The role of *Serratia marcescens* OmpF and OmpC porins in antibiotic resistance and virulence

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ABSTRACT

Serratia marcescens is a microorganism that constitutes one of the primary causes of nosocomial outbreaks in hospitals. One characteristic of *S. marcescens* clinical isolates is the high resistance to antimicrobials used in the clinic. Recent reports have attributed antibiotic resistance to altered porin expression.

In this study, *S. marcescens* Db11 isogenic porin mutants were generated using the generalized transducing phage IF3 to move marked target-genes between isogenic strain backgrounds, prior to removal of the antibiotic resistance cassette by Flp-FRT strategy. Mutants for three classical porins were obtained and the effect of *ompF* and *ompC* deletion on antimicrobial resistance was evaluated by MIC. The use of this method avoided the incorporation of additional resistance markers and is an alternative strategy to create clean unmarked *Serratia* mutant strains. The lack of OmpF, but not OmpC, significantly increased MIC values to the β-lactam drugs such as ampicillin and cefoxitin as well as to nitrofurantoin. Genetic deletion of both *ompF* and *ompC* did not compromise the integrity of the bacterial cell envelope in optimal growth conditions, suggesting that other outer-membrane porins may function in a compensatory role to facilitate nutrient uptake and cell envelope integrity.

S. marcescens is a pathogen of *C. elegans* and can be used to study host response to bacterial infections. The host model *Caenorhabditis elegans* was used in this study to investigate if porin deficits affected bacterial virulence. When porin mutants were evaluated in the *C. elegans* host model, the virulence of the single porin mutant strains increased in comparison to the wild-type. This study demonstrated that mutations of *ompF* and *ompC* did not attenuate *S. marcescens* virulence, but rather demonstrated a hypervirulent phenotype when they were

assessed in *C. elegans*. The absence of OmpF and OmpC porins in *S. marcescens* appeared to increase the bacterial invasion of *C. elegans* nematode tissue. Further studies are required to fully investigate the hypervirulent phenotype of these mutant strains. This study reveals that decrease of outer membrane permeability due to porin mutation alters antimicrobial resistance and does not generate virulence attenuation in *S. marcescens* Db11.

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
Amp	Ampicillin
AMPs	Antimicrobial peptides
ATCC	American Type Culture Collection
ATP	Adenosine 5'-triphosphate
BAM	β-barrel assembly machinery
bp	Base pairs
CAA	Casamino acids
Car	Carbenicillin
Chl	Chloramphenicol
Da	Dalton (unit for molecular mass; 1 Dalton = 1 gram/mole)
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl phosphate
EDTA	Ethylenediamine-tetra-acetic acid
ESBL	Extended spectrum β-lactamases
kDa	Kilodalton (unit for molecular mass; 1 kiloDalton = 1000 Daltons)
LPS	Lipopolysaccharide (lipid found in the outer leaflet of the outer membrane of Gram-negative bacteria)
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug Resistance
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
mg	Milligram(s)
MIC	Minimum inhibitory concentration
min	Minute (s)
ml	Millilitre
mM	Millimolar
M	Molar
µm	Micrometer
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
nt	Nucleotides
OM	Outer membrane
OMP	Outer membrane protein
ORF	Open reading frame
PDB	Protein Data Bank (database of 3D known protein structures)
POTRA	Polypeptide transport associated
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

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Figure 1.1. *Microbiol Mol Biol Rev.* 2003 Dec;67(4):593-656. Title: Molecular basis of bacterial outer membrane permeability revisited (Figure 2). ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation.

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Figure 1.9. Adv Drug Deliv Rev. 2005 Jul 29;57(10):1486-513. Title: Bacterial resistance to antibiotics: active efflux and reduced uptake. (Figure 1) Order detail ID: 65448606. Order License Id: 3420850152141

Figure 1.10. *Clin Microbiol Rev.* 2009 Oct;22(4):582-610. doi: 10.1128/CMR.00040-09. Title: Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. (Figure 3B). ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation.

Figure 1.13. *Nat Rev Immunol.* 2010 Jan;10(1):47-58. doi: 10.1038/nri2689. Title: Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. (Figure 1) Order License Id: 3384980589432. Order detail ID: 64871385

Chapter 4. *Microbiology.* 2014 Sep;160(Pt 9):1882-92. doi: 10.1099/mic.0.081166-0. Title: The lack of OmpF, but not OmpC, contributes to increased antibiotic resistance in *Serratia marcescens*. (Entire paper). Order detail ID:66605675, Confirmation Number: 11329583.

CHAPTER 1. LITERATURE REVIEW.

1.1. *SERRATIA MARCESCENS.*

1.1.1. *Serratia marcescens*.

S. marcescens is a Gram-negative rod shaped bacterium (Hejazi & Falkiner, 1997). It is a saprophytic microorganism found in diverse ecological niches. This microorganism produces DNAse, lipase, and gelatinase; it is lactose negative and does not produce indole. It is lysine and ornithine decarboxylase positive, ferments sucrose and D-sorbitol, but is L-arabinose and raffinose negative (Grimont & Grimont, 1978).

First described in 1823, *S. marcescens* was thought to be a harmless microorganism and was used for several years as a tracer organism in different studies. One of the studies described was the monitoring of vulnerability of New York City subway system to biological warfare using *S. marcescens* as a tracer agent (Mahlen, 2011). *S. marcescens* produces a red pigment that has linked this Enterobacterium with a colorful history (Mahlen, 2011). This bacterium was first isolated from an infected individual in 1913 (Woodward & Clarke, 1913). It was not until the early 1950s that *S. marcescens* started being recognized as an opportunistic pathogen associated with urinary tract infections (Mahlen, 2011; Wheat *et al.*, 1951).

Among *Serratia* species, *S. marcescens* represents the most common pathogen in human infections (Mahlen, 2011; Stock *et al.*, 2003b). This bacterium has been also recovered from hospital-acquired infections (Laupland *et al.*, 2008; Samuelsson *et al.*, 2014). This pathogen causes meningitis, bloodstream infections, urinary tract infections, endocarditis as well as wound infections (Mahlen, 2011). Its outbreaks are associated with contaminated medical equipment, disinfectants, milk and the hands of healthcare workers (Voelz *et al.*, 2010). *S. marcescens* is

considered an emerging opportunistic pathogen able to develop antimicrobial resistance (Laupland *et al*., 2008; Suh *et al*., 2010; Voelz *et al*., 2010; Mahlen, 2011).

S. marcescens can be found in a variety of environmental niches and natural hosts (Grimont & Grimont, 1978). *S. marcescens* infects insects such as *Drosophila melanogaster* (Flyg *et al.*, 1980). In plants this microorganism produces the Cucurbit Yellow Vine Disease, a bacterial infection that affects the phloem of crops such as watermelon, pumpkin and squash (Zhang *et al.*, 2003).

1.1.2. *S. marcescens* virulence factors.

S. marcescens produces hemolysin, proteases, lipase, nuclease, chitinase, peroxidase and multiple enzymes that facilitate invasion as well as factors that facilitate the infectious process (Hejazi & Falkiner, 1997). *S. marcescens* also grows as a biofilm when grown on different surfaces (Labbate *et al.*, 2004, 2007; Shanks *et al.*, 2007). Biofilm, as a mode of growth, protects the bacterial community when grown in the presence of disinfectants, antibiotics and the hostile immune system (Kalivoda *et al.*, 2008; Labbate *et al.*, 2004; Rice *et al.*, 2005; Shanks *et al.*, 2007). This opportunistic pathogen forms biofilm on contact lenses that can produce keratitis (Hume *et al.*, 2003; Zhou *et al.*, 2012). *S. marcescens* biofilm has also been reported as the causing agent of adverse transfusion reactions of platelet concentrates (Greco-Stewart *et al.*, 2012).

Another virulence factor in Gram negative bacteria like *S. marcescens* is lipopolysaccharide (LPS). The LPS is one of the main components of the outer membrane and represent an additional impermeable envelope that protects the cell and blocks the entrance of hydrophilic solutes. The LPS, is found exclusively in the outer leaflet of the Gram-negative outer membrane and is a physical barrier that protects the bacterium from host defences, and mediates

direct interactions with host cell receptors and antibiotics (reviewed in Ramachandran 2013; Samuelsson *et al*. 2014). These molecules act as endotoxins, which are potent signalling molecules that elicit events that lead to host tissue damage and bacteraemia (Ramachandran, 2013; Samuelsson *et al.*, 2014). The structural composition of the LPS molecule is divided into three parts: the lipid A component, the core and the O-polysaccharide portion, which have been well studied in opportunistic pathogens such as *Pseudomonas aeruginosa* (Lam *et al.*, 2011). The O-polysaccharide is formed by repetitive units of sugars also responsible for the O-serotypes of Gram-negative bacteria. According to the nature of the O-antigen, a Gram-negative bacterium strain can be classified as having a rough or smooth phenotype (Coderch *et al.*, 2004). The LPS is important in the resistance to host defences (Tan & Kagan, 2014). As part of innate immunity, the Toll-like receptor 4 (TLR4) in the host recognizes the LPS molecule and elicits a potent immune response (Park & Lee, 2013). LPS activates monocytes/macrophages to secrete cytokines that lead to the production of endogenous inflammatory mediators (Park & Lee, 2013; Salomao *et al.*, 2012; Tan & Kagan, 2014). However, modifications in the O-polysaccharide chain do not always produce attenuated phenotypes, other alterations, such as Lipid A modification by deacylation and palmitylation decrease recognition by TLR2 and TLR4 (Portnoy, 2005; Matsuura, 2013).

Other factors that can contribute to virulence are those molecules that play a role in bacterial motility. *S. marcescens* produces the biosurfactant serrawettin, which aids in the colonization of surfaces. There are three peptides in *Serratia* that have been reported: serrawettin W1 (serratamolide), serrawettin W2 and serrawettin W3. These are potent extracellular products that enhance spreading bacterial growth on a surface (Matsuyama *et al.*, 1992; Pradel *et al.*, 2007; Matsuyama *et al.*, 2011; Soo *et al.*, 2014).

S. marcescens also produces the hemolysin ShlA, which is a pore-forming toxin that, with ShