The first porins to be functionally characterized were OmpC, OmpF and PhoE in *E. coli* by Nikaido and Rosenberg (1981) using a simple liposome swelling assay. These three proteins have since been categorized as part of Type 1 General Bacterial porin family, which is ubiquitous among Gram negative species. Other porins have also been identified, with functions beyond just diffusion of material through the outer membrane. Of particular interest are the Gram negative

porins OmpA, OmpX and LamB due to the wealth of existing research on these proteins and importance to cell function.

1.2.2. Porins in *S. marcescens*

1.2.2.1. OmpF

The outer membrane porin OmpF is one of the three porins first identified by Nikaido & Rosenberg in 1981. It is a 41 kDa protein, which is folded as a 16 stranded antiparallel β barrel (Cowanit *et al.* 1995; Yamashita *et al.* 2008). There are two distinct surfaces to the pore; the periplasmic "smooth" side with 8 short hairpin turns, and the "rough" extracellular side with 8 long loops. The extracellular loops 1, 4, 5, 6, 7 and 8 partially cover the pore entrance and confer some of the size selectivity. OmpF assembles as a tight homotrimer in the outer membrane, and extracellular loop 2 serves to hold each subunit together. The third loop is folded into the OmpF pore to reduce its diameter. The inner pore residues are hydrophilic, and produces a size exclusion limit of the OmpF pore is ~600 Da, which lets key nutrients through but keeps out larger compounds. This porin was first characterized by Nikaido *et al.* in 1981, but has since been identified in *S. marcescens*. The OmpC and OmpF orthologues were found in *S. marcescens* clinical isolate UOC-51 by Hutsul and Worobec in 1994. Both orthologues are approximately 70% homologous to the *E. coli* versions and maintain the conserved –PEFGGD- amino acid sequence in extracellular loop 3.

The OmpF pore does not exhibit any substrate specific gating. Compounds larger than 600Da are excluded on the basis of size, and the pore has difficulty transporting hydrophobic molecules. Research on *S.* Typhimurium OmpF by Benz, Schmid, and Robert (1985) observed a twofold preference for cations over anions. Liposomes containing OmpF were prepared, and treated with anion or cationic solutions, and evaluated for electrical membrane potential. Treating liposomes

permeated with OmpF have significantly reduced membrane potential following treatment with KCl, indicating a heightened flow of ions across the liposome membrane. The *ompF* gene is located in the Db11 genome at the SMDb11_RS10455 locus, and shares 68% identity with the *E. coli* orthologue.

Due to OmpF's relatively large pore size for a bacterial porin, its primary role is the influx of nutrients in low nutrient conditions. OmpF expression is heightened in low solute environments (high water availability) in order to increase membrane permeability to any available ions and nutrients (Dupont *et al.* 2007; Liu and Ferenci 1998). Furthermore, OmpF's high pore flow rate makes it a common point of entry for reactive oxygen species, H+ ions or salts, so in damaging oxidative or pH conditions its synthesis is decreases in order to reduce membrane permeability (Begic and Worobec 2005; Yamashita *et al.* 2008).

1.2.2.2. OmpC

OmpC is typically studied in parallel with OmpF, due to their shared regulatory relationship through the EnvZ/OmpR two component system (TCS) (Liu and Ferenci 1998; Oshima *et al.* 2002). The pore formed by OmpC is smaller than that in OmpF, at 10 Å diameter compared to OmpF's 20 Å diameter (Baslé *et al.* 2006; Lou *et al.* 2011). Like OmpF, OmpC is composed of 16 stranded β- barrel fold, linked into a tight trimeric structure. Extracellular loop 4 of OmpC curves over the perimeter of the pore entrance, constricting its entrance diameter. In *E. coli* K12, DNA sequences of *ompC* and *ompF* share 69% identity. Amino acid homology in the pores of the two proteins is slightly higher, at 74%.

Observations by Begic & Worobec in 2005 illustrated the control of OmpF and OmpC expression in *S. marcescens* via two systems: the first is the TCS EnvZ/OmpR and the second is

the small non coding RNA micF. The snRNA micF is stimulated under high stress conditions, and will bind OmpF mRNA to cause its destruction through the bacterial response to double stranded RNA. Using a β galactosidase fusion reporter, Begic & Worobec were able to photometrically quantify expression of each porin under a variety of conditions. Expression of *S. marcescens* OmpF and OmpC increased as the concentration of sucrose or salicylate increased, as well as increasing pH from 6 to 8. High temperatures appear to inhibit expression of *S. marcescens* OmpC and OmpF, with the greatest promoter activity at 28^{o}C, and the least at 42^{o}C.

1.2.2.3. PhoE

PhoE is very similar in structure to OmpC and OmpF, in that it forms a 16 stranded antiparallel β barrel. Like OmpC and OmpF, it forms a tightly bound homotrimer in the outer membrane (Hagge *et al.* 2002). The PhoE pore displays affinity for phosphate containing compounds. Unlike OmpF, which displays a twofold preference for cationic compounds, PhoE is anion selective, as illustrated in the same liposome swelling experiment performed by Benz *et al.* (1985).

Unlike OmpC and OmpF, PhoE expression is controlled by the PhoB/PhoR TCS, which is stimulated by phosphate starvation (Chekabab, Harel, and Dozois 2014). In low phosphate conditions, the PhoB response regulator binds the PhoE promoter to drive expression. A common feature of OmpF and PhoE is the role of micF snRNA, which post transcriptionally represses their expression (Hagge *et al.* 2002).

1.2.2.4. LamB

S. marcescens LamB forms a homotrimer in the outer membrane like OmpC, OmpF and PhoE. However, it is structurally and phylogenetically distinct, and does not fall into the same

family. The LamB monomer is an 18 β strand β barrel (Gelder *et al.* 2002). Like OmpC, it has an outer loop that folds over the pore entrance to constrict its diameter, thus increasing its size specificity. The internal pore residues are largely aromatic, which goes to facilitate the transport of carbohydrates (Bert van den 2012).

S. marcescens lamB is annotated as SMDb11_RS18465. It shares 78% homology with the multispecies maltoporin consensus sequence (WP_074188961.1). While *lamB* is annotated as a maltoporin in many organisms, it is also used to import a multitude of necessary sugars (Gelder *et al.*, 2002). Depletion of glucose and cAMP is a key signal for induction of *lamB*, increasing permeability to much needed carbohydrates.

The *lamB* gene is regulated by the *malEFG* operon and the *malK lamB malM* operon, which achieves maltodextrin import through control of LamB and a series of ABC type transporters (Gelder *et al.*, 2002).

1.2.2.5. OmpA

In the *S. marcescens* Db11 genome, *ompA* is locus tagged as SMDb11_RS05155, and shares 79% homology with the *E. coli* orthologue on the amino acid level (NP_415477.1). No specific studies of OmpA have been conducted in *S. marcescens*.

The role of OmpA protein as a porin is primarily the maintenance of structural integrity between the outer membrane and the peptidoglycan layer (Mittal *et al.* 2011). While OmpA has a pore diameter approximately equal to that of OmpF, at about 20 Å, it has a substantially lower role in membrane permeability. In studies of *Acinetobacter baumannii* OmpA, it had an observed permeability ~70 fold less than OmpF (Smani, Roca, and Vila 2014).

OmpA's primary role is the interface of the outer membrane and the peptidoglycan layer. This is achieved either as a monomer or dimer. The protein's N terminus is embedded in the outer membrane, where it serves as a linker for potential dimerization. The C terminal domain is non-covalently linked to the peptidoglycan layer. The dimer linkage can serve as a hinge, pulling the protein barrel and the peptidoglycan layer towards or away from the outer membrane (Tamm *et al.* 2003).

OmpA appears to have a role in host cell adhesion in *K. pneumoniae* and *E. coli*, assisting virulence (Mittal *et al.* 2011). In a murine meningitis model, OmpA was shown to be necessary for infection, and appeared to be binding host epithelial surface glycoproteins. The expression of OmpA was required for successful invasion of HBMEC cells by binding Ecgp96, an HSP90 related protein.

1.2.2.6. OmpX

Integral outer membrane protein X (OmpX) is structurally reminiscent of typical outer membrane porins, but instead has a major role in host cell adhesion as opposed to membrane permeability. The *E. coli* homolog was crystalized in 1999 by Vogt & Schulz to a 1.9 Å resolution, and observed an 8 stranded β sheet that extends into the extracellular space to present 4 loops for binding to host substrates. These host targets are yet to be determined.

Whilst OmpX is upregulated by *Enterobacter aerogenes* in high osmolarity media, as observed by (Dupont *et al.* 2004), the OmpX pore is too small for even water molecules to pass. Structural analysis o of OmpX with the software HOLE observed tight hydrogen bonding between Lys27, Tyr80 and Asp124 in the β barrel, creating a strong permeability barrier (Caflisch and Bo 2005).

S. marcescens OmpX was first characterized by Guasch *et al.* (1995), where they exogenously expressed a 17kDA *S. marcescens* protein called Omp4, which conferred resistance to bacteriocin N28. This protein was identified as an orthologue of *E. coli* OmpX. The *ompX* gene is locus tagged in *S. marcescens* Db11 as SMDb11_0761.

1.2.3. Assembly

Bacterial outer membrane porins must be exported from the cytoplasm through the inner membrane, then assembled and inserted into the outer membrane. The OMP proteins are synthesised by cytoplasmic ribosomes, and the Sec system translocates them across the inner membrane. The model for the Gram negative Sec translocation system is made up of 3 proteins: SecYEG, SecA and SecB (Green and Mescas 2016). The SecYEG complex is composed of 3 different subunits to span the inner membrane and form a flexible channel through which nascent membrane proteins can move. The SecA protein serves as an ATPase and provides energy to the SecYEG heterocomplex to move substrates across the membrane. The SecB protein serves as a cytoplasmic chaperone that primes substrate proteins in a translocation compatible state. SecB has been shown to bind and facilitate translocation of OmpC, OmpF, OmpA, OmpX, PhoE and LamB, as well as variety of other membrane proteins (Baars *et al.* 2006).

Once through the inner membrane, the assembly and outer membrane insertion of OMPs is performed by the β barrel assembly machinery (BAM) complex. This complex is composed of BamABCDE. Its BamA subunit is shaped by the other 4 subunits into an active conformation. The active sites of BamA reduce the lipid viscosity in the outer membrane and make insertion of new outer membrane proteins more energetically favorable (Galdiero *et al.* 2012; Green and Mescas 2016).

Additional proteins assist in both translocation through the membrane and assembly of nascent proteins. For example, the DnaK/DnaJ/GrpE can act in place of SecB to successfully translocate proteins through SecYEG (Altman, Kumamoto, and Emrl 1991). These proteins and others chaperone translated OMPs to their destination.

1.3. *S. marcescens* and Antimicrobials

1.3.1. Antibiotics used in *S. marcescens* Treatment

Antibiotics are an umbrella term for compounds that are acutely effective at either halting bacterial growth (bacteriostatic) or killing bacterial cells (bactericidal) when applied. Many of these compounds are naturally secreted by organisms as self defense mechanisms, as is the case with penicillin which was first observed as a natural product of the ascomycetous fungi *Penicillium*. Since the discovery of penicillin in 1928 by Dr. Alexander Fleming, countless related compounds have been discovered or synthesised with the same β lactam ring structure. The family of β lactam antibiotics have been the drugs of choice for Gram positive and negative infections for decades. Many other families of drugs have been developed in the decades since. For example, two classes of drugs commonly used in the treatment of *S. marcescens* are the aminoglycosides and the fluoroquinolone antibiotic classes. These are the preferred method of treatment because *S. marcescens* has high inherent resistance to a variety of β lactam antibiotics. Furthermore, this organism is developing resistance to multiple classes of antibiotics more and more frequently due to the species' genetic plasticity and abundance of compatible plasmids (Iguchi *et al.* 2014; Moradigaravand *et al.* 2016). Mechanisms of resistance can vary from drug to drug, and from species to species.

1.3.2. Drug Inactivating Enzymes

1.3.2.1. Beta Lactam Resistance

Beta lactam resistance is achieved in *S. marcescens* through four mechanisms 1) inactivating enzymes, 2) efflux of drugs, 3) alteration of penicillin-binding proteins (PBP,); and 4) reduction in membrane permeability (Munita and Arias 2016; Yang *et al.* 2012; Zaman *et al.* 2017).

The β-lactam inactivating enzymes are referred to as β-lactamases, and often target the amide bond of the β-lactam ring to halt its activity. One such group of β lactamases are the extended spectrum β-lactamases (ESBL), which are often plasmid encoded. There are over 500 different enzymes in this group. The ESBL group belongs to the class A group "2be" of the β lactamases, which are effective against carboxypenicillins, ureidopenicillins, cephalosporins and Aztreonam. However, these enzymes do not affect carbapenems and are vulnerable to the inhibitory molecules clavulanic acid and tazobactam. *S. marcescens* clinical isolates frequently carry plasmids with genes encoding such enzymes (Yang *et al.* 2012).

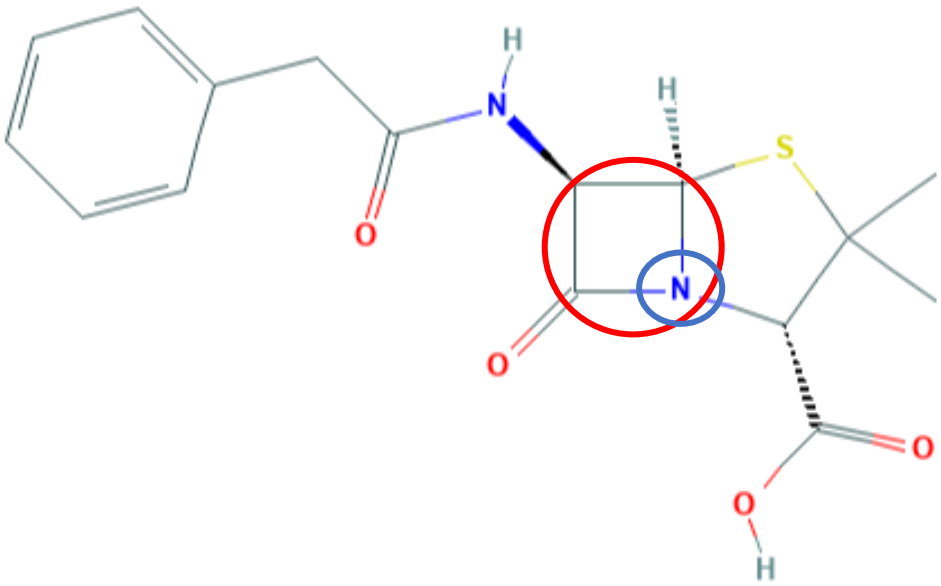

Figure 1: 2D line structure of Penicillin-G. The β lactam ring is circled in red, and the amide targeted by β lactamases is circled in blue (Pubchem CID 5904).

All strains of *S. marcescens* carry a chromosomally encoded β-lactamase from the AmpC class. It was the first ESBL enzyme to be discovered, and confers resistance to the majority of cephalosporin drugs. Mutations in the active site of this enzyme can significantly modify its spectrum of activity. Amino acid substitutions in an *S. marcescens* AmpC collected in southern Taiwan displayed a 100 fold increase in activity against ceftazidime compared to the wild type AmpC (Yang *et al.* 2012; Yu *et al.* 2019). However, the AmpC enzyme cannot confer phenotypic resistance on its own, and required two other gene products. These proteins are AmpR (regulator), AmpG (permease), AmpD (amidase). AmpC and AmpR are transcribed in opposite directions from an intervening promoter. The induction of AmpC by certain β lactam drugs can only be achieved in the presence of AmpR. (Munita and Arias 2016; Zaman *et al.* 2017). In the absence of β-lactam drugs, AmpR is binds to free peptidoglycan subunits, keeping AmpR from binding the AmpC promoter. When β-lactams break down the peptidoglycan layer, the resulting production of cell wall synthesis peptides compete for the peptidoglycan precursors, thereby releasing AmpR. This free AmpR can now bind the AmpC promoter and induce *ampC* expression.

A class of β-lactamases of particular concern are the carbapenemases, which can hydrolyze the majority of β lactams including carbapenems (Zaman *et al.* 2017). These have been detected in *S. marcescens* isolates worldwide (Bush 2010; Yang et al. 2012). Carbapenems are typically drugs of last resort, and are used when causative organisms are already highly resistant to other drugs. Molecular class A, B and D carbapenemases have been detected in many *S. marcescens* strains (Yang *et al.* 2012). The *Serratia marcescens* enzymes (SME) and *Klebsiella pneumoniae* carbapenemase (KPC) type enzymes are part of molecular Class A and are the more frequently encountered enzymes in clinical isolates. They confer potent resistance to penicillins, aztreonam, cephalosporins and carbapenems. However, SME type enzymes can be inhibited only by

clavulanic acid, whereas KPC type enzymes can be inhibited by both clavulanic acid and tazobactam. The imipenemase (IMP) type enzymes of the class B carbapenemases are increasingly prevalent in clinical isolates of *S. marcescens* worldwide. The IMP enzymes are metallo-β lactamases that confer near ubiquitous resistance to β lactams, with the exception of aztreonam. They were initially detected in Japan in the early 2000s but have since spread around the globe (Datta *et al.* 2014; Kong, Shneper, and Mathee 2011).

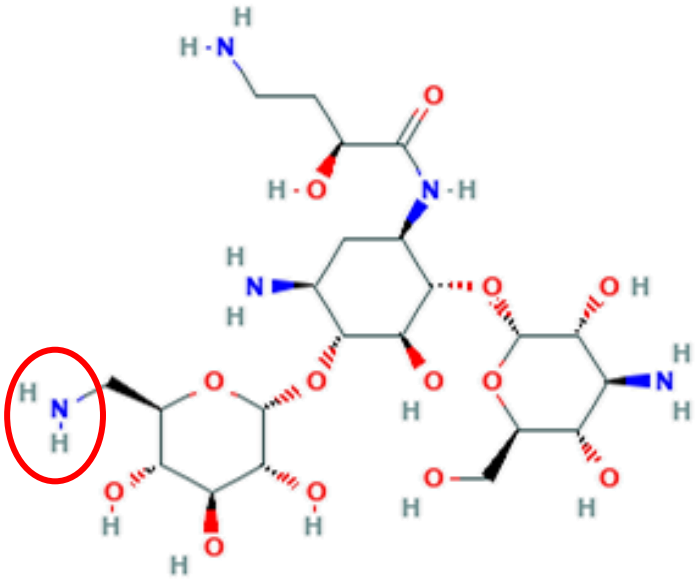

Figure 2: 2D line structure of Amikacin. The 6" Amine that is acetylated by AAC6" is circled in red (PubChem CID 37768).

1.3.2.2. Aminoglycoside Resistance

Because of its predisposition to β lactam resistance, *S. marcescens* is frequently treated with aminoglycoside drugs. These drugs target the 30S subunit of cytosolic ribosomes to block protein synthesis (Krause *et al.* 2016; Yang *et al.* 2012). However, *S. marcescens* isolates can carry enzymes that can modify aminoglycosides to reduce their activity. The most common enzyme of this class in *S. marcescens* is the Aminoglycoside 6-N-acetyltransferase (AAC 6") enzymes. It can acetylate aminoglycosides at the 6" amine position (Figure 2).

AAC6" activity can confer resistance to tobramycin, dibekacin, amikacin, netilimicin, ethynetilimycin and sisomycin. However, a second class of bifunctional enzyme has been observed in *S. marcescens* that is capable of both adenylating and acetylation of aminoglycosides at different positions. This leads a greater spectrum of activity and resistance. The final class of aminoglycoside modifying enzymes in *S. marcescens* are the AAC (6")-lc proteins. These are encoded by a separate gene, and can be found in the genome of certain *S. marcescens* strains.

1.3.2.3. Fluoroquinolone Resistance

The third class of antibiotic drugs often used in the treatment of *S. marcescens* are the fluoroquinolones (Yang *et al.* 2012). These drugs target DNA gyrase to destabilize genome replication and inhibit bacterial proliferation. Resistance can be achieved by mutations in GyrA or by efflux of the drug (Ezelarab *et al.* 2018). Mutations granting resistance are common, but are not themselves transmissible by plasmids. However, plasmid mediated quinolone resistance (PMQR) sequences can confer resistance through a variety of mechanisms. Quinolone resistance genes (QNR) encode repeat protein structures that interfere with ciprofloxacin's binding to DNA gyrase. The QNR proteins also increase the prevalence of genomic mutations that could lead to changes

in DNA gyrase that no longer allow fluoroquinolone activity. The more potent resistance gene found on plasmids are the AAC(6")-lb-cr enzymes, which can acetylate both aminoglycosides and fluoroquinolones resulting in resistance (Ezelarab *et al.* 2018; Yang *et al.* 2012).

While fluoroquinolone can be inactivated by enzymes, changes in permeability or efflux are also common mechanisms of resistance in *S. marcescens* (Inato *et al.* 2008; Wozniak *et al.* 2012).

1.3.3. Porins and Antimicrobial Resistance (AMR)

The primary role of porins in the outer membrane is to facilitate permeability, and are therefore the point of entry for the majority of hydrophilic antimicrobials (Galdiero *et al.* 2012; Yang *et al.* 2012). In *S. marcescens*, there has been limited research into porins and their role in antimicrobial resistance (Yang et al. 2012). The focus has been on OmpC and OmpF, as they are the least specific and most highly expressed in the cell. A study by Moya-Torres *et al.* (2014) observed heightened resistance to β lactam drugs ampicillin and cefoxitin, as well as nitrofurantoin in *S. marcescens* OmpF deletion mutants. This was not observed in *S. marcescens* OmpC mutant strains. This observed OmpF dependant resistance to β lactams is also observed in *E. coli* and *K. pneumoniae* (Garcia-Sureda *et al.* 2011; Sugawara, Kojima, and Nikaido 2016). There are several other porins in *S. marcescens* that have been implicated in antimicrobial resistance in closely related species. A recent study by Choi & Lee, 2019 investigated each of the major outer membrane porins in *E. coli*. They generated mutant strains defective for OmpC, OmpF and OmpA, and measured subsequent changes to growth, membrane integrity and antibiotic resistance. By testing the single, double and triple mutant strains for growth rate under various stress conditions, the researchers were able to hypothesise functions for each of these three porins. Mutants deficient in *ompC* and/or *ompA* were shown to have increased susceptibility to NaCl, but not to sodium dodecyl

sulfate (SDS) and ethanol. Susceptibility to NaCl in these OmpC and/or OmpA mutants was partially reduced by deletion of OmpF, but lack of all three did not display wild type phenotypes. This indicated that the OmpC and OmpA proteins are involved in osmoregulation and membrane stability, which is supported by structural studies of both OmpA and OmpC. Furthermore, the Δ*ompC*Δ*ompF*Δ*ompA* mutant exhibited increased permeability to fluorescent DNA dye SYTOX green as compared to the wild type strain. Heightened permeability to SYTOX green in absence of these three important OMP proteins suggests a compromised outer membrane. This triple OMP mutant strain also displayed significant growth defects in lysogeny broth (LB) medium growth conditions, indicating substantial changes to osmoregulation and membrane integrity as a result of the genotype (Choi and Lee 2019).

Choi and Lee (2019) observed that deletion of *ompF* resulted in heightened resistance to ampicillin, cefalotin, cefoxitin, ceftazidime, aztreonam, tetracycline, chloramphenicol, clindamycin and cinoxacin. Single deletion of *ompC* resulted did not affect the resistance profile, but did result in heightened susceptibility to imipenem and puromycin. Loss of OmpA resulted in increased susceptibility to cefalotin, imipenem, vancomycin, chloramphenicol, clindamycin, puromycin, mupirocin and trimethoprim. Loss of both *ompA* and *ompC* resulted in heightened susceptibility across all tested antimicrobials except for cinoxacin, novobiocin and nitrofurantoin. Interestingly, the deletion of *ompC*, *ompF* and *ompA* resulted in a resistance profile similar to that of *ompF* mutant strains but with a massive increase in susceptibility to all non-β lactam drugs tested (Choi and Lee 2019).

An earlier study by Dupont, James, Chevalier, & Page (2007) investigated the relationship between OmpF and OmpX in the context of antimicrobial challenge and adverse growth conditions. They did so by measuring expression of *ompX* via a β galactosidase assay. Dupont *et*

al. (2007) observed a significant difference in expression of *ompX* when challenged with a variety of antimicrobials, harsh solutes and solvents. The three conditions that drove *ompX* expression the most were the presence of novobiocin, the presence of dipyridyl, and the presence of salicylate. Furthermore, significant increases in OmpX expression were observed in the presence of paraquat, norfloxacin, nalidixic acid, ethanol, phenethyl alcohol (PEA), and growth at 42^{o}C. The MarA protein appears to be an activator of *ompX*, illustrated by a lack of overexpression of *ompX* in MarA deletion mutants under the same stressful conditions. Induction of the regulator MarA has been shown occur in response to various antimicrobials, and control the expression of genes involved in antimicrobial resistance. While the pore diameter of OmpX is very small, and its contribution to membrane permeability is debated, it does appear to be part of the cell's response to various antimicrobials and membrane stressors.

1.3.4. Efflux and Antimicrobial Resistance

Efflux pump systems are a significant contributor to antimicrobial resistance, and are a growing subject of interest in the research community. *Serratia* species have many efflux pumps that are well studied in related species. The major families of efflux pumps in *S. marcescens* are the resistance nodulation division (RND), major facilitator superfamily (MFS), ATP-binding cassette (ABC) and small multidrug resistance (SMR) efflux pumps (Kumar and Worobec 2002, 2005; Li, Plésiat, and Nikaido 2015).

The export of fluoroquinolones by RND efflux pump SdeAB was first observed by Kumar & Worobec in 2002. They cloned the *sdeAB* gene into *E. coli* strain AG102MB, and observed increased resistance to all fluoroquinolones. Furthermore, efflux of chloramphenicol, SDS, ethidium bromide and n-hexane by SdeAB was observed in the *E. coli* system. The SdeAB pump is composed of the SdeA periplasmic adaptor component, and the SdeB transmembrane

transporter. This system appears to be regulated by an upstream regulatory gene product *sdeR*, which itself is a homolog of the antimicrobial sensitive *E. coli* transcriptional regulator *marA*. There are however two other efflux systems in *S. marcescens*: SdeCDE and SdeXY. Deletion of the SdeCDE efflux system does not appear to increase the MIC of any common antimicrobials, either because it did not recognize the substrates or because it is not a functional efflux system. The final RND system is SdeXY, identified by GenBank searches of the NUSM8906 *S. marcescens* genome, contributes to resistance. Deletion of the genes coding for the SdeXY system resulted in significant decreases in resistance to tigecycline, tetracycline, ciprofloxacin and cefpirome.

The second category of efflux pump in *S. marcescens* is the MFS family system exemplified by SmfY. The gene for this system was cloned from *S. marcescens* NUSM8903 into hypersensitive *E. coli* strain KAM32 to evaluate the impact of SmfY on antimicrobial resistance. The KAM32 strain lacks *acrB, ydhE* and *hsd*, leading to a significant reduction in efflux capability. The introduction of plasmid borne *smfY* resulted in significant increases in resistance to norfloxacin, benzalkonium chloride, acrifavine and ethidium bromide. Some strains of *S. marcescens* harbor multiple orthologues of this SmfY system.

The first *S. marcescens* ABC system identified was SmdAB in *S. marcescens* NUSM8906 (Matsuo *et al.* 2008). The two components, SmdA and SmdB, did not individually contribute to drug resistance in the KAM32 hypersensitive *E. coli* strain. However, when combined, this system results in significantly increased resistance to DAPI, norfloxacin, ciprofloxacin, tetracycline, and Hoescht 33342 (a nucleic acid stain). A particularity interesting note on ABC efflux pumps is that they are highly conserved between both prokaryotes and eukaryotes. In human cancers, they can contribute to chemotherapy resistance by pumping drugs out of the cell. The ABC transporter

systems are highly conserved in both bacteria and in humans, and inhibitors are not pursues as potentially treatments due to high cytotoxicity in human cells (Sun *et al.* 2012).

The final efflux system to be discussed is the SMR efflux pump systems. It was initially investigated by Inato *et al.* (2008). Much like this research group's work in ABC type pumps, they cloned the SMR type *ssmE* efflux pump into the KAM32 *E. coli* strain. Expression of this pump in KAM32 resulted in significant increases in resistance to norfloxacin, ciprofloxacin, acriflavine, chlorohexidine, ethidium bromide, and methyl viologen. Of these, the expression of *ssmE* resulted in the largest increases in resistance to acriflavine and ethidium bromide. This finding has also been reported in studies of the *E. coli* SMR efflux pump EmrE.

The high inherent resistance to β-lactams displayed by *S. marcescens* has led to treatment protocols that rely heavily on later generation β-lactams such as carbapenems, aminoglycosides and fluoroquinolones. However, the growing number of genomic and plasmid borne resistance mechanisms observed in clinical isolates is worrying, and will present significant challenges for health care providers going forward (Yu *et al.* 2019).

1.4. The Role of Porins in Bacterial Pathogenesis

The first challenge a pathogen needs to overcome in its attempt to colonize the host environment are physical barriers. The most basic defense an organism has is physically blocking the movement of invaders. These can be prebuilt, or put together in response to attack (Wang, 2014). For example, plants encase infected tissues in carbohydrate polymers, whereas mammals will block off infected areas with a mesh of fibrin and platelets (Acimovic *et al.* 2015; Rey *et al.* 2013; Wang 2014). Walls of epithelial tissue can be a serious impediment to bacterial colonization, should the pathogen lack any way to stick to the host cells. This adhesion is core to many

microorganisms' ability to cause disease (Galdiero *et al.*, 2012). The OMP found in *S. marcescens* that has been shown to play a role in adhesion in other species are OmpA. OmpA like proteins are present in all genera of the Gram negatives. A study by Mittal *et al.*, (2011) investigated the binding target of *E. coli* K1 OmpA on the surface of neonatal mouse neurons. Furthermore, they mutated the four extracellular loops of K1 OmpA to test the impact on virulence. Based on their findings, OmpA is capable of binding Fc-γ, Ecgp96 and gp96 to initiate invasion of macrophages and neurons. Mutation of loops 1, 2 and 4 caused *in vitro* increases in serum susceptibility. In mouse infection models, only the loop 1 and loop 2 mutants displayed reduced virulence. The K1 OmpA loop 4 mutant displayed significant increases in inflammation and mortality. The K1 OmpA loop 3 mutant was also hyper virulent, and was better able to invade and survive inside of macrophages and neutrophils. Many pathogens use immune cells as carriers across surfaces normally impermeable to foreign cells, but can be infiltrated by host immune cells (Ishii *et al.* 2014; Mittal *et al.* 2011; Newton *et al.* 2010).

The second line of defense against infections are chemicals secreted into bodily fluids or stored in cells. These molecules can interact with conserved pathogenic peptides and carbohydrates to result in white blood cell chemo attraction, pathogen opsonisation or pathogen death. In humans, the primary innate enzymatic defenses are the classical and alternative complement pathways. The classical pathway begins with C1 binding IgG antibodies that have opsonized a microbe. This begins a 9 protein cascade that results in the formation of a poly-C9 pore, complexed with C6, 7 and 8. This massive pore results in loss of bacterial membrane integrity and cell death. The alternative pathway begin with mannose binding lectin (MBL) binding conserved bacterial mannose residues, which recruits C1 homologs MASP-1 and 2. The intermediate steps of the alternative pathway differ slightly from the classical, but result in the same outcome; immune cell

attraction and C9 pore formation. Bacteria have evolved ways to overcome complement. With respect to the porins found in *S. marcescens*, the OmpX protein is hypothesised to play a role in host defense subversion (Nishio *et al.* 2005; Vogt and Schulz 1999). Many bacteria have a 17kda outer membrane protein with high total homology to *S. marcescens* OmpX, ranging from 50% to 99%. The OmpX homolog found in *S.* Typhimurium, called PagC, was cloned into *E. coli* by Nishio *et al.* (2005) to measure the level of swine serum resistance. When *pagC* was expressed in *E. coli*, the strain's growth in active porcine serum was equivalent to its growth in inactivated porcine serum. Mutagenesis of PagC resulted in loss of serum resistance. Structural studies of *E. coli* OmpX have identified external loop 1 as a potential antibody and complement binding site. Similar analysis of *S.* Typhimurium Rck and *Yersinia enterocolita* Ail have identified residues involved in virulence and serum resistance were identified (Nishio *et al.*, 2005).

Besides complement, potential hosts deploy a wide array of antimicrobial peptides (AMP) to kill or slow down microbes (Wang 2014). Bacteria can detect and respond to these molecules through sensor kinase systems, changing metabolism to overcome or halt the damage. Two component systems such as PhoP/Q and EnvZ/OmpR are responsible for changes in transcription upon exposure to AMPs (Barchiesi *et al.* 2012; Wang 2014). In *S.* Typhimurium, the trimeric porin OmpD forms a complex with the PhoP/Q regulated YdeI protein to achieve heightened resistance to cathelicidin; a macrophage stored polypeptide critical in the phagosome. Research by Pilonieta *et al.* (2009)observed that the loss of YdeI alone results in the greatest loss of cathelicidin resistance. OmpD disruption causes a ~60% increase in sensitivity to both polymyxin B and to cathelicidin. Strangely enough, loss of OmpD and YdeI results in the smallest reduction in resistance. This suggests that without YdeI, the OmpD porin somehow facilitates the cathelicidin mediated killing of *S.* Typhimurium. Pilonieta *et al.*, (2009) suggests the interaction of outer

membrane porins and other proteins to block entry of foreign molecules or maintain membrane integrity.

OMPs play an important role in the regulation of virulence, by controlling the diffusion of compounds subsequently detected by inner membrane sensor kinases, which then differentially regulate virulence factors (Oshima *et al.* 2002). While PhoP/Q can respond to AMPs and Mg+ scarcity, it is also a core virulence regulator in many of the enterobacteriaceae. Other notable TCS are EnvZ/OmpR and PhoB/R, whose primary roles are responding to changes in osmolarity and phosphate starvation respectively. A 2017 study by Tipton and Rather (2017) observed the regulation of both colony phase variation, motility and virulence in the enterobacteriaceae *A. baumannii* by the TCS EnvZ/OmpR (Tipton and Rather 2017). Genes controlling conversion of colonies from opaque to translucent variants were screened by high throughput transposon sequencing. Colonies the exhibited heightened or significantly reduced phase variation had their transposon insertion regions sequenced. One such transposon insertion locus was the *ompR* gene, which had resulted in ~50% more translucent colonies. Isogenic mutants of *A. baumannii* were made for both *envZ* and *ompR*, and both the single and combinatorial mutants exhibited significantly heightened switching. The translucent biased strains exhibited reduced virulence compared to opaque colonies when injected into a *G. mellonella* moth larva model. A degree of feedback exists between the function of outer membrane porins and their corresponding regulators. Without proper porin function, signals cannot reach the periplasm and inner membrane sensor kinases. Should porin function become dysregulated or perturbed, the upstream TCS function could be effected (Begic and Worobec 2005; Srividhya and Krishnaswamy 2004). This appears to be species dependant. While OmpR has been shown to regulate the genes *ompC* and *ompF* in *E. coli* and *S. marcescens*, the research by Tipton & Rather (2017) noted that *A. baumannii* OmpC

homology with *E. coli* OmpC is only 27%, and the lack of OMP regulation by OmpR was confirmed by quantitative polymerase chain reaction (qPCR).

Vaccine Development: Several publications in the field of vaccine development have observed the immune system's robust response to bacterial outer membrane porins. The ubiquity of OMPs, and the high degree of conservation between OMPs of different species makes them a perfect signal to the immune system for the presence of bacteria. Innate immunity is keyed to recognize conserved bacterial peptide domains, and (Chaplin 2010; Wetzler 2010) observed OMPs as a potential pathogen associated molecular pattern (PAMP). The immune system recognizes PAMPs via Toll-like receptors (TLR) presented on the surface of host cells. Wetzler (2010) observed that *Neisseria meningitides* trimeric β-barrel porin PorB stimulated a significant upregulation CD86, mediated by PorB binding of TLR2-TLR1 heterodimer binding and MyD88 activation. Such a cascade serves to alert the host immune system that infection is underway, and would attract additional macrophages, neutrophils and T cells to the area for clearance. In this way, OMPs can hinder a pathogen's virulence as they can be potent immune stimulants. This immunogenicity, combined with the correlation between porin function and antimicrobial influx serve as significant selective pressures on clinically relevant strains to perturb the function of their porins.

In the context of human infections, antimicrobial resistance is a constant concern. Patient outcomes are massively dependant on the resistance profile of their particular infection. This is particularly true of organisms like *S. marcescens* that display high genetic plasticity, and a resultant eagerness to take on new and useful resistance genes from other strains or species. Porins such as *A. baumannii* OmpA, *K. pneumoniae* OmpK36 and *E. coli* OmpF have been found mutated or deleted in clinical isolates, and are responsible for part of their heightened resistance

The relationship between host and pathogen is complex, but begins with the cell surface. Embedded in the surface of bacteria are OMP proteins, which both help and hinder the bacteria in its colonization of the host. Outer membrane porins assist infection by facilitating adhesion to host cells via their extracellularly exposed moieties, as is the case in OmpX and OmpA. Porins can control influx of host defense molecules in conjunction with other proteins, as is the case with *S. enterica* porins PagC and OmpD. Some large, trimeric porins can determine antimicrobial susceptibility, as they are the largest potential entry points in the membrane. Dysregulation or loss of these genes can result in clinically significant increases in drug resistance. On the other hand, OMPs can act as early warnings for the host immune system, where their highly conserved surface structures can be recognized as clearly bacterial and marked for destruction by both humoral and cellular immunity. Unfortunately, whilst many Gram negative porins have homologs in *S. marcescens*, only a very small number have received significant investigation. The growing clinical relevance of this organism should hopefully lead to greater scrutiny of its ability to overcome therapies and host defenses by regulating its membrane permeability.

1.5. *C. elegans* as a Model for Bacterial Pathogenesis

1.5.1. C. elegans

Caenorhabditis elegans is a model organism heavily studied and made popular by Dr. Sydney Brenner in the 1970s (Brenner 1974). It is a nematode roundworm ubiquitous to soil environments around the world (Powell and Ausubel 2008; Zhang *et al.* 2017). It is small, transparent bodied organism that hatched at a size of 0.25 mm in length and grows to 1 mm at adulthood. The nematode develops from an egg to reproductive adult in only 3 days, making proliferation of populations extremely rapid in a research setting. Nematodes live approximately

3 weeks in laboratory conditions and have a well understood life cycle. The *C. elegans* adult is eutelic, meaning that mature adults all contain the same number of cells; 959 somatic cells and 2000 germ cells. This feature has proven incredibly useful in the study of *C. elegans* development. Individual cells can be destroyed early in development or made to express a visible product, and the consequences can be tracked as development progresses (Corsi, Wightman, and Chalfie 2015). The consistency between adult organisms makes it an excellent model organisms for toxicological or infectious disease research, where variation between individuals would normally have an impact on results (Hellinga *et al.* 2015; Taffoni and Pujol 2015).

The *C. elegans* life cycle can be broken down into the following stages: embryo, eggs, Larval (L)1, L2, L3, L4, and finally gravid (egg laying) adult. Embryonic development lasts approximately 150 minutes after fertilization of the oocyte. Once developed, the egg is released through the vulva and develops through the remaining 5 stages. Occasionally, developmental defects or mutations in the parent can result in failure to develop the vulvar opening or otherwise release the eggs. The consequences of such a developmental defect are eggs hatching inside the gravid parent, resulting in the colloquially named "bag of worms" phenotype (Corsi *et al.* 2015; Powell and Ausubel 2008).

C. elegans nematodes are normally hermaphroditic, meaning they develop both male and female gametes. As such, they are capable of self-fertilization. Hermaphrodite gametes develop as a pair of U shaped tubular structures alongside the intestine on either side of the vulvar opening. Both of these tubes contain both male and female germline cells. An adult hermaphrodite *C. elegans* can produce several hundred eggs during their lifetime. Approximately 1 in every 1000 nematodes will be born male, and will develop different anatomical structures. Male nematodes develop only one gonadal tube, and do not develop a vulva. Furthermore, they develop a fan shaped

tail that houses a mating spicule responsible for release of sperm into the hermaphrodite's uterus. This process is marked by differences in behaviour, such as the male using touch to scan the hermaphrodite's reproductive opening (Brenner 1974; Corsi *et al.* 2015).

1.5.2. Anatomy

The *C. elegans* body plan is broken down into the following categories: epidermis, musculature, digestive tract, nervous system and reproductive tissue (Corsi *et al.* 2015). The epidermis of the *C. elegans* nematode consists of a basal layer of multinucleate epidermal cells that secrete specialized collagens, lipids and glycoproteins to form the cuticle. The cuticle is shed and rebuilt during development to allow the nematode to grow. In the case of damage to the cuticle, either through physical methods or colonization by a pathogen, the wound tissue changes actin structure to stitch the area shut and begin production of new cuticle polymers (Corsi *et al.* 2015; Taffoni and Pujol 2015). The cuticle also serves to anchor the nematode to its immediate environment and allow muscle contractions to move the whole body.

C. elegans muscle cells are mono-nucleate, and do not fuse together like they would in vertebrates. These muscles contract in regular waves that result in the nematodes undulating wave like movements. Genetic studies of *C. elegans* mutants resulting in muscle defects have led to advances in our understanding of degenerative muscle diseases, such as Duchenne muscular dystrophy. This fact underlines the fundamental shared pathways between the *C. elegans* nematode and more complex organisms. This is reflected in many systems in *C. elegans*, such as the nervous and immune systems (Corsi *et al.* 2015).

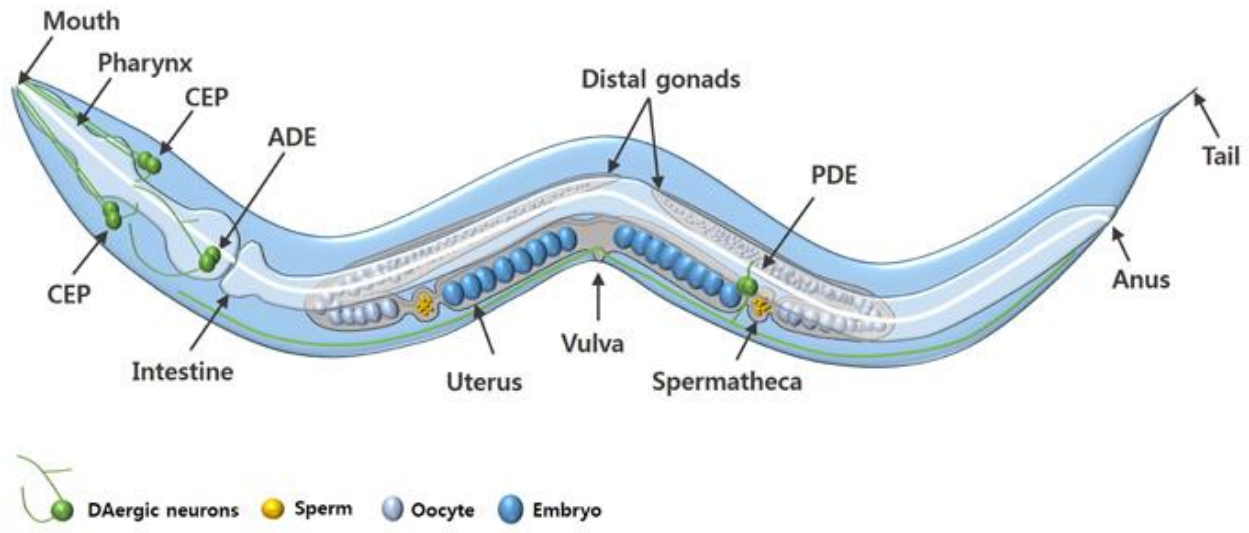

Figure 3: Diagram of adult *C. elegans* hermaphrodite. Important structures are indicated by arrows. Four cephalic (CEP) neurons, two anterior deirid (ADE) neurons, and two posterior derid (PDE) neurons are indicated in green (Chege and McColl 2014).

The digestive tract is an incredibly important and highly visible structure in the nematode. It is visible with minimal microscope magnification, and changes in its health due to disease are rapidly apparent (Powell and Ausubel 2008). The digestive tract begins at the pharynx, where food material enters and passes to the "grinder". The grinder is a muscular lobed structure that contracts to crush and pump bacteria into the intestinal lumen. The grinder structure's ability to destroy bacteria and prevent colonization decreases with age, which leads to increasing bacterial colonization over time. The intestine continues the breakdown of bacteria initiated by the grinder by chemically degrading food with a variety of proteases, lysozymes and surfactants. Available nutrients are subsequently absorbed into the intestinal epithelia by endocytosis. Starvation or dangerous food sources can be detected by a rudimentary nervous system, which can change feeding behaviour (Corsi *et al.* 2015).

The *C. elegans* nervous system consists of 302 neurons in an adult hermaphrodite (Chege and McColl 2014; Corsi *et al.* 2015). These neurons are concentrated in the head, ventral cord, and the tail. Due to the small number of total neurons, many express receptors that would be displayed by many different neurons in vertebrates. For example, individual *C. elegans* neurons produce multiple odorant receptors, instead of one receptor in human neurons. An olfactory receptor important in the detection of harmful bacteria Is the *tol-1* receptor and pathway (Glater, Rockman, and Bargmann 2014; Pradel *et al.* 2007). This receptor is homologous to vertebrate Toll-Like Receptors (TLR) but does serves a different function. Loss of function *tol-1* nematodes display increased feeding on pathogenic bacteria and increased infection.

1.5.3. Immune System

The *C. elegans* nematode has a wide array of genes involved in immune response. They exhibit would healing, chemical defenses and antimicrobial peptides using many homologs of

those used by vertebrates. However *C. elegans* does not have roving cellular immunity, such as macrophages or haemocytes. They also do not have an adaptive immune system, and so must rely exclusively on innate systems (Taffoni and Pujol 2015).

C. elegans is bacterivorous, and is therefore frequently exposed to a wide array of benign and pathogenic bacterial species. Pathogen recognition by *C. elegans* cells is not fully understood. Many pathways that serve core functions in the innate immune system in, for example, *Drosophila* do not serve an anti-microbial function in *C. elegans* (Taffoni and Pujol 2015). For example, the important immune molecules of the Toll like receptor (TLR) family have a homolog in *C. elegans* which seems to control feeding behaviour but not immune defense (Pradel *et al.* 2007). Furthermore, proteins in the *Drosophila* TLR signalling cascade such as MYD88 and NF-kB do not have homologs in *C. elegans*. *C. elegans* also does not use tumor necrosis factor (TNF), Pelle and IL-1R associated kinase (PIK1) or inhibitor of NF-kB in any antimicrobial responses. Deletion of a *C. elegans* protein called TIR-1 contains TLR binding domains results in massive increase in mortality when challenged with pathogenic bacteria. This finding indicates that TLR binding domains have evolved in *C. elegans* and serve a function in the immune system, but the recognition cascade is different than in more complex organism and the mechanism is unclear.

There are fundamental differences in the signaling pathways between *C. elegans* and more complex species, but an important similarity is the production of antimicrobial peptides. A study by Alper, Mcbride, Lackford, Freedman, & Schwartz (2007) used RNA interference technology to disrupt genes they suspected were responsible for antimicrobial response due to their homology to genes in other species. Lysozymes are first line of defense in vertebrates. It attacks the peptidoglycan by hydrolyzing the bonds between its N-acetylglucosamine (NAG) and N-acetylmuranic acid (NAM) subunits, leading to heightened permeability and cell death. Alper *et*

al. (2007) identified 3 lysozyme coding genes in *C. elegans*, as well as the tissues in which they were highly expressed. Other antimicrobial compounds such a caenopores, defensin-like peptides, caenecins and neuropeptide-like proteins have homologs in *C. elegans* that are important to innate immunity. Furthermore, *C. elegans* relies on the basic defense mechanism of reactive oxygen species (ROS) production. The antimicrobial effect of ROS is well studied; for example the use of hydrogen peroxide as an antiseptic. The cells lining the *C. elegans* intestine have been observed to produce ROS using NOX DUOX1/BLI-3 in response to *E. faecalis* infection (Chaplin 2010; Powell and Ausubel 2008; Taffoni and Pujol 2015). However, the exact stimulus causing the *C. elegans* cells to produce antimicrobial ROS is unclear. The basic homology between *C. elegans* defenses and those of other species contributes to its value as a model.

1.5.4. Benefits of *C. elegans* as an Infection Model

Due to its easy management, proliferation and observation, *C. elegans* has become a prolific model for pathogenesis of bacterial infections (Corsi *et al.* 2015; Powell and Ausubel 2008). Pathogens tested in a *C. elegans* host model are diverse, and range from mycobacterium, pseudomonads and a variety of enterobacteriaceae. Disease progression in the nematode varies significantly between pathogen species. For example *Microbacterium nematophilum*, a natural nematode pathogen, colonizes the rectal cuticle and stimulates inflammation of the local tissue whereas *Pseudomonas aeruginosa* colonizes the intestinal tract by forming a thick biofilm. Infection assays are extremely simple to perform, as *C. elegans* nematodes will naturally feed on available bacterial populations propagates on growth media. Furthermore, counting of live and dead worms can be performed quickly and with minimal training, meaning assays have high throughput.

Another useful feature of *C. elegans* is that the body is transparent, meaning internal changes due to infection can be visualized using dissection or confocal microscopes (Corsi *et al.* 2015; Hellinga *et al.* 2015). This method can be made even more powerful through the use of fluorescent pathogenic strains, such as those made to express green fluorescent proteins (GFP). Fluorescent strain infections can provide valuable information on tissue tropism in the host nematode.

Furthermore, infected nematodes can be collected post infection, washed, and mechanically homogenized to release the ingested bacteria. The CFU of the infecting bacteria can then be enumerated by dilution series, or have expression levels evaluated by qPCR methods. Such methods can illustrate the pathogen's growth and metabolism in the host, which serves to contextualize the infecting organism's virulence and pathogenesis. For example, should a strain appear highly virulent, but produce a low number of CFUs during pathogenesis, it could be said that the heightened virulence is not due to increased ability to replicate or colonize the host environment.

1.5.5. *S. marcescens* and *C. elegans*

The first time an inducible antibacterial defense was observed in *C. elegans* was a in a study of *S. marcescens* virulence using a *C. elegans* host model (Mallo *et al.* 2002). Both *C. elegans* and *S. marcescens* are incredibly common in the environment, and are in frequent contact. *S. marcescens* is capable of causing intestinal infections in *C. elegans*. The bacterium is capable of surviving destruction by the pharyngeal grinder, and colonizing the intestinal lumen. The infection results in intestinal bloating, followed by bacterial colonization of non-gastrointestinal tissues. Mortality typically occurs at ~7 days following initial infection. The *C. elegans* response to *S.*

marcescens infection is similar to response to other Gram negative bacteria; secretion of lysozymes, lectins and ROS (Mallo *et al.* 2002).

1.6. Genomics of *S. marcescens*

The genome of *S. marcescens* is approximately 5.2 Mb in size, with a GC composition of about 60%. Of the full sequenced strains available, there are typically 4700 to 4900 coding sequences. There can be substantial genomic variability between strains. A study by Iguchi et al, in 2014 compared two available whole genomes of *S. marcescens*; Db11 and SM39. The Db11 strain is a streptomycin resistant mutant of a *Drosophila* melanogaster isolate, whereas SM39 is a multidrug resistant clinical isolate first characterized by Iguchi *et al.*, 2014. There are 3970 coding sequences shared between the two strains, with 860 and 728 being unique to SM39 and Db11 respectively. Beyond differences in genome contents, the topology of operons common to the two strains varied as well. For example, O-antigen biosynthesis genes were significantly different with respect to the individual genes present and the location of said genes. Notable virulence factors were present in one strain but not in the other, such as a Type II Secretion System (T2SS) and multiple Type 5 Secretion Systems (T5SS) unique to strain SM39. These two secretion systems are implicated in virulence mechanisms and their presence in the genome of SM39 is a reflection of its adaptation as a human pathogen and the plasticity of the *S. marcescens* genome.

2. Outer Membrane Porins in *S. marcescens*

2.1. Materials and Methods

2.1.1. Bacterial Strains and Plasmids

The plasmids and strains used in this research are indicated in Table 2 and Table 5 respectively. The primary strain used in this study is *S. marcescens* type strain Db11 for which the genome sequence is publically available. This strain is a spontaneous streptomycin mutant derived from the Db10 strain, a bacterial pathogen originally isolated from *Drosophila melanogaster* (Flyg, Kenne, and Boman 1980). The *E. coli* strains utilized for cloning and allelic exchange approaches were DH5 alpha and DH5alpha λ pir respectively.

Table 2: Plasmids used in the course of this research.

Plasmid	Genotype/Phenotype	Source
pUFR-GFP	pUFR047::*gfpmut3* (EcoRI/PstI)	(Kurz *et al.* 2003)
pKAS32	Positive selection vector for allelic exchange	(Skorupski and Taylor 1996)

Table 3: Primers used for qRT-PCR.

Primer	Sequence 5' to 3'	Gene Target	Product Size
Sm *rpoB*(L)1	TACGCACAGACTAACGAG	*rpoB*	103 nt
Sm *rpoB*(R)1	CTTCTTCAATAGCAGACAGG		
Sm *ompA*(L)1	TGGTCAGTGCGTATTCAAC	*ompA*	129 nt
Sm *ompA*(R)1	CTCGTCTGGGTGGTATGG		
Sm *ompC*(L)1	TGGACATACCGTAGCCTTC	*ompC*	145 nt
Sm *ompC*(R)	GCGACCTACCGTAACAAC		
Sm *ompF*(L)1	TGGCGACACCTACACCTAC	*ompF*	113 nt
Sm *ompF*(R)1	GCAGAGCGAAGTTCAGACC		
Sm *ompX*(L)1	TACGGTCTGGTTGGTCTG	*ompX*	167 nt
Sm *ompX*(R)1	CTACGAATGCGGTTCTGC		
Sm *phoE* (L)1	AATCACGGTTACGGTAGG	*phoE*	130 nt
Sm *phoE* (R)1	TACGGTGTGGTGTATGAC		
Sm *lamB*(L)1	CTCTTGTCGCCTTCTTTC	*lamB*	197 nt
Sm *lamB*(R)1	GGTGTTCTCTCTACTCAGG		

Table 4: Primers for PCR amplification of *S. marcescens* Db11 genes.

Primer	Sequence 5' to 3'	Product	Product Size
Gm^R HindIII-F	CGCATAAAGCTT CGAATTAGCTTCA AAAGCGCTCTGA	Gentamycin Cassette flanked by FRT inside of pPS856 and terminal HindIII recognition sites	1054 bp
Gm^R HindIII-R	CGCATAAAGCTT CGAATTGGGGAT CTTGAAGTTCCT		
ompA Kpn1 - Upstream F	CGCATAGGTACC TCACAAAAATCA TC	OmpA Flanking regions with terminal Kpn1 recognition sites	1965 bp
ompA Kpn1 - Downstream R	TATGCGGGTACC CCAGATACTCTCG C		
PF*ompA*(Seq)01	TCTATAACGTCAG AAAAACT	Flanking region of *ompA* spanning the crossover point to confirm gene deletion	306 bp
PR*ompA*(Seq)01	TGCGTGAGAGCG CCTTTGTG		
PF*ompA*(Seq)02	TTAAACCTTGGCG AAGGAAT	Flanking region of *ompA* spanning the crossover point to confirm gene deletion	160 bp
PR*ompA*(Seq)02	GGCCTTGAAGGG TTGTGGCA		

Table 5: Bacterial strains used in the course of this research.

Strain	Genotype/Phenotype	Source
Db11	Db10 Spontaneous Sm^R mutant	(Flyg *et al.* 1980)
20C2	*wzm*:: Tn5 transposon. Cm^r. O-Ag biosynthesis. ABC-2 transporter specialized in the translocation of LPS O-antigen.	(Kurz *et al.* 2003)
Db11-GFP	Db11 with pUFR047::*gfpmut3* (pUFR-GFP)	(Kurz *et al.* 2003)
ATF101	Unmarked Db11 Δ*ompF*	(Moya-Torres *et al.* 2014)
ATC101	Unmarked Db11 Δ*ompF*	(Moya-Torres *et al.* 2014)
ATFC01	Unmarked Db11 Δ*ompC*Δ*ompF*	(Moya-Torres *et al.* 2014)
ATE101	Unmarked Db11 Δ*phoE*	(Moya-Torres *et al.* 2014)
ATFCE1	Unmarked Db11 Δ*ompC*Δ*ompF*Δ*phoE*	(Moya-Torres *et al.* 2014)
CBS11	Recovered by Canadian Blood Services from contaminated platelet concentrate	(Greco-Stewart *et al.* 2012)
CBS12	Recovered by Canadian Blood Services from contaminated platelet concentrate	(Greco-Stewart *et al.* 2012)

2.1.2. Media and Growth Conditions

S. marcescens strains were grown in Luria-Bertani (LB) media (Difco) at 28^{o}C, 37^{o}C and 40^{o}C based on the procedure being implemented. Where needed, media was to be supplemented with 10% NaCl, or the antibiotics ampicillin (100 µg/ml), streptomycin (1000 µg/ml), kanamycin (40 µg/ml) and carbenicillin (500 µg/ml).

All nematodes were propagated on NGM agar, and survival assays were conducted on NGM2 medium. NGM contains Bacto-Peptone (2.5 g/L), NaCl (3 g/L), Bacto-Agar (17 g/L), cholesterol (5 mg/L), $MgSO_4$ (1 mM), $CaCl_2$ (1 mM) and KH_2PO_4 (25 mM). NGM2 contains 3.5 g/L of Bacto-peptone instead of 2.5 g/L (Brenner 1974).

Liquid NGM2 to be used for growth of *S. marcescens* strains uses the same recipe as above, but omitting the use of Bacto-Agar.

2.1.3. Genomic DNA Isolation

Genomic DNA of *S. marcescens* isolated to be used for PCR amplification was collected by phenol-chloroform extraction. Isolated bacterial colonies were used to inoculate 3 mL of sterile LB broth, and were allowed to grow at 37^{o}C in a mechanical rotator overnight. Overnight cultures were collected and centrifuged at 12000 rpm for 2 minutes in a Sorvall Legend Micro 21 at room temperature to produce a dense cell pellet. The supernatant was drawn off, and the pellet was re-suspended with 440 µl of Tris-EDTA buffer (TE). Then, 40 µl of 25 mg/mL Proteinase K (NEB) and 10 µl of 10% SDS were added to the cell suspension and mixed by inverting. The solution was incubated at 37^{o}C for 3 hours for complete lysis of bacterial cells in solution. Once the incubation time elapsed, 50 µl of 10M Ammonium acetate was added to the mixture, and was mixed by inverting. In a fume hood, 550 µl of phenol:-chloroform isoamyl alcohol (25:24:1) (Sigma Life

Technologies) was added to the mixture and vortexed to homogenize, followed by centrifugation at 14800rpm for 30 minutes. The aqueous phase is collected, transferred to another micro centrifuge tube. This phenol:-chloroform isoamyl alcohol addition, mixing, centrifugation and separation is repeated two addition times to generate a pure DNA containing aqueous solution. Once a final aqueous fraction is collected, its total volume is approximated and add two equivalent volumes of ice cold 100% ethanol. This mixture is gently mixed by inverting, and centrifuged at 4^{o}C, at 14800 rpm for 30 minutes. Ethanol is removed by pipetting, the resulting DNA pellet is rinsed with 70% ethanol, and then allowed to air dry at 50^{o}C. Once dry, it is rinsed again with 70% ethanol. This pellet is then re-suspended in 100 µl of TE buffer + 1 µl of RNaseA (1 mg/ml) (NEB). The DNA sample is then quantified by NanoDrop ™ One/One© Microvolume UV-Vis Spectrophotometer (ThermoFisher). Master DNA stocks were frozen at -20^{o}C, and working 5 µg/mL stocks were prepared and frozen as well.

2.1.4. Preparation of Competent Cells

2.1.4.1. Chemical Competent Cell preparation using Rubidium chloride

E. coli DH5alpha or DH5alpha lambda *pir* are struck out onto LB agar and incubated at 37^{o}C overnight. Individual colonies are used to inoculate 3 mL of LB broth, which is allowed to grow overnight at 37^{o}C with agitation. After incubation, 0.5 mL of overnight culture was used to inoculate 125 mL of fresh LB in 250 mL baffled flasks. These flasks were grown at 37^{o}C with shaking, checking the levels of 600 nm wavelength light ($OD_{600)}$ hourly, until a reading of ~0.5 was reached. Cultures were then transferred to sterile 250 mL centrifuge bottles, and allowed to chill on ice for 10 minutes. Once chilled, bottles are centrifuged at 3000rpm, at 4^{o}C, for 15 minutes. Supernatant is removed, and cells were re-suspended in 200 mL of 10 mM RbCl. This cell suspension was then centrifuged once again, and the pellet was re-suspended in 20 mL of 10 mM

RbCL + 10% Glycerol. Aliquots of 200 µl were dispensed into 1.5 mL micro centrifuge tubes, and flash frozen in a dry ice ethanol bath then stored at -80^{o}C.

2.1.4.2. Preparation of *S. marcescens* Db11 Electrocompetent Cells

Cells to be transformed with plasmid constructs were made electro competent, as opposed to chemically competent, as to achieve high transformation efficiency. *S. marcescens* strains were cultured in 3 mL of LB broth at 37^{o}C for 24 hours. In two 250 mL baffled flasks, 125 mL of LB broth is inoculated with 0.5 mL of overnight culture. Inoculated broth is incubated at 37^{o}C with shaking. Additional OD_{600} readings were taken every hour until an OD_{600} of ~0.5 was achieved. Cultures were then transferred to sterile 250mL plastic centrifuge bottles and chilled to 4^{o}C for 10 minutes. Chilled bottles were then centrifuged at 3000rpm for 20 minutes at 4^{o}C (Sorvall Legend Micro 21). Culture media supernatant was then removed, and the cell pellet was re-suspended in ice cold sterile water. This centrifugation and washing process is repeated three additional times. After the fourth and final centrifugation, the cell pellet was re-suspended in 10mL of ice cold 10% glycerol. Micro centrifuge tubes were loaded with 100 µl of the glycerol cell mixture, then flash frozen in a dry ice ethanol bath and stored at -80^{o}C.

2.1.5. Restriction Digest and Cleanup of DNA products

All primers designed to contain restriction enzyme cut sequences on the end received an additional six nucleotides as mismatched sequences (Table 4). Amplified PCR products were isolated using a 1.5% agarose electrophoresis gel, and desired bands were excised with a clean razor blade. Gel pieces then had DNA extracted using a QIAquick Gel extraction kit (Qiagen). Resulting DNA samples were measured for concentration and purity by NanoDrop. For restriction enzyme digestion of purified PCR products and cloning vectors, 10 µl of sample is combined with

1.5 µl of enzyme and 37 µl of 1X reaction buffer. Restriction digestion reactions are allowed to incubate for 3 hours at 37°C, and are then isolated and cleaned in the same way as PCR reaction samples.

2.1.6. Design of Unmarked Mutants

Attempts to produce unmarked isogenic mutants in the Db11 was modeled on Moya-Torres *et al.* (2014) and work by Skorupski & Taylor (1996). Approximately 1 kbp upstream and downstream flanking regions for genes of interest were amplified by PCR with restriction enzyme cut sites at each end. These flanking regions were then joined to an intervening fragment containing a Gm^R selectable marker and flippase recognition target (FRT) sequencess. This would allow us to both select for the presence of this construct using gentamycin containing media, and eventually remove the resistance marker through the use of a plasmid borne flippase.

This produced a single large fragment that could then be inserted into a π dependant vector pKAS32, which would not successfully replicate in a Db11 host and would necessitating the integration crossover of the Gm^R fragment for growth on selection media. The pKAS32 vector contains a wild type *rpsL* gene, which confers sensitivity to streptomycin. If the exchange vector is present, Db11 cells will fail to grow on LB streptomycin 1000 µg/mL media. Patching of colonies from LB gentamycin 20 µg/mL to LB streptomycin 1000 µg/mL will identify strains that have successfully crossed over the Gm^R cassette and lost the plasmid backbone.

2.1.7. Preparation of total RNA

Total RNA was collected for gene of interest expression quantification by qPCR. The strains were first inoculated into 3mL of fresh sterile LB broth from isolated colonies. Cultures were grown at 37°C with agitation until an OD_{600} of ~0.65-0.75 was achieved. This cell

concentration was chosen as it is representative of mid log phase, based on kinetic growth curves performed using each *S. marcescens* strain of interest. Once an OD_{600} of ~0.65-0.75 was achieved, cell cultures were transferred to sterile micro centrifuge tubes and centrifuged at 12000 rpm for 2 minutes to produce a dense pellet. Cells were then frozen for 2-3 hours at -80^{o}C. After the freezing period is complete, pellets were thawed on ice, and subsequently treated with 100 µL of 1 mg/mL lysozyme. This mixture is allowed to incubate at room temperature for 10 minutes to allow for lysis of bacterial cells. After lysis is complete, cell mixtures are treated with 250 µl of lysis buffer and 3.5 µl of beta-mecaptoethanol (BME), and mixed by vortex. Once mixed, samples are combined with 250 µl of 100% EtOH, and mixed by pipetting. Samples were then loaded into an RNeasy MinElute Spin column (QIAGEN), and spun at 10000rpm for 30 seconds. The eluent is disposed of, and 350 µL of Buffer RW1 (QIAGEN) is added to the membrane and spun at 10000rpm for 15 seconds. Flow through was discarded, and 500 µL of RPE buffer was added to the column and centrifuged at 10000 rpm for 2 minutes. The flow through was again discarded, and centrifuged at a further 1 minute at 10000 rpm. The column was transferred to a sterile microcentrifuge tube. The column membrane was then treated with 40 µL of RNAse free water and allowed to stand at room temperature for 5 minutes. Columns were centrifuged at 10000 rpm for 2 minutes to elute the RNA. These samples were then stored at -80^{o}C until needed. Prior to preparation for cDNA syntheses, 5 µL of thawed RNA sample was mixed with 1 µL of RDR buffer, 1 µL TURBO DNAse (ThermoFisher) and 7 µL RNAse free water. This mixture was heated to 37^{o}C for 30 minutes, then further heater to 62^{o}C for 15 minutes. The RNA in this mixture would then be quantified by Nanodrop and used for cDNA synthesis.

2.1.8. Quantitative Real-Time PCR (qPCR)

Quantification of mRNA transcripts was accomplished using BioSystems SYBR Select and Invitrogen Superscript VILO protocols (ThermoFisher). The first step to this process was the synthesis of complementary DNA (cDNA) that is from mRNA transcripts in each sample. The mRNA samples were loaded into plastic PCR tubes with DEPC- treated water, VILO Enzyme reaction mix and Superscript Enzyme Mix, such that the final RNA added was 2.5 µg (Table 6). Tubes were gently mixed, and incubated at 25^{o}C for 10 minutes, then at 42^{o}C for 60 minutes. The reaction was terminated by heating to 85^{o}C for 5 minutes. Samples were stored at -20^{o}C for future use. Once cDNA synthesis is complete, transcripts were quantified by qPCR.

Quantitative real time PCR was accomplished using SYBR Select Master Mix fluorescence reagent (ThermoFisher). Primers designed to target the 5' end of desired mRNA transcripts were diluted to 10mM. The SYBR select Master Mix, primers, cDNA template and RNase free water were combined in PCR reaction tubes, then mixed well and centrifuged. Samples were then transferred to reaction wells on a MicroAmp™ Optical 96-Well Reaction Plate (Applied Bio Systems). Plates were loaded into an Applied Bio Systems StepOne Real Time PCR system. The PCR amplification protocol is described in Table 7.

Table 6: Invitrogen Superscript VILO cDNA Synthesis reaction components used in this study.

Reaction Component	Quantity
5X VILO Reaction Mix	4 µl
10 Superscript Enzyme Mix	2 µl
RNA Sample	2.5 µg
DEPC Treated H_2O	Added to achieve total 20 µl volume

Table 7: BioSystems SYBR Select qPCR reaction components used in this study.

Reaction Component	Volume
SYBR Select Master Mix (2X)	10 µl
Forward Primer	1.25 µl
Reverse Primer	1.25 µl
cDNA Template	3.00 µl
RNase Free Water	5.375 µl
Total Reaction Volume	20 µl

Table 8: Applied BioSystems StepOne Real Time PCR system cycle profile.

Step	Temperature	Duration	Cycles
UDG Activation	50°c	2	Hold
AmpliTaq Fast DNA Polymerase	95°c	2	Hold
Denature	95°c	15	40
Anneal/Extend	60°c	1	

2.1.9. Microscopy

Approximately ten nematodes in the L4 age group were collected from assay plates every day, for five days. Nematodes are washed in M9 Nematode Buffer on an NGM agar plate, and then transferred to a 2% agarose pad atop a microscope slide. Nematodes first were immobilized with 10 mM levamisole anesthetic, and then treated with 10mM NaN_3. The NaN_3 fully immobilizes bacterial cells by blocking electron transport chain function and ATP synthesis, with would otherwise be motile within the nematode and interfere with imaging. A cover slip was applied over the immobilized nematodes, and sealed around the edges with silicon grease and molten agar. Samples could then be visualized with both DIC and fluorescence (455nm) using a Zeiss LSM 700 confocal microscope.

2.1.10. Antibiotic Susceptibility Assay

Strains were evaluated for antimicrobial resistance by broth microdilution. A working stock of Muller Hinton Broth (MHB) (Difco) was prepared, and loaded with 2 times the highest concentration of the drug to be tested. The first column of wells is first loaded with 100 µl of 2X drug MHB. All other wells received 50 µl of sterile MHB. To create a twofold dilution series, 50 µl is drawn from the 2X drug MHB wells, and transferred to the next column (A2). This is repeated for the remaining columns, with 50 µl discarded from well A12. Sub cultured bacterial strains are collected mid log phase. Cultures are standardized to 0.5 McFarland (Densichek Plus Standards Kit), and 60 µl are combined with 3 mL of MHB. Each well is then inoculated with 50 µl of this diluted culture. Plates are incubated at 37^{o}C for 18 hours. The drug's MIC is the concentration in the lowest concentration to produce no visible pellet.

2.1.11. *C. elegans* Survival Assays

2.1.11.1. Nematode Growth and Maintenance

C. elegans N2 nematodes were used in this study. This is the reference strain and wild type for the *C. elegans* species. All nematodes were propagated on nematode growth medium (NGM) agar, and survival assays were conducted on NGM2 medium. NGM contains Bacto-Peptone, NaCl, Bacto-Agar, cholesterol, MgSO4, CaCl2 and KH2PO4. NGM2 contains 3.5 g/L of Bacto-peptone instead of 2.5 g/L (Brenner 1974). The *C. elegans* feeding strain of *E. coli* was OP50; a uracil auxotroph that is extremely attenuated and produces no significant nematode death. To maintain nematodes, 6 cm NGM agar plates were spotted with *E. coli* OP50 and spread, then incubated at 37^{o}C overnight. This produces a lawn of OP50 bacteria, which is then seeded with N2 gravid nematodes. Gravid nematodes will lay up to 300 eggs overnight, after which the adults are removed and killed. Once adults are removed, plates are incubated at 16^{o}C refrigerators, where they are allowed to mature to the L4 stage. This L4 stage is the age chosen to begin survival killing assays.

2.1.11.2. Preparation of *S. marcescens* assay plates

To produce *S. marcescens* assay plates, 3 mL of LB broth inoculated with a single colony was grown at 37^{o}C with agitation. After 8hrs, 50 µl of culture was spotted onto 6cm NGM2 and spread to the ~1/3 of the agar surface area. Plates are subsequently incubated at 37^{o}C overnight to produce a bacterial lawn.

2.1.11.3. Survival assays in *C. elegans*

After incubation at 37^{o}C, assay plates are allowed to cool. Once at room temperature, each plate is seeded with 30 L4 stage hermaphrodite nematodes. With 3 plates per strain tested, this

results in 90 L4 nematodes per strain. Plates are incubated at 25^{o}C, with live vs. dead nematodes recorded every 18 and 24 hrs. Dead nematodes were removed and incinerated using an ethanol lamp as to avoid double counting. Because the assay plate nematodes are able to reproduce, their progeny will quickly overtake the plate. To avoid losing track of assay animals, adult nematodes are transferred to fresh NGM plates seeded with test strains every two days. Nematodes that crawl off of the agar and up the sides of the plate are counted as lost instead of dead for the purposes of analysis. Data was used to generate Kaplan-Meier survival curves (Graphpad Prism). Each assay was performed in triplicate for each strain tested.

2.1.11.4. Survival Killing Assay with *Galleria mellonella*

A second host model system, *G. mellonella* larvae were infected with *S. marcescens* porin mutant strains. *G. mellonella* waxworm moth larvae were ordered from The Worm Lady® (McGregor, Ontario) and shipped via Canada Post to the University of Manitoba. Larvae were packaged in permeable plastic containers containing woodchips for insulation as a substrate. Larvae were used for all assay work within 1 week of being received. Prior to use, larvae were cleaned with 70% ethanol and a cotton swab to remove surface contaminants. Test strains to be used were subcultured in LB broth until an OD_{600} of ~0.8 was achieved. These were then diluted to 0.5 MacFarland in PBS. This solution would contain approximately $1.5X10^{8}$ CFU/ml, and was further serially diluted in PBS to $1.5X10^{4}$ CFU/ml. A syringe sterilized with 70% ethanol was rinsed with sterile H_2O, and then used to inject 10 µl of diluted bacterial culture into the right hind pro-leg of each larvae for a given strain. The needle was then re-sterilized and rinsed before moving on to the next strain.

2.2. Results

2.2.1. *S. marcescens* Porin Mutant Growth Rate

Porins serve a crucial role in maintenance of homeostasis in *S. marcescens*, and in bacteria more generally. Porin deletion strains have been shown to significantly hinder growth in optimal growth conditions across several related entobacteraceae species. To determine if this is the same for *S. marcescens* porin mutant strains, strains were evaluated for growth kinetics in LB broth, M9 minimal media, and NGM2 broth at different temperatures (28^{o}C, 37^{o}C and 40^{o}C). The LB broth conditions were rich and complex, and served as an ideal growth environment for both the parental and mutant strains. Liquid M9 medium contains glucose, $MgSO_4$, $CaCl_2$ and KPO_4 buffer. Such an environment provides simple sugars, salts and stable pH for growth, but is not rich and contains defined components as opposed to the complex yeast lysate found in LB broth. The NGM2 broth is a variation on the NGM2 medium used for nematode infection assays in that the agar is omitted. This medium serves as a minimum nutrient source thus ensuring that nutrients are obtained from the nematode host. Establishing the growth kinetics of the porin mutant strains in NGM2 medium will determine that any avirulent phenotype observed is not attributed to poor growth.

Despite the substantial presence of these OMPs in the cell membrane, no significant difference in growth profiles were observed between *S. marcescens* Db11 and its isogenic porin mutants. This is the case across all media types, and all temperature conditions tested .

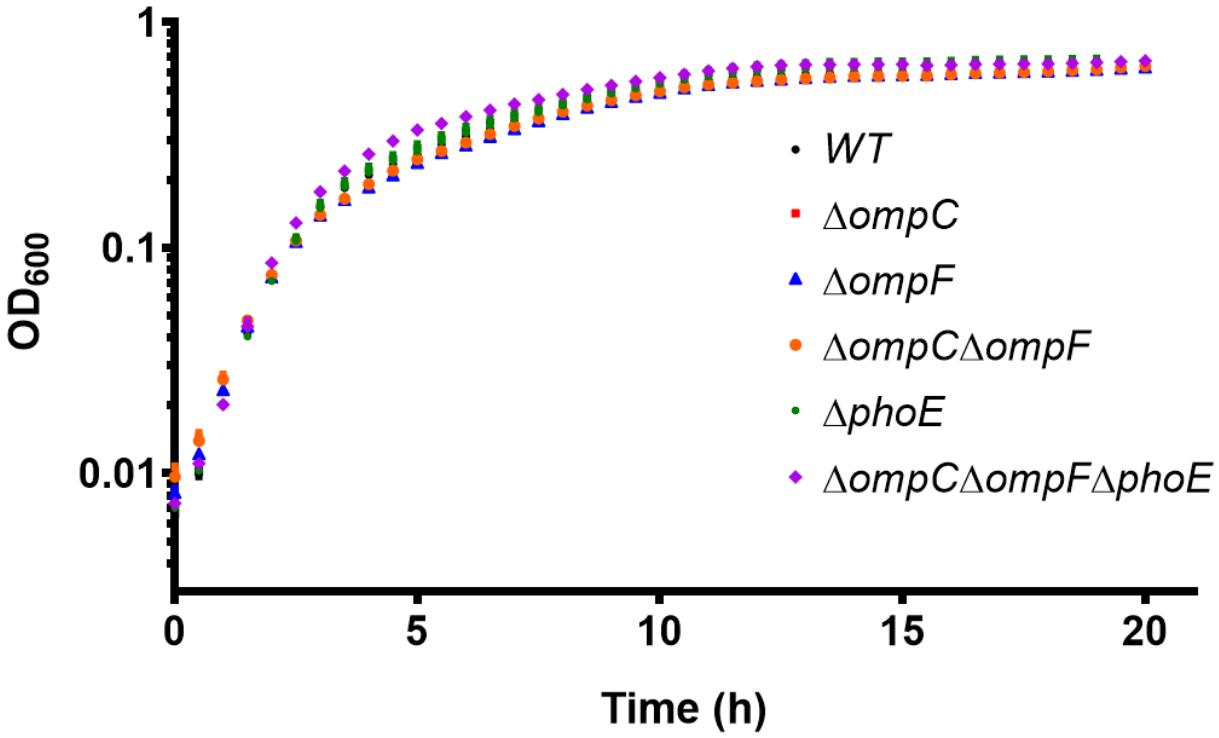

Figure 4: Growth curves of *S. marcescens* WT Db11 and isogenic porin mutants. Bacteria were grown in LB medium at 37°C over a time period of 20 hours. Data and error bars are representative of one of three independent experiments. Error bars are very small and not visible.

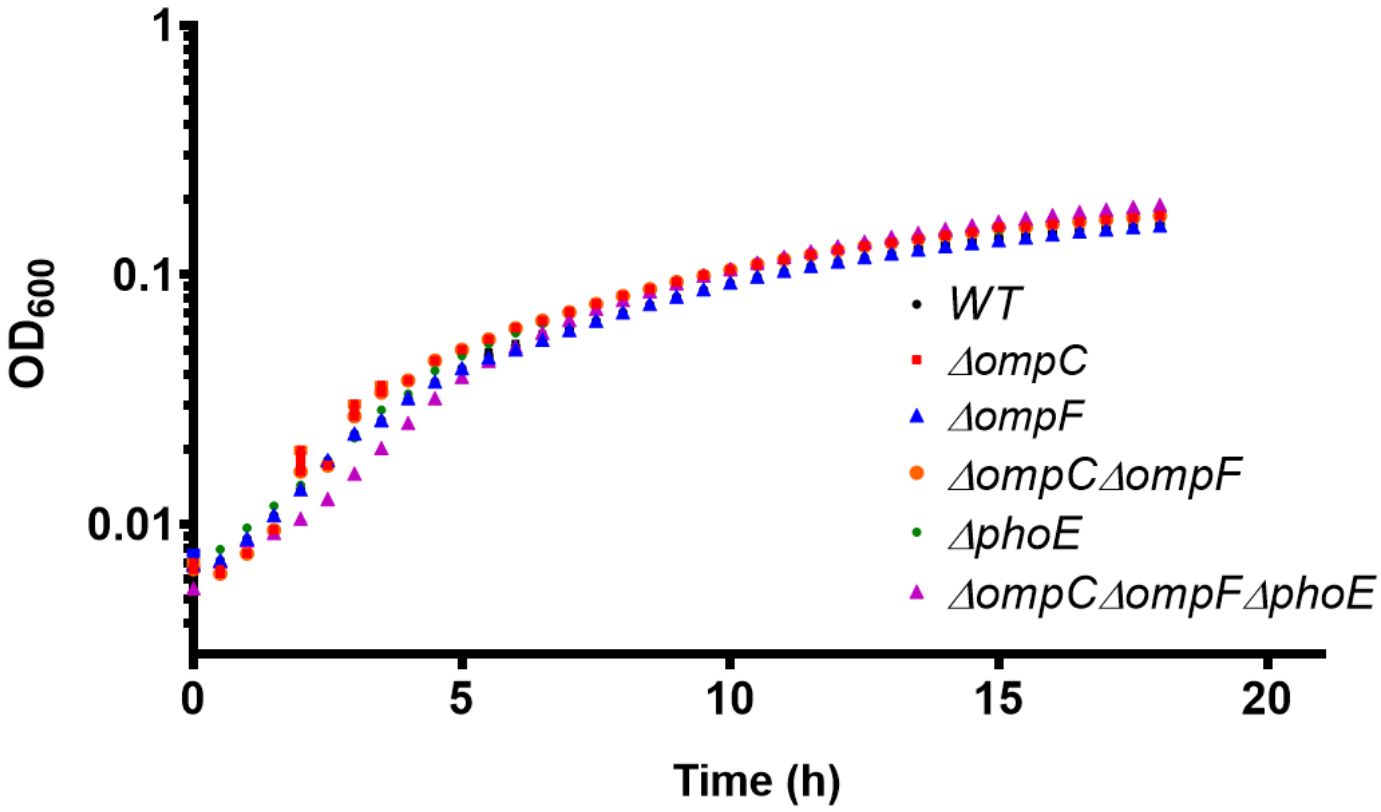

Figure 5: Growth curves of *S. marcescens* WT Db11 and isogenic porin mutants. Bacteria were grown in M9 media +10% Sucrose at 37ºC over a time period of 18 hours. Data and error bars are representative of one of three independent experiments. Error bars are very small and not visible.

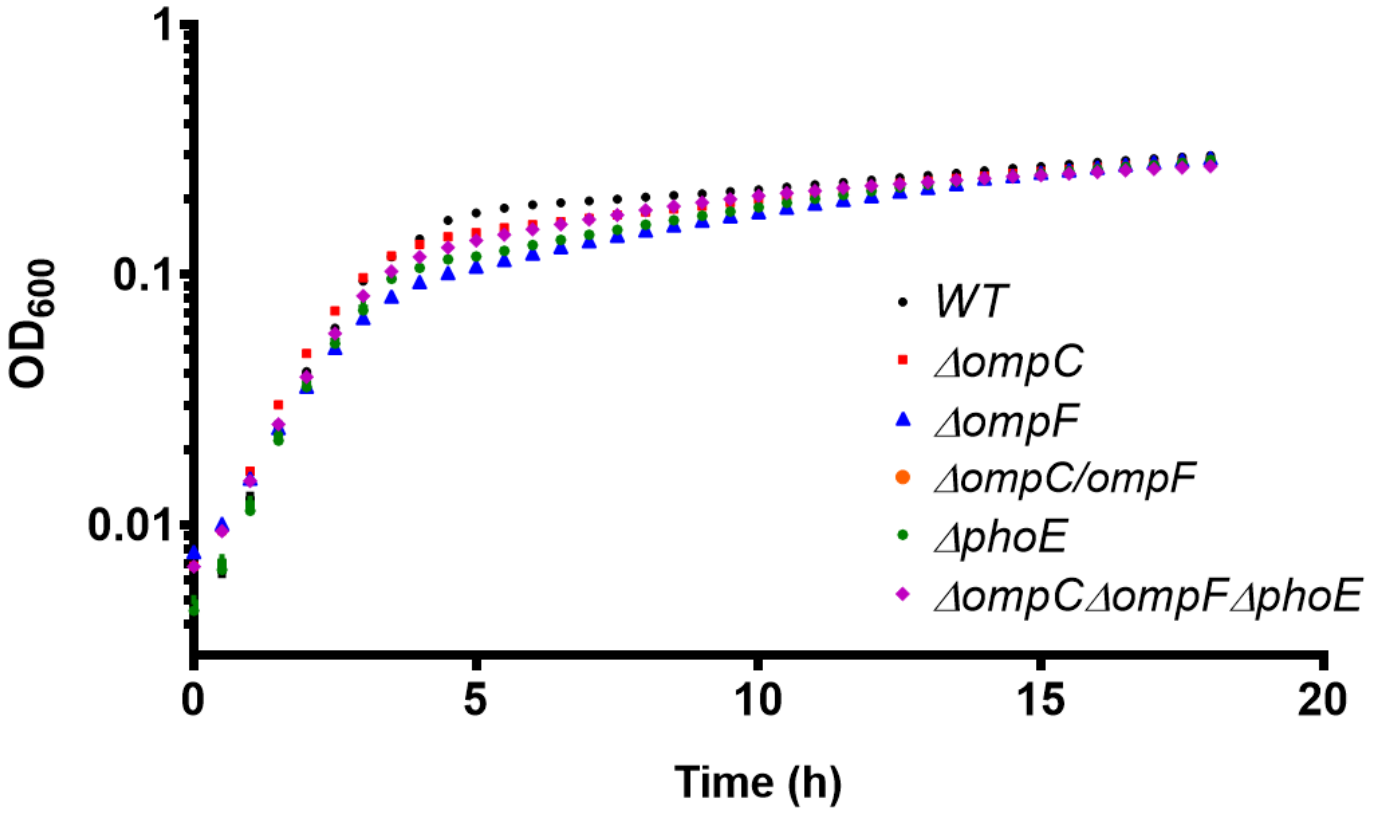

Figure 6: Growth curves of *S. marcescens* WT Db11 and isogenic porin mutants. Bacteria were grown in NGM2 liquid medium at 37°C over a time period of 18 hours. Data and error bars are representative of one of three independent experiments. Error bars are very small and not visible.

2.2.2. *S. marcescens* Porin Mutant Strain Virulence

Nematode survival assays served to illustrate the relative virulence of each *S. marcescens* porin mutant strain. The pathogenesis of *S. marcescens* in the *C. elegans* nematode begins with colonization of the anterior intestinal tract once the nematode grinder has been evaded. Innate immune defenses are triggered in the nematode, which may serve to slow or reduce the bacterial infection. In these assays, L4 nematodes were selected due to their increased resistance to infection relative to the older adults, and their increased susceptibility to infection relative to younger individuals. Phalangeal grinder structures are more robust and efficient in younger individuals, which may serve to explain this decreasing resistance as the life cycle progresses (Kurz and Tan 2004). In the L4 nematodes, this balance makes for an ideal environment to observe increases or reductions in virulence.

To ascertain whether deletion of one or more porin types compromised the virulence of *S. marcescens*, nematode survival assays were conducted with single (Δ*ompC*, Δ*ompF* and Δ*phoE*) and combinatorial (Δ*ompC*Δ*ompF* and Δ*ompC*Δ*ompF*Δ*phoE*) strains. In the case of the single porin deletion mutants, neither Δ*ompC* ($p = 0.504$) nor Δ*phoE* ($p = 0.567$) deletion mutants display virulence different than that of the parental strain. However, the Δ*ompF* deletion mutant does display a statistically significant ($p = 0.044$) increase in virulence is observed as compared to the parental strain, however the magnitude of this change is too small to be considered biologically relevant.

Both the Δ*ompC*Δ*ompF* mutant strain and the Δ*ompC*Δ*ompF*Δ*phoE* mutant strains displayed significant increases in virulence. Combinatorial mutants achieved 50% mortality approximately 8 hours earlier than the parental strain. Of these combinatorial mutant strains, the Δ*ompC*Δ*ompF*Δ*phoE* displays greater virulence than the Δ*ompC*Δ*ompF* strain.

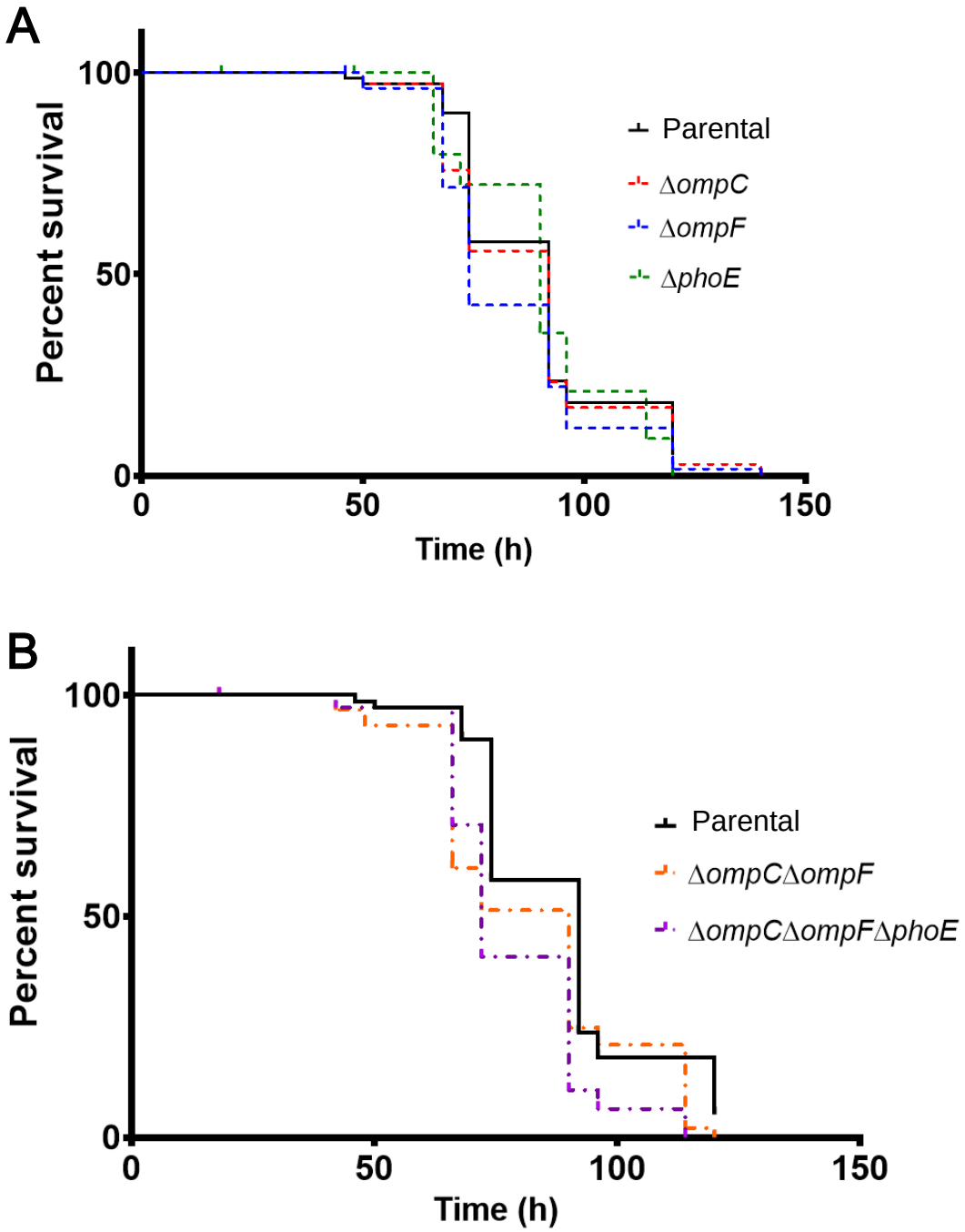

Figure 7: Survival of *C. elegans* nematode infected with *S. marcescens* strains. Kaplan-Meier survival curve of *C. elegans* N2 nematodes infected with Db11 (Black, n=90), Δ*ompC* (Red, n = 90, $p = 0.504$), Δ*ompF* (Blue, n = 90, $p = 0.044$), Δ*phoE* (Green, n = 90, $p = 0.57$), Δ*ompC*Δ*ompF* (Orange, n = 90, $p = 0.004$) and Δ*ompC*Δ*ompF*Δ*phoE* (Purple, n = 90, $p < 0.0001$). All *p* values were determined by log rank analyses.

2.2.3. Visualization of *C. elegans* Colonization by *S. marcescens* Mutant Strains

Observation of the infection of *C. elegans* by Db11 and isogenic porin mutants was achieved with confocal microscopy. The *C. elegans* body is auto fluorescent which increases in response to infection and as the individual ages; particularly in the gut walls. The autofluorescence is easily visible in the background, but is ablated using digital manipulation of florescent signal gain and image contrast.

Nematodes infected with parental and mutant strains carrying pUFR-GFP plasmids were observed every 24 hours for 96 hours. Past 96 hours, the nematode population was insufficient to provide a number suitable for study. The head, intestine and reproductive tissue were the primary focus of microscope imaging, as these locations are most frequently colonized by *S. marcescens*.

The progress of each strain's infection of the *C. elegans* host is reflected in Table 9. In all strains but the Δ*ompC*Δ*ompF* mutant, fluorescent bacteria were visible in the intestine at 24 hours p.i. At 48 hours p.i, all strains had colonized the nematode intestine. All but the double mutant began display varying degrees of grinder colonization. The parental strain displayed the greatest colonization of the grinder at 48 hours. Of all the strains observed, only the Δ*ompC*Δ*ompF*Δ*phoE* mutant displayed colonization of the uterine tissues. Several nematodes infected with the triple mutant exhibited "gonadal explosion" following preparation for microscopy, likely due to the buildup of pressure and bacteria in the uterus. At 72 hours p.i, the parental strain displayed significant colonization of the grinder and intestinal lumen. It was at this time that uterine colonization was beginning to be observed by the parental strain. In the single porin mutants, colonization of the grinder, intestine and uterus was observed, but noticeable less significant than the level of colonization by the parental strain. The Δ*ompC*Δ*ompF* mutant colonization was further reduced compared to both the parental and the single mutant strains. Uterine colonization of the

nematode by the double mutant was not observed at any time point. Colonization by the triple mutant was reduced compared to the parental strain, and was more similar to the colonization by the single porin mutants. Whereas the colonization by the parental strain increased from 72 to 96 hours in those nematodes that did survive, colonization by the mutant strains did not increase significantly between 72 hours and 96 hours. While virulence was not much greater or lower than parental, those nematodes that did survive to 96 hours did display less colonization by porin mutants. In all strains, at 72 hours the nematode intestine became bloated from bacterial buildup. In the parental, this increase in intestine lumen volume was mostly filled with additional bacteria. However, in the nematodes infected by porin mutants this increase in intestinal volume did not correspond with increase in visible GFP signal to indicate additional bacterial growth.

Table 9: Observation of nematodes colonized with GFP-expressing *S. marcescens* strains over a period of 120 hours. The infectious process was monitored using DIC and fluorescence microscopy. Anatomical locations analyzed were found to be colonized or invaded. * Low colonization, ** Median colonization, *** High colonization. N=10 worms examined daily. The experiment was repeated 3 times. The result shown represent one of three independent experiments.

Strains	**Infection**	**Hours fed on *S. marcescens* lawns**			
		24 h	48 hr	72 hr	96 hr
Db11-GFP	Grinder		**	***	***
	Intestine	*	*	***	***
	Uterus			*	*
Δ*ompF*-GFP	Grinder		*	*	*
	Intestine		*	**	**
	Uterus			*	*
Δ*ompC*-GFP	Grinder		*	**	**
	Intestine	*	*	**	**
	Uterus			*	*
Δ*phoE*-GFP	Grinder		*	**	**
	Intestine	*	*	**	**
	Uterus			*	*
Δ*ompF*Δ*ompC*-GFP	Grinder			*	*
	Intestine		*	*	*
	Uterus				
Δ*ompF*Δ*ompC* Δ*phoE* -GFP	Grinder		*	**	**
	Intestine	*	*	*	*
	Uterus		*	*	*

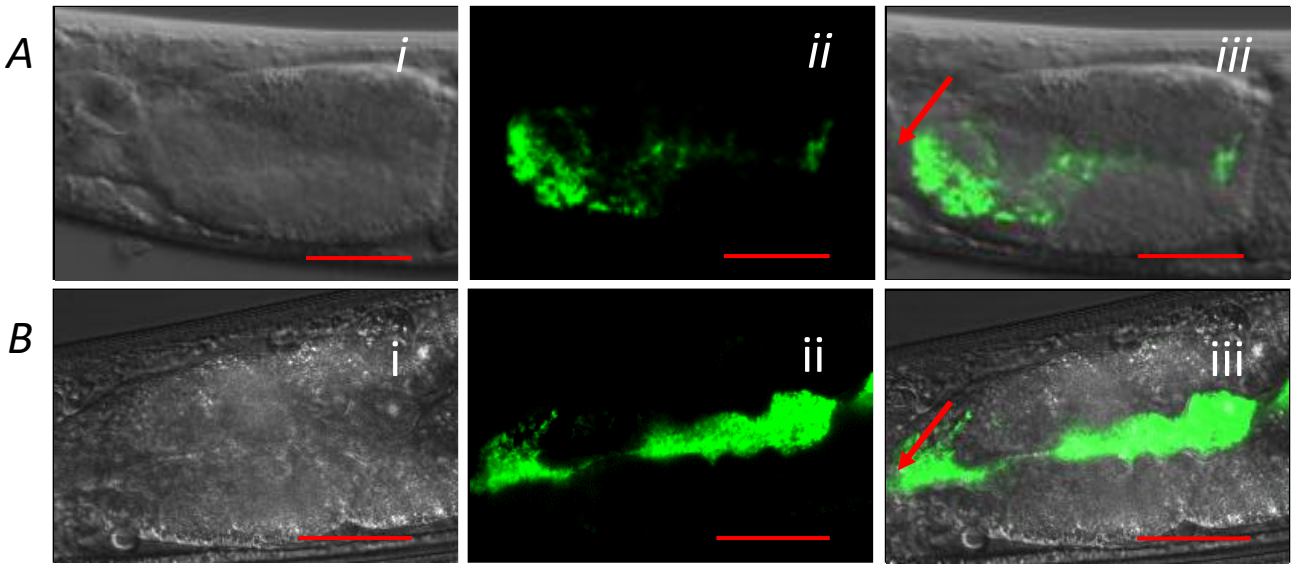

Figure 8: Upper intestinal colonization of *S. marcescens* Db11 in *C. elegans*. Accumulation of GFP-tagged Db11 in the intestinal tract at: (A) 24 hours and (B) 48 hours post infection. Accumulation of bacteria distends the intestinal lumen affecting reduction in intestinal cell volume. Panels i corresponds to DIC signal and panel ii corresponds to GFP signal. Red arrows in A and B indicate the beginning of the nematode intestine. Panel iii corresponds to the merged signal image. Scale bar is 20 µm.

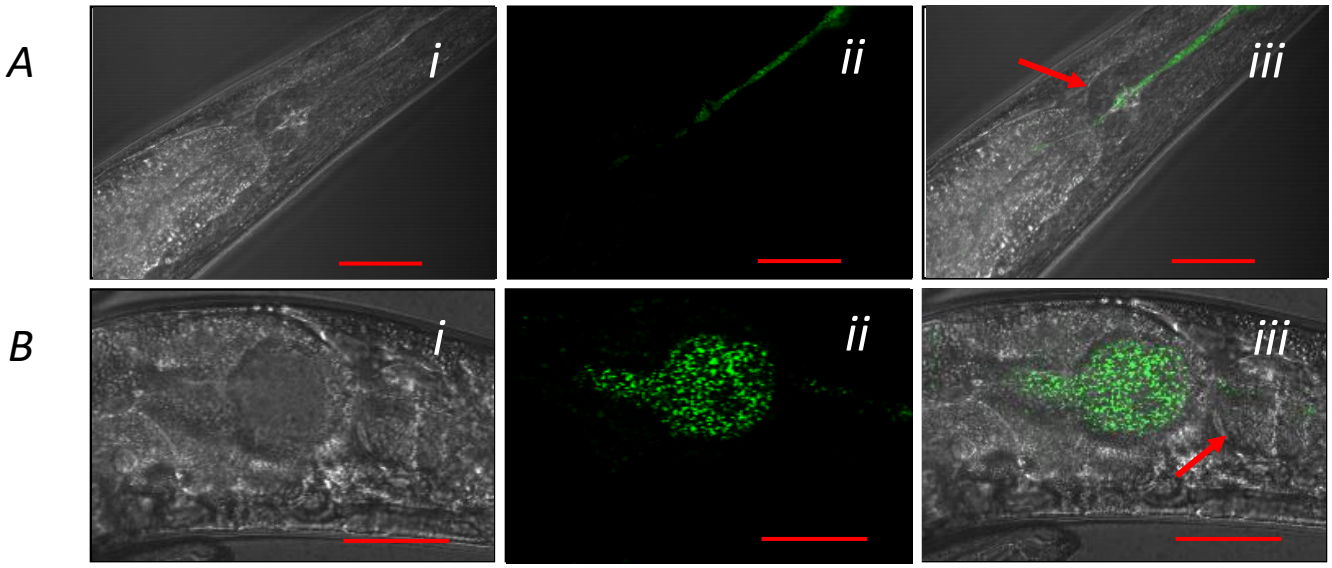

Figure 9: Upper intestinal colonization of *S. marcescens* Δ*phoE* mutant in *C. elegans*. Accumulation of GFP-tagged Db11 in the intestinal tract at: (A) 48 hours and (B) 72 hours post infection. Panels i corresponds to DIC signal and panel ii corresponds to GFP signal. Red arrows in A and B identify the nematode grinder. Panel iii corresponds to the merged signal image. Scale bar is 20 µm.

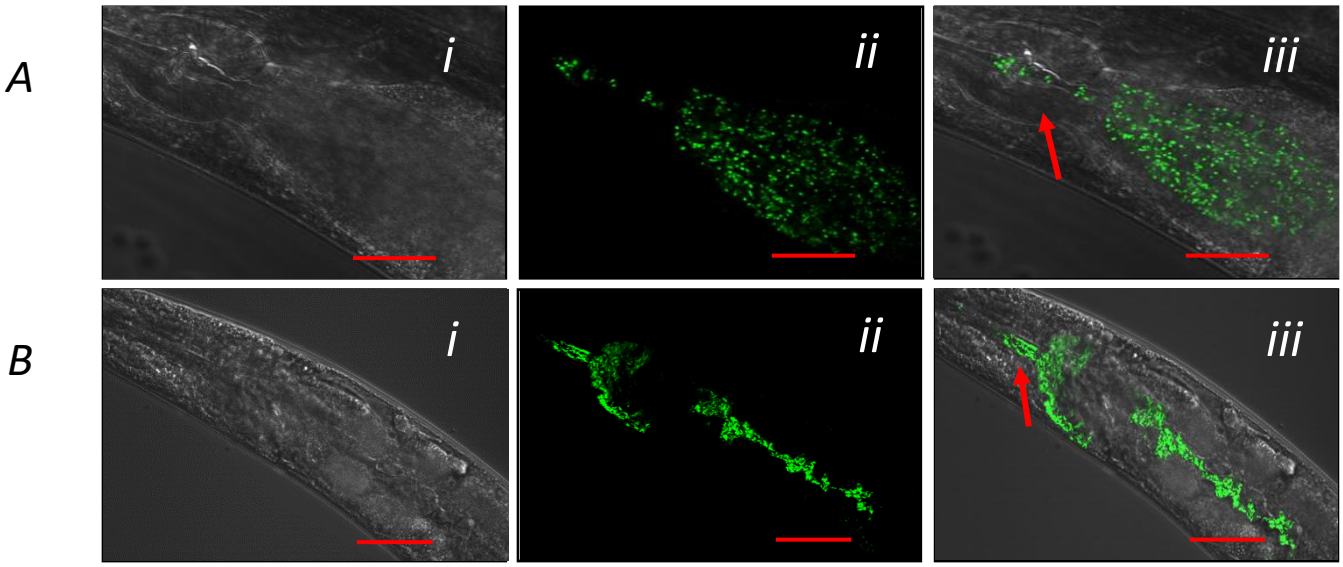

Figure 10: Upper intestinal colonization of *S. marcescens* Δ*ompC*Δ*ompF*Δ*phoE* mutant in *C. elegans*. Accumulation of GFP-tagged Db11 in the intestinal tract at: (A) 48 hours and (B) 72 hours post infection. Panels i corresponds to DIC signal and panel ii corresponds to GFP signal. Red arrows in A and B identify the nematode grinder. Panel iii corresponds to the merged signal image. Scale bar is 20 µm.

2.2.4. *S. marcescens* Porin Mutant Strain Virulence in *Galleria mellonella*

One of the many meaningful differences between *C. elegans* and humans is that the nematode model cannot be studied at 37°C. A popular model organism used for virulence assays at a temperature closer to that of the human body is the larvae of *G. mellonella*. This insect can grow comfortably at 37°C. All single and combinatorial mutant strains were evaluated for virulence in *G. mellonella* larvae at 25°C and 37°C. In both conditions, no mutants deviated from the parental strain's virulence phenotype. All larvae were dead between 10 and 12 hours post infection. No strains exhibited a significant change in virulence relatively to the wild type (log rank test). All control larvae survived past 15 hours and were sacrificed by freezing to -20°C.

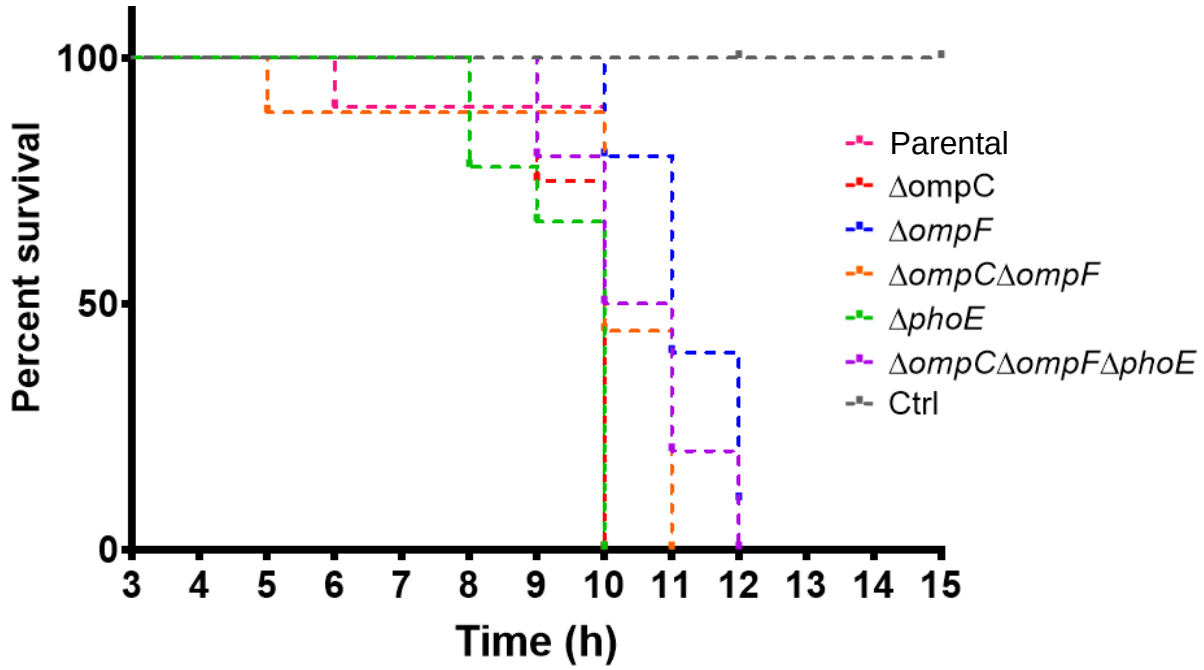

Figure 11: Survival of *G. mellonella* larvae infected with *S. marcescens* strains. Kaplan-Meier survival curve of *G. mellonella* moth larvae infected with Db11 (Pink, n=30), Δ*ompC* (Red, n=30, $p = 0.268$), Δ*ompF* (Blue, n=30, $p = 0.083$), Δ*phoE* (Green, n=30, $p = 0.268$), Δ*ompC*Δ*ompF* (Orange, n=30, $p = 0.079$) and Δ*ompC*Δ*ompF*Δ*phoE* (Purple, n=30, $p = 0.087$). Survival curve is representative of one of three independent experiments. All *p* values were determined by log rank analyses.

2.2.5. Expression of OMP Genes in *S. marcescens* Mutant Strains

Because of the differences in both virulence and colonisation of *C. elegans* by the porin mutant strains, combined with a lack of growth defect across multiple conditions, we theorized that the cells were somehow changing their expression profile to offset the disadvantages imposed by loss of key OMPs. In general, we did observe changes in OMP expression in *S. marcescens* porin mutant strains. The cut-offs values for significance was set at 2 fold differences. All gene target expression was compared to the parental strain using the Pfaffl method (Pfaffl 2001). The expressions profiles measured are reflective of mid log growth at 37^{o}C in LB broth.

Elevated expression of *ompC* gene was observed in the Δ*ompF* deletion mutant. This increase was not observed in the Δ*phoE* strain; the only other strain containing *ompC*. Two fold increases in *ompX* were achieved by the Δ*ompF*, the Δ*ompF*Δ*ompC* and the Δ*ompF*Δ*ompC*Δ*phoE* strains. In the triple mutant, *ompX* expression reached 4 fold overexpression compared to the parental strain. Overexpression of *ompA* was observed in both the double mutant and the triple mutant; both approaching 6 fold greater than the parental strain.

The *phoE* gene was suppressed in the Δ*ompF* and the Δ*ompC* deletion mutants. Both mutants displayed reduced the *phoE* expression to below 50%. However, in the Δ*ompF*Δ*ompC* deletion mutant, *phoE* was overexpressed two fold more than the wild type strain. No *phoE* signal was observed in either the Δ*phoE* or the Δ*ompF*Δ*ompC*Δ*phoE* strain, reflective of the genes deletion in both strains.

The *lamB* gene was under expressed in all deletion mutant strains. In the Δ*ompF* and in the Δ*ompF*Δ*ompC*Δ*phoE* mutants, *lamB* expression was reduced by 50% compared to the parental

strain. In the Δ*ompC*, the Δ*phoE*, and the Δ*ompF*Δ*ompC*Δ*phoE* strains the expression of *lamB* was reduced by nearly 100%.

In summary, our *ompF* mutant displayed significant increases in expression of both *ompC* and *ompX*. The increase in *ompX* was also observed in both the Δ*ompF*Δ*ompC* and the Δ*ompF*Δ*ompC*Δ*phoE* strains. The *ompA* gene was overexpressed in both the double and triple deletion strains. The *lamB* gene was depressed in all mutant strains.

2.2.6. Generating additional porin mutant strains

Three genes were identified as promising candidates for deletion in the Db11 background. Both *ompA* and *ompX* are significantly upregulated in both the double and triple deletion mutants. The *lamb* gene was significantly or fully repressed in all mutant strains. Attempts were made to delete each in the parental background. This project did not result in successful deletion of any of these genes, but did progress to the point of producing a merodiploid Db11 containing both the native version of *ompA* and an allelic exchange cassette composed of OmpA flanking regions and a gentamycin resistance cassette. This merodiploid was identified by successful growth on Gm20 µg/mL LB agar, and confirmed by end point PCR with primers PF*ompA*(Seq)01 and PF*ompA*(Seq)01.

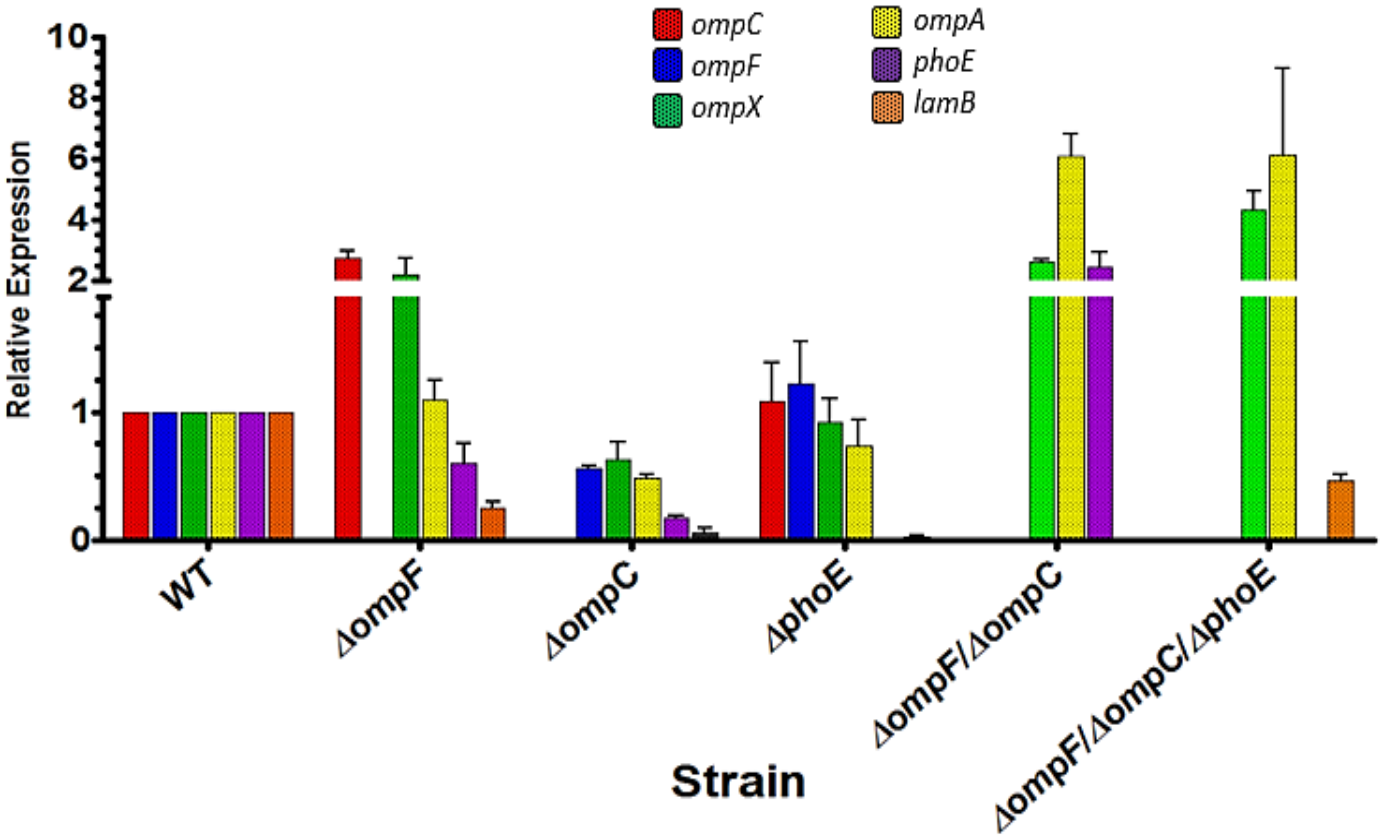

Figure 12: qRT-PCR of *S. marcescens* Db11 and mutant strains at the late log growth phase (OD~0.8) at 37ºC. Graph represents one of three biological replicates performed to analyze transcriptional levels of *ompF*, *ompC*, *ompX*, *ompA*, *lamB* and *phoE*. The *rpoB* gene was selected as a housekeeping gene. Data and standard deviations are an average of three biological replicates.

3. *S. marcescens* Clinical Isolates

3.1. Materials and Methods

3.1.1. Preparation of Genomic DNA for Sequencing

Genomic DNA to be used for whole genome sequencing must have both excellent purity and integrity. DNA cannot be highly sheared due to over handling and cannot contain large amounts of purification reagents. The two isolates that had been detected in Platelet Concentrate (PC) by Canadian Blood Services (CBS), CBS2010-11 and CBS2010-12, were plated out on LB agar plates and incubated at 37^{o}C to generate isolated colonies. After incubation, colonies were used to inoculate 6mL of LB broth. Cultures were allowed to grow until an OD_{600} of ~0.8 was achieved, at which point both were centrifuged at 12000rpm for 10 minutes to produce a dense pellet. The pellet is re-suspended in 180 µl of Purelink Genomic Digestion Buffer (ThermoFisher), and mixed with 20 µl Proteinase K (ThermoFisher) in order to lyse the cells. The samples are incubated at 55^{o}C with occasional gentle vortexing until the solution is fully translucent (approximately 4 hours). After incubation, 20 µl RNase A (ThermoFisher) is added to the solution, then mixed well and allowed to incubate at room temperature for 2 minutes. The sample is then treated with 200 µl of PureLink Genomic Lysis/Binding Buffer and mixed well to produce a homogenous solution. Next, 200 µl of 100% ethanol is added to the sample and mixed by vortexing. The sample lysate (~640 µl) is loaded into the PureLink Spin Column and centrifuged at 10,000rpm for 1 minute at room temperature. The DNA bound to the column is then washed with Wash Buffer 1, then Wash Buffer 2. Samples are eluted from the column by the addition of 25 µl of pH 8.0 TRIS buffer, then incubation for 1 minute followed by centrifugation at 14800 rpm for 1 minute. Samples were evaluated for purity by Nanodrop, with ideal 260/280 absorbance range of ~1.9. The quantification

of DNA was accomplished using the Qubit 4 Fluorimeter (Invitrogen), with a minimum concentration of 150 ng/µl required for PacBio Massively Parallel sequencing.

3.1.2. PacBio Next Generation Genome Sequencing

We aimed to generate a high quality whole genome sequence for both CBS11 and CBS12 for comparison with the reference strain and other available genomes. Once quality and concentration were confirmed to be within the desired range, clinical isolate DNA samples were sent to Genome Quebec (Montreal, QC) for PacBio Massively Parallel sequencing on a fee based service.

3.1.3. Assembly and Annotation

Once sequencing was complete, the contiguous fragments needed to be assembled into a single genome sequence, and then annotated with identified coding sequences and gene products. This was accomplished by the Canadian Centre for Computation Genomics (C3G), in association with Genome Quebec and McGill University, on a fee-based service. Contigs were circularized and polished by C3G. Once polished, sequences were annotated with Prokka, an open source command line program that is widespread in the annotation of bacterial genomes. Clinical isolate genomes were annotated by referencing a genus specific database (NCBI: ASM78391v2) produced by the University of Maryland School of Medicine and Institute for Genome Science (IGS).

3.1.4. Comparison and Analysis

3.1.4.1. Phylogeny of Clinical Isolates

Phylogenetic placement can be informative in identifying lineages or ancestral ecological niches. Once assembly and annotation was completed, analysis and comparison of the CBS11 and CBS12 genomes could begin. First, both genomes were aligned against 9 other completed *S. marcescens* genome sequences (Table 10) using a Mauve Progressive Global alignment. This method identifies large segments of DNA that are consistent between genome with few rearrangements. These are referred to as local collinear blocks (LCB), and can be extracted as alignments. This method is robust in the building of phylogenies between closely related species, as it compares kilobase sized fragments with potentially dozens of loci, instead of only a few SNPs in the case of single gene tree building. Trees were built using the Tamura Nei DNA evolution model, and bootstrap values were produced from 100 iterations (Hall 2013).

Table 10: Published *S. marcescens* chromosomes used for phylogenetic comparison.

Strain	**Chromosome Accession Number**
B3R3	NZ_CP026702.1
Db11	NZ_HG326223.1
RSC-14	NZ_CP012639.1
CAV1492	NZ_CP011642.1
FDAARGOS_65	NZ_CP026050.1
SM39	NZ_AP013063.1
SmUNAM836	NZ_CP012685.1

3.1.4.2. Mobile Elements

Because of the plasticity of *S. marcescens* genomes, mobile elements are a frequent and important factor in this species evolution. Clinical isolate genomes were analyzed using IslandViewer 4 (Bertelli et al. 2017). This tool integrates four genetic island prediction systems: IslandPath-DIMOB, SIGI-HMM, IslandPick and Islander. It also implements annotation and prediction algorithms that can identify virulence factors or AMR genes. Detected genetic islands are labelled with corresponding colors in circularized genome diagrams (Figure 15), as well as the individual genes in those islands.

3.1.4.3. Antimicrobial Resistance Genes

To identify putative antimicrobial resistance genes in the CBS2010_11 and CBS2010_12 genomes, both were submitted to the Comprehensive Antimicrobial Resistance Database (CARD). The database is integrated into the Resistance Gene Identifier (RGI) tool for resistome prediction from genome sequences. The RGI analyzes DNA sequences using three different algorithms for AMR gene discovery: perfect, strict and loose. Perfect hits are genes with 100% identity with clinically confirmed AMR genes or mutations. Strict hits are genes with homology to known AMR genes, and takes SNPs into account in predicting whether or not the hit is likely functional or not. Loose hits have partial identity with known AMR genes, but contain differences that put them outside the predictive models used in the strict detection algorithm. Search criteria were set to detect perfect, strict, and loose hits. Hits characterized as loose that had >95% identity were not moved into the Strict hit pool.

3.2. Results

3.2.1. Genomics of Clinical Isolates

The PacBio genome sequencing of CBS11 generated 6 contiguous fragments (Contigs), with a maximum contig size of 5,650,352 base pairs. Assembly of CBS11 contigs by C3G produced a 5,352,713 bp chromosome and 139,859 bp plasmid. The total GC nucleotide content of the CBS11 genome is 59.39% (3,262,061bp). Both the chromosome and plasmid were successfully circularized following assembly. Once identified, the plasmid was given the nomenclature CBS11p1 to differentiate it from the strain's chromosome.

Regarding CBS12, the PacBio sequencing produced a single continuous fragment, with a total length of 5,650,352 bp. After trimming and assembly, a single contig of the same length was generated, representing the isolate's chromosome. No plasmid was detected in *S. marcescens* CBS12.

3.2.2. Comparison and Analysis

3.2.2.1. Genomics

Global alignment of CBS11 and CBS12 against a cohort of completed *S. marcescens* whole genome sequences indicates these two isolates are not clones and are unique strains. This method generates large collinear blocks of DNA. These blocks are defined as conserved segments that appear to be free of internal rearrangements. Two such collinear block neighbor joining trees are shown in Figure 14. Both trees are highly similar, and indicate that CBS11 and CBS12 are not clones and cluster with distinct groups of *S. marcescens* isolates.

Further investigation of CBS11's plasmid, labelled CBS11p1, using whole plasmid BLAST search reveals several large CBS11p1 fragments have high identity (>90%) with an

existing *S. marcescens* plasmid PWNM146. Besides PWN146, all high confidence BLAST hits are in other enterobacteriaceae, particularity *K. pneumoniae*. Coverage of PWN146 against the query CBS11p1 sequence is high, but does not cover a large ~7kb fragment in the CBS11 plasmid. Further BLAST comparison of CBS11p1 against its host strain and other *S. marcescens* strains reveals that this ~7kb region is also present in CBS11's chromosome, as well as the *S. marcescens* UMH7 strain. The annotations of this region contain several type 1 pilus assembly genes, a peroxide resistance gene and an integrase.

Table 11: Pairwise nucleotide identity of different porin genes to their homolog in the reference strain Db11.

OMP Gene	Nucleotide Homology to Db11		Accession number of gene in *S. marcescens* Db11
	CBS11	***CSB12***	
ompF	93.5%	97.5%	SMDB11__1014
ompC	93.6%	97.5%	SMDB11_2659
phoE	87.1%	95.1%	SMDB11_RS10455
ompA	96.5%	98.2%	SMDB11_RS05155
ompX	99.6%	88.1%	SMDB11_0761
lamB	96.0%	97.1%	SMDB11_RS18465

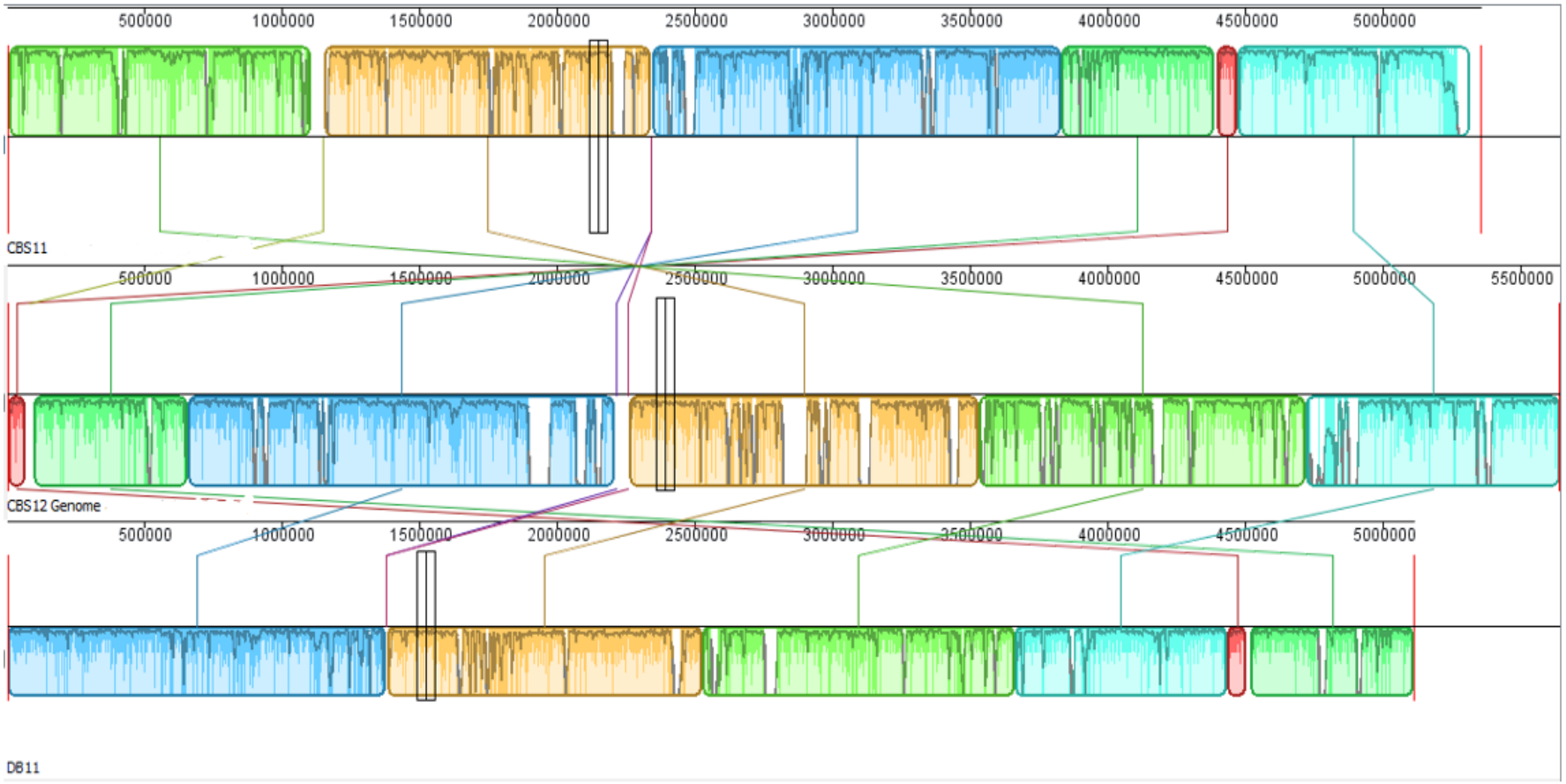

Figure 13: Example Mauve Progressive Global alignment of *S. marcescens* CBS11, CBS12 and Db11 chromosomes. Colored regions indicate unique local collinear blocks. Amplitude of block regions indicates degree of homology, with gaps indicating no homology in that region. Number values above each alignment indicate nucleotide position in each genome.

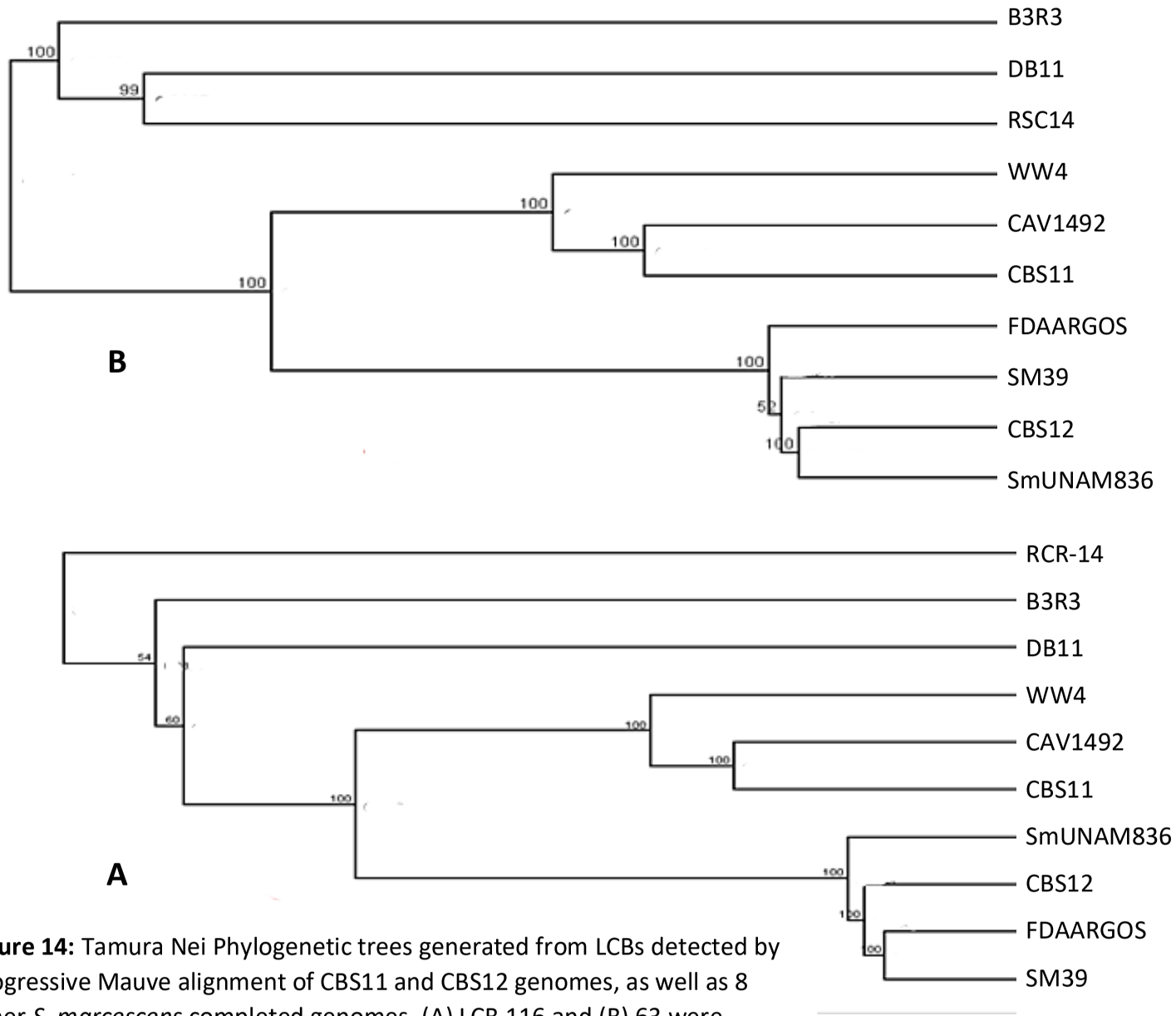

Figure 14: Tamura Nei Phylogenetic trees generated from LCBs detected by progressive Mauve alignment of CBS11 and CBS12 genomes, as well as 8 other *S. marcescens* completed genomes. (A) LCB 116 and (B) 63 were chosen as examples due to their large size and lack of internal rearrangements.

3.2.2.2. Genetic Islands in CBS11 & CBS12

Genetic islands are regions of DNA that contain evidence of horizontal transfer. Such regions often contain features important for virulence and survival (Land et al. 2015). Both CBS11 and CBS12 were processed using IslandViewer in order to identify such genetic islands(Bertelli *et al.* 2017). This bioinformatics platform integrates various databases to detect and label genetic islands and mobile elements present in submitted DNA sequences. The generated island maps are shown in Figure 15. Color coding indicates the number of different screening methods that detected a particular island, described in the figure text. Both strains' chromosome contain a number of genetic islands. There are no islands homologous between the two strains, but many islands contained genes with similar functional annotations. Many genetic islands contain toxin secretion systems, genetic replication or repair genes, phage components, transposases or integrases.

Using IslandViewer, a ~7kb integron was detected in both the CBS11 chromosome and CBS11p1 plasmid sequence.

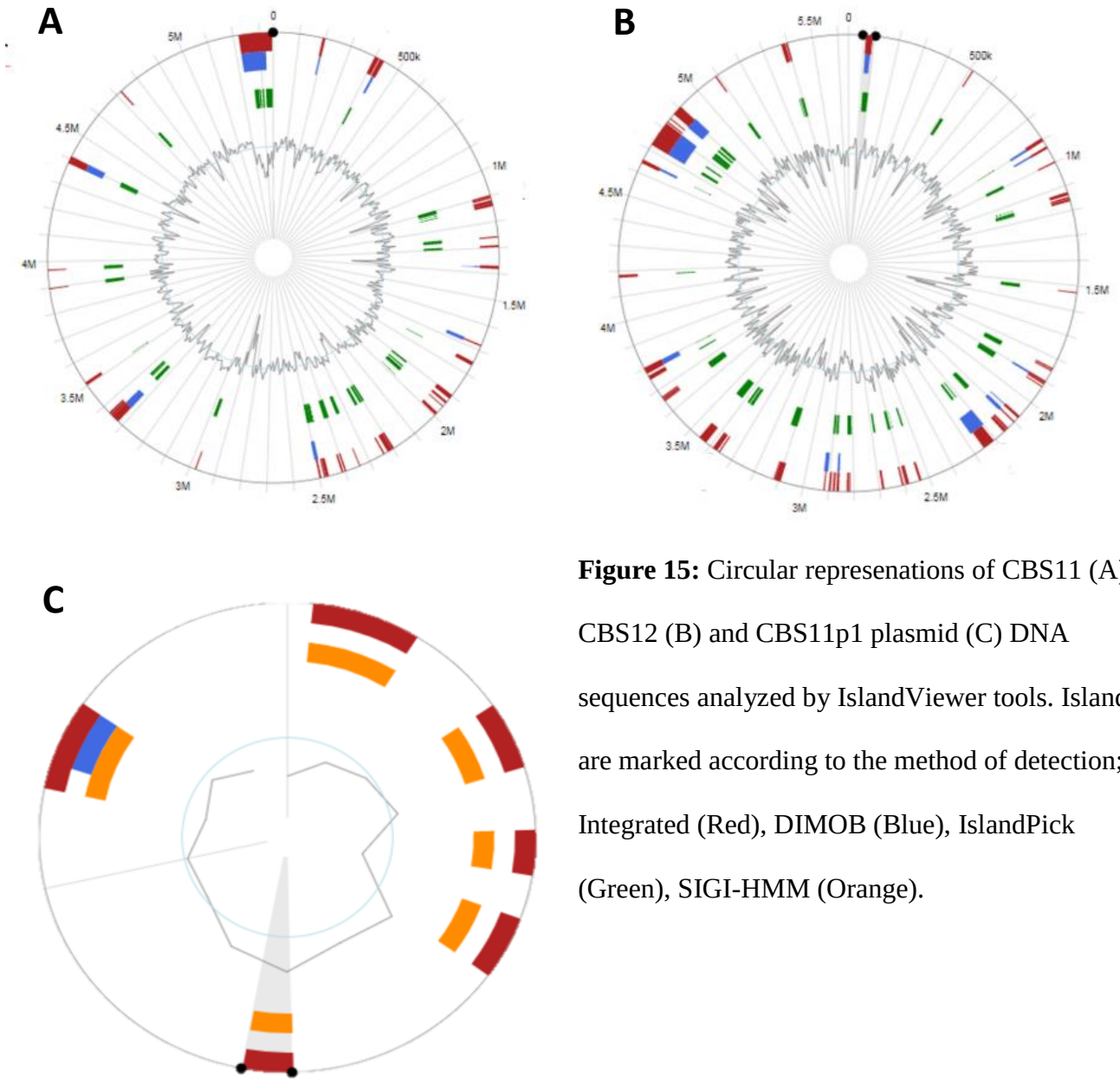

Figure 15: Circular represenations of CBS11 (A), CBS12 (B) and CBS11p1 plasmid (C) DNA sequences analyzed by IslandViewer tools. Islands are marked according to the method of detection; Integrated (Red), DIMOB (Blue), IslandPick (Green), SIGI-HMM (Orange).

3.2.2.3. Antimicrobial Resistance Profile

Resistance gene identifier program hits from the "Strict" detection system that appeared in both CBS11 and CBS12 are shown in Table 12. The CBS11 strain produced 2 more hits than CBS12, shown in Table 13. The CBS11p1 sequence generated no perfect or strict hits, and only a single loose hit, shown in Table 14. Those hits detected by the strict RGI varied in % identity to CARD database references, ranging from >99% to 42%. The alleles in CBS11 and CBS12 that were detected by the strict tool had high % coverage; greater than 95%. The two strict RGI hits unique to CBS11 were a *glpT* mutant allele conferring fosfomycin resistance, and an MFS antibiotic efflux pump *tet(41)* that can confer tetracycline resistance.

Some drugs were evaluated for MIC in both clinical isolates (Table 15). Determinations on the resistance or susceptibility of each strain were made according to the Clinical & Laboratory Standards Institute (CLSI) microbiology guidelines (Georgi *et al.* 2012). According to the MIC values collected, CBS11 exhibits an ampicillin (MIC = ≥256 µg/ml) resistance phenotype. For contrast, CBS12 exhibits intermediate resistance to ampicillin (MIC = 16 µg/ml), but did not display resistance according to CLSI.

While the CBS11p1 sequence did produce a single loose criteria RGI hit; a chloramphenicol phosphotransferase. This hit shared 88.76% of its length with the CARD reference sequence, but only had a percent identity of 26.06%. Despite the low identity, microdilution MICs were measured for both CBS11 and CBS12 to see if either displayed intermediate resistance or resistance to chloramphenicol. Despite the presence of the putative chloramphenicol phosphotransferase in the CBS11 strain, it did not display intermediate resistance or resistance to chloramphenicol with an MIC of 4 µg/ml according to CLSI guidelines. This value was the same as that observed in CBS12.

Table 12: Resistance Gene Identifier program hits common to both *S. marcescens* CBS11 and CBS12. All % identity values and % length values displayed are based on CBS11 alleles. All hits shown were detected by the strict detection system.

RGI Criteria	ARO Term	SNP	Detection Criteria	AMR gene family	Drug Class	Resistance Mechanism	% Identity	% Length
Strict	AAC6'		Protein Homolog Model	AAC6'	Aminoglycosides	Inactivation	96.6	100
Strict	*adeF*		Protein Homolog Model	RND Efflux	Fluoroquinolone, Tetracycline	Efflux	60.1	98.87
Strict	*adeF*		Protein Homolog Model	RND Efflux	Fluoroquinolone, Tetracycline	Efflux	42.18	98.68
Strict	*adeF*		Protein Homolog Model	RND Efflux	Fluoroquinolone, Tetracycline	Efflux	42.26	98.86
Strict	CRP		Protein Homolog Model	RND Efflux	Macrolides, Fluoroquinolone, Tetracycline, Penam	Target alteration	99.05	100.00
Strict	*E. coli* EF-Tu mutant	R234F	Protein Variant Model	Elfamycin Resistant EF-Tu	Elfamycin	Target alteration	95.17	96.33
Strict	*H. influenza* PBP3	D350N	Protein Variant Model	PBP mutations conferring resistance to β lactam antibiotics	Carbapenem, cephamycin, penam, monobactam, cephalosporin	Inactivation	52.91	96.23
Strict	SRT-2		Protein Homolog Model	SRT Beta-lactamases	Cephalosporin	Inactivation	98.41	100.00

Table 13: Uniquely identified RGI program hits of *S. marcescens* CBS11 detected as "strict".

RGI Criteria	ARO Term	SNP	Detection Criteria	AMR gene family	Drug Class	Resistance Mechanism	% Identity	% Length
Strict	*E. coli* GlpT mutant	E448K	Protein Variant Model	GlpT	Fosfomycin	Inactivation	96.6	100
Strict	Tet(41)		Protein Homolog Model	MFS Efflux	Tetracycline	Efflux	60.1	98.87

Table 14: Uniquely identified RGI program hits of *S. marcescens* CBS11p1 detected as "loose".

RGI Criteria	ARO Term	SNP	Detection Criteria	AMR gene family	Drug Class	Resistance Mechanism	% Identity	% Length
Loose	cmlV		Protein Homolog Model	Chloramphenicol Phosphotransferase	Phenicol	Inactivation	26.06	88.76

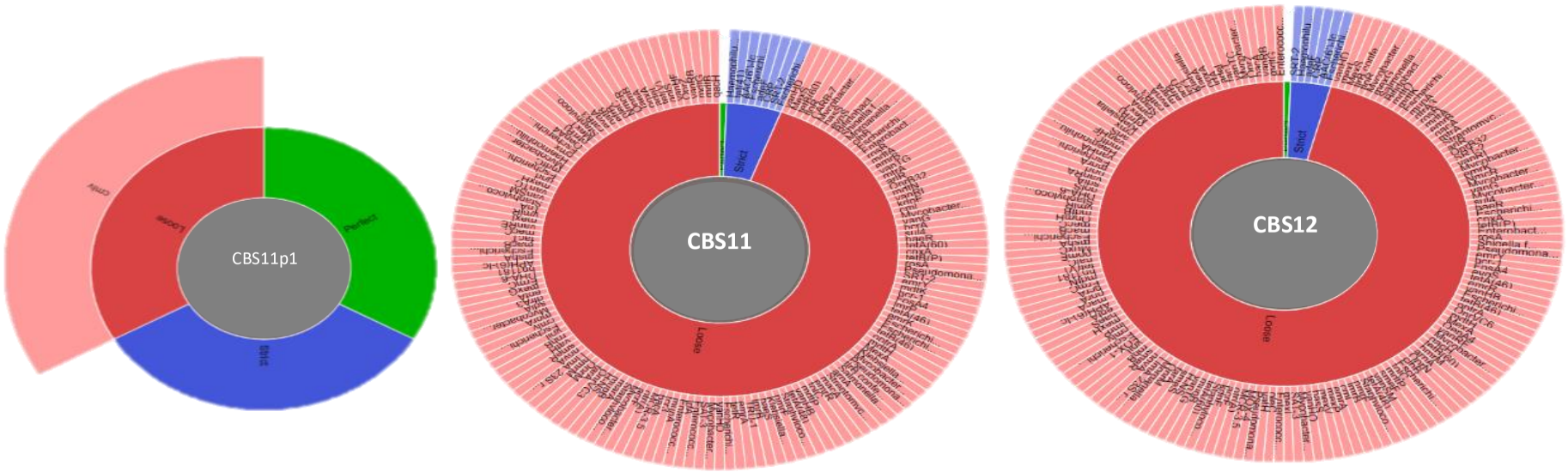

Figure 16: CARD Resistance Gene Identifier program hits in the CBS11 chromosome, plasmid and CBS12 chromosome. Perfect (Green), Strict (Blue) and Loose (Red) gene hits are represented as slices of this circle diagram.

Table 15: MIC of *S. marcescens* strains CBS11 & CBS12.

	***S. marcescens* CBS11**		***S. marcescens* CBS12**	
	Test Result	Interpretation	Test Result	Interpretation
Amikacin (AK)	2	S (S ≤ 16)	3	S (S ≤ 16)
Ampicillin (AM)	**>256**	**R (R ≥ 32)**	16	I (I = 16)
Ceftriaxone (TX)	0.25	S (S ≤ 1)	0.0094	S (S ≤ 1)
Gentamicin (GM)	1	S (S ≤ 4)	1.0	S (S ≤ 4)
Chloramphenicol (CPL)	4	S (S≤8)	4	S (S≤8)

*Interpretations indicating resistance are bolded

3.2.3. Virulence of Clinical Isolates

In the same way that virulence was evaluated for OMP mutant strains of *S. marcescens* Db11, we evaluated the virulence of the CBS11 and CBS12 isolates in the *C. elegans* nematode host model.

The CBS11 isolate achieved 50% mortality at approximately 72 hours after initial seeding, and 100% mortality at approximately 114 hours after initial seeding. Mortality was much greater than both strains tested alongside CBS11. These results indicate substantial hyper virulence by *S. marcescens* CBS11 in the *C. elegans* host model as compared to those for Db11.

This is in contrast to the CBS12 isolate, which was largely the same as the Db11 wild type. Both CBS12 and Db11 achieved 50% mortality at approximately 114 hours after initial plate seeding, and 100% mortality at approximately 138 hours after initial seeding.

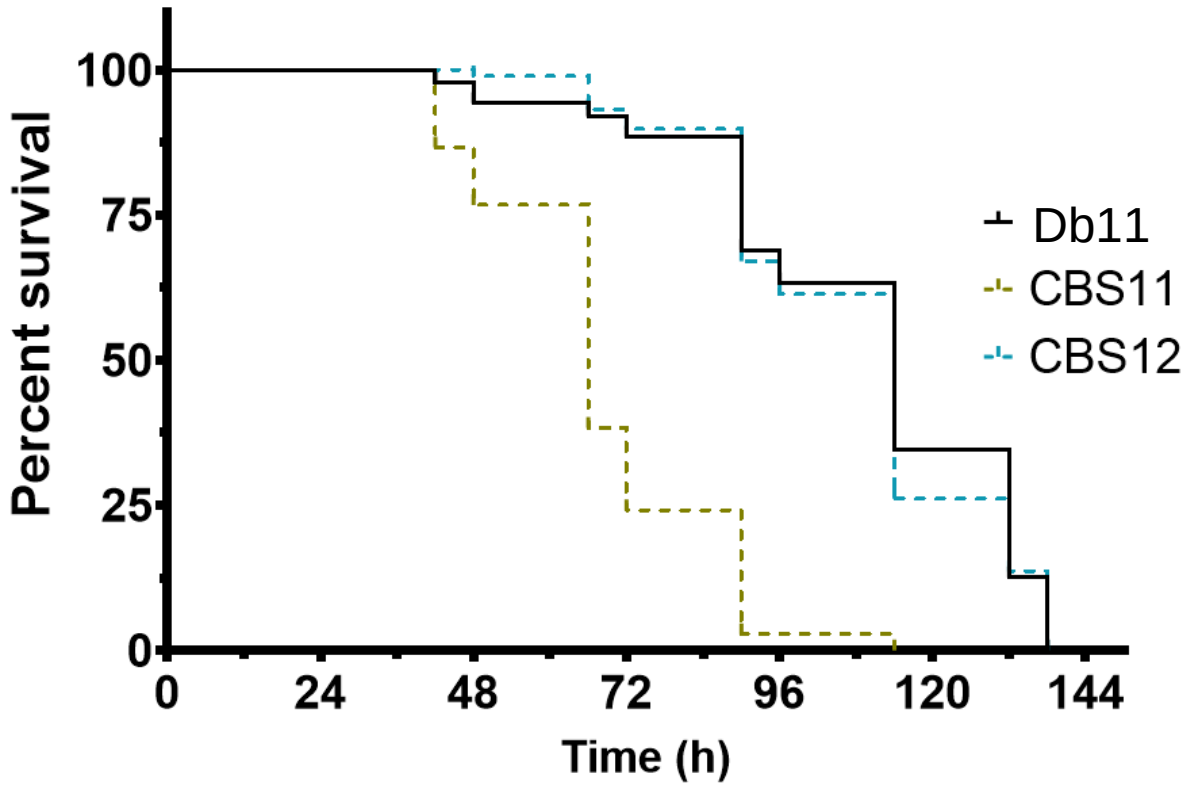

Figure 17: Survival of *C. elegans* nematode infected with *S. marcescens* strains. Kaplan-Meier survival curve of *C. elegans* N2 nematodes infected with Db11 (Black, n=90), CBS11 (Orange, n=90, $p < 0.001$), CBS12 (Blue, n=90, $p = 0.700$). Survival curve is representative of one of three independent experiments.

4. **Discussion:**

The alteration of OMP function in various Gram negative species has been established as having a significant impact on cell physiology (Begic and Worobec 2005). Depending on the organism in question, the alteration of OMP genes may lead to changes in growth rate, fitness, virulence or resistance to a range of antimicrobial compounds. This study investigated the role of *S. marcescens* OMPs in response to environmental changes.

Growth of OMP mutants: Using mutants previously generated by Moya-Torres (2014), this study aimed to evaluate the impact of single and combinatorial porin loss of function on virulence and expression profile.

Based on the results of growth in various conditions, OMP mutants with one or multiple porins deleted did not exhibit a reduction in growth rate. In minimal or rich media, there was no significant difference in growth between mutants and the parental strain. This was also the case at room temperature, 27^{o}C or 40^{o}C. No difference between mutants and the wild type was observed when mutants were exposed to high concentrations of sucrose (10%). This concentration of solute was observed to reduce maximal growth of the parental strain by approximately 50%. Such a significant osmotically stressful environment should serve to illustrate deficiencies in a particular strain's ability to tolerate high osmolarity. That all porin mutants evaluated did not exhibit differences in growth indicates that some level of compensation is occurring in these strains to offset the loss of function of these crucial proteins; *ompF*, *ompC* and *phoE*. High temperatures have also been shown to be disruptive to the bacterial outer membrane, and these conditions did not produce any differential changes in growth of any mutant strains (Bystritskaya *et al.* 2016).

Infection models: In a nematode host model, the Δ*ompF* mutant, Δ*ompF*Δ*ompC* mutant, and the Δ*ompF*Δ*ompC*Δ*phoE* mutants displayed statistically significant increases in virulence compared to the parental strain. Since there was no significant change in growth rate of the bacteria in liquid broth, this increase in virulence could not be immediately attributed to an enhanced growth rate by these mutants following infection. Prior research by Moya Torres (2014) had established the hyper virulence of *S. marcescens* Db11 Δ*ompF*, but had not investigated the effects of *phoE* deletion in this background or the Δ*ompF*Δ*ompC*Δ*phoE* genotype. The impact of porin deletion has been studied in several other enterobacteriaceae but has been only recently investigated in *S. marcescens* (Galdiero *et al.* 2012). The PhoE protein has been linked to virulence in *Vibrio cholera* (Chekabab *et al.* 2014). In that species, PhoE permits entry of phosphate into the periplasm where it can be detected by the inner membrane PhoB/R TCS, which acts to regulate transcription of several virulence factors such as motility and adhesion. This system has not been investigated in *S. marcescens*, but such a role could be similarly played by the *S. marcescens* PhoB/R TCS. The increase in virulence of the triple porin mutant compared to the double porin mutant could be attributable to the loss of the *phoE* gene. However, a study by Kurz *et al.* (2003) found that transposon insertion into *phoE* in *S. marcescens* resulted in attenuated virulence in a *C. elegans mode*. Combined with the findings of this thesis, the findings of Kurz *et al.* suggest some degree of cross talk between the ompC/ompF and phoE regulatory networks.

Despite the significant but biologically irrelevant differences in virulence observed using a *C. elegans* host model, we did not observe and differences in virulence when using a *G. mellonella* waxworm moth larvae model. This was the case 21ºC, and at 37ºC. The differences between these two host models are significant, and as such significant differences in the virulence of *S. marcescens* strains in each model are not surprising. The *G. mellonella* immune system

includes multitudes of systems not present in *C. elegans*. Melanisation is one such system of defense, which is responsible for the intense black coloration of sick or dying *G. mellonella* larvae. This system is triggered by exposure to pathogen signal molecules, which leads to activation of prophenoloxidase. This enzyme's activity leads to the tanning of tissue through the deposition of melanin, which contributes to clot stiffness and aids to block fluid loss or further bacterial invasion (Marmaras, Charalambidis, and Zervas 1996). Such a complex cascade leading to tissue healing or clotting is lacking in *C. elegans*. Furthermore, *G. mellonella* contains motile phagocytic cells called haemocytes that seek and destroy invading bacteria. This form of cellular immunity is lacking in *C. elegans*, which must rely on chemicals or physical barriers for defense against pathogens. These differences are just a few of those differentiating *C. elegans* and *G. mellonella*, and may have contributed to the differences in virulence when testing in each model organism.

Regulation of porins: Because there was no significant difference in growth between porin mutants and the Db11, there is likely a compensatory mechanism acting to offset the deletion genotype. That there are differences in virulence between the parental strain and the OMP mutants may be due to such a compensatory mechanism. As such, we investigated the expression profile of three additional known outer membrane porins important in a variety of enterobacteriaceae species. These genes are *ompA, ompX* and *lamB*. Upon quantification of these genes' expression levels in the parental and porin mutant strains, we observed several significant differences. The first is a 2-fold increase in expression of *ompC* by the Δ*ompF* mutant strain. This is largely consistent with results observed in other studies of these two OMP genes (Begic and Worobec 2005). It has long been understood that both genes are controlled by an upstream TCS known as EnvZ/OmpR (Harlocker, Bergstrom, and Inouye 1995). The upstream promoter of *ompF* has high affinity for the OmpR response regulator, and will therefore bind OmpR at even very low

concentrations. There is also a suppressor site upstream of *ompF* that has much lower affinity. The promoter upstream of *ompC* has affinity for OmpR more similar to that of the *ompF* suppressor. As such, when OmpR is in very high concentrations, OmpR will bind both the *ompF* promoter and suppressor blocking its expression, and bind the *ompC* promoter leading to its expression. Previous research has observed that loss of OmpF function results in a compensatory increase in OmpC expression. The two genes expression can be driven by OmpR after it is phosphorylated by the sensor kinase EnvZ. The inability to produce functional OmpF will result in high OmpR phosphorylation resulting in increased expression of OmpC (Viveiros *et al.* 2007). The Δ*ompF* mutant also displayed a two fold increase in expression of *ompX*. Research in an *E. coli* model illustrated that regulation of *ompF* and *ompX* are linked through the regulators MarA and H-NS (Dupont *et al.* 2007). In that study a significant decrease in OmpF production was observed in an *ompX* overexpression background. That would suggest that OmpX serves to suppress OmpF production.

The results of this thesis suggest the same relationship going in the opposite direction; that *ompF* serves to suppress expression of *ompX*. This overexpression of *ompX* appears to be exacerbated by the loss of *ompC* in addition to *ompF*. Why this is the case is unclear. However, due to the regulatory relationships that exist between *ompC* and *ompF*, it is possible that these regulatory changes are triggered through similar pathways.

The overexpression of *ompA* in the double and triple mutants was not shared by the Δ*ompF* mutant strain. This indicates that *ompA* overexpression is the result of combined *ompF* and *ompC* deletion. The difference in *ompA* expression between the double and triple mutants does not rise to the level of statistical significance. Therefore, the role of *phoE* in the overexpression of *ompA* in the triple mutant is unclear at this time.

Interestingly, the *lamB* gene's expression was reduced in all mutants tested as compared to the parental strain (Figure 12). While there is evidence linking the expression of *ompC* and *lamB*, the relationship between *phoE* and *lamB* is unclear at this time. With respect to *ompC*, very early research by Diedrich and Fralick (1982) observed that in SDS polyacrylamide gel electrophoresis (PAGE) gels of *E. coli* strains, increased LamB presence was strongly linked to depressed OmpC protein. They observed a negative linear trend between the fractions of total protein content represented by OmpC versus LamB. No evidence has been found to support a direct link between OmpF activity and LamB, nor does evidence support a direct link between the OmpF regulators EnvZ/OmpR and the regulators of LamB. All this taken together makes the findings of our research interesting in that OmpC, OmpF and PhoE deletion mutants all displayed reduced expression of LamB.

S. marcescens clinical isolates: The second arm of this project aimed to relate the observations of OMP mutants from the Db11 background to clinical isolates of *S. marcescens*. This began with the genomic analysis of two clinical isolates of *S. marcescens* provided by Dr. Sandra Ramirez of Canadian Blood Services (CBS). Both of these isolates, CBS11 and CBS12, were identified by routine BacT/ALERT screening of platelet concentrates. In the initial study of these two strains by Brown *et al.* (2012), growth of CBS11 and CBS12 were the same in LB broth but CBS12 did have a slower growth rate in platelet concentrate. A study by Drago *et al.* (2013) investigating the antimicrobial effects of platelet concentrate found that such products are effective inhibitors of various oral pathogens such as *E. faecalis, C. albicans, S. agalactiae* and *S. oralis.* A recent literature review by Varshney, Dwivedi, and Pandey (2019) found that across several different types of platelet concentrate products available to medical practitioners, all were moderately to extremely inhibitory to a variety of Gram negative and Gram positive organisms.

Taken together, these two studies support that platelet concentrates are a difficult growth environment for many different bacterial species. That CBS12 is less able to grow in PC than CBS11, it is very possible that CBS12 is less well adapted to growth and infection of mammalian hosts.

Infection model: Upon investigation of CBS12 and CBS11's virulence in a *C. elegans* host model, we did observe that CBS11 exhibited hyper virulence with respect to both CBS12 and Db11. Whether this is due to the same factors that resulted in greater serum resistance in CBS11 than CBS12, as shown by Greco-Stewart *et al.* (2012) is an avenue for further investigation. One difference that is immediately evident between the two strains is the presence of the CBS11p1 plasmid in the CBS11 strain. This plasmid does in fact contain a genomic island with several fimbria synthesis and assembly coding sequences. These plasmid borne fimbriae genes are all annotated as Type 1 Fimbriae components. Fimbriae are a common virulence mechanism across many bacterial species. Within the enterobacteriaceae, they are frequently implicated in host cell adhesion and colonization. A review by Dahlberg *et al.* (2009) describes *E. coli* Type 1 Fimbriae as being responsible for crucial in binding host cell surface mannose substrates. The fimbriae proteins then serve to anchor bacteria to the host cell, promoting colonization and infection.

Phylogeny of clinical isolates: To determine the phylogenetic placement of these two strains among available *S. marcescens* isolate genome, we followed methods previously described by Zhang and Lin (2015). Their research aimed to build phylogenetic trees of closely related species with genomes that can be largely aligned to one another. A challenge with building the phylogeny of close relatives is that conventional genes with low mutation rates may have too few SNPs to determine divergence at the strain level. As such, fragments of two genomes that align to one another can be used to build a phylogeny based on many SNPs, so long as there are few internal

rearrangements in such a fragment. These fragments are referred to as “local collinear blocks” in the study by Zhang and Lin (2015). Using a Mauve Progressive Global alignment tool, 9 completed *S. marcescens* genomes were aligned to one another. An example alignment of Db11, CbS11 and CBS12 is shown in Figure 13. Two of the largest LCBs were selected to construct phylogenetic trees of CBS11, CBS12 and the 9 selected strains. The two trees constructed from LCB 116 and LCB 63 are shown in Figure 14. Both trees are extremely similar, grouping CBS11 and CBS12 in separate clusters. The species reference strain Db11, also clusters separately. These three clusters are distinct and the strains within were isolated from unique sources. The Db11 cluster also contains *S. marcescens* RCS-14, isolated from the roots of *Solanum nigrum*. The cluster also appears to contain *S. marcescens* B3R3, which was identified in China as a possible causative agent of Whorl Rot; an infection in corn that results in browning and necrosis of leaves and stalks (Wang *et al.* 2015).

The cluster associated with CBS11 contains two other strains: *S. marcescens* WW4, and *S. marcescens* FDAARGOS_65. The WW4 strain was originally isolated from a paper mill, along with a strain of *P. aeruginosa* (Kuo *et al.* 2013). The two phylogenetic trees shown highlight the potential disagreement produced when using multiple collinear blocks. The FDAARGOS_65 strain was part of a large United States federal government database of genomes to be used for diagnostic purposes. This strain was collected from Children’s National Hostpital (Washington, DC, U.S) in 2013. In the tree generated from LCB 63, the FDAARGOS_65 strain clusters with CBS11. However in the LCB 116 tree (Figure 14), FDAARGOS_65 instead clusters more closely with CBS12. In this second tree, CBS11 instead clusters with WW4 and *S. marcescens* CAV142. The CAV142 strain is a human pathogen like FDAARGOS_65, and was also originally isolated

from a human respiratory infection. This indicates that CBS11 clusters closely with both pathogenic strains and environmental isolates.

The close relatedness of CBS11 to both environmental and pathogenic isolates is particularly interesting when we begin to look at the CBS11p1 plasmid's similarity to other sequenced plasmids. Upon BLAST search of CBS11p1, the highest identity hit is against the *S. marcescens* plasmid PWN146 (Accession # LT575492), with 98% nucleotide identity against 32% of the CBS11p1 plasmid. The PWN146 plasmid was isolated from the pathogenic nematode *Bursaphelenchus xylophilus*, responsible for Pine Wilt Disease (Vicente *et al.* 2016). This cluster highlights the melding of several very different niche environments in the three strains discussed above and the CBS11 plasmid. It seems CBS11 is closely related to both clinical isolates and environmental isolates with pathogenic capabilities in non-human hosts. It is more distantly related to the species references sequence Db11.

The CBS12 strain consistently clusters with *S. marcescens* SM39 and *S. marcescens* SmUNAM836. The SM39 strain was a clinical isolate described in a comparative genomic study by Iguchi *et al.* (2014). The SmUNAM836 strain was also a pathogenic clinical isolate, investigated by Sandner-miranda *et al.* (2016). It is a multidrug resistant isolate from a Mexican patient suffering from obstructive pulmonary disease. Interestingly, *S. marcescens* CBS12 clusters with these two highly virulent isolates, yet displays reduced growth in human serum according to Mastronardi and Ramirez-Arcos (2007) and reduced virulence in *C. elegans* according to our research. It is possible that the closer relationship to environmental isolates by CBS11 than CBS12 may indicate that it is better adapted to non-human infection than human infection. To relate the virulence of these two strains to their virulence in humans, cell culture or animals models could be considered for infection studies.

Neither CBS11 nor CBS12 displayed very different drug resistance profiles. The CBS11 strain displayed an ampicillin MIC of >256 µg/ml, as compared to the 16 µg/ml MIC value for CBS12. A putative chloramphenicol resistance gene was detected by the CARD RGI tool, albeit among the loose hit output. After testing the CBS11 and CBS12 strains for chloramphenicol resistance, neither strain displayed inhibition or resistance to this drug, suggesting that the chloramphenicol phosphotransferase in question is not functional under these conditions.

Conclusions and future directions: It appears that OMPs play a role in the virulence of *S. marcescens* as they do in other enterobacteriaceae. Some degree of cross talk between different porin regulatory networks is suggested by qPCR results of OMP mutant strains.

In regards to the two clinical isolates that we sequenced, they are not clonal and represent two distinct lineages. The virulence of CBS11 is significantly greater than that of CBS12 in a *C. elegans* host model. The CBS11 isolate has a greater resistance to Ampicillin than CBS12, but no genes were detected by the CARD RGI that would explain such a difference. Beside the ampicillin resistance, no significant difference in drug profiles were observed. These strains provide an excellent opportunity to study *S. marcescens* strains recovered as contaminants from a health care environment. Existing genetic tools developed by Moya-Torres *et al.* (2014) would allow manipulation of OMP genes in these strains, letting us observe the effects in a new genetic background.

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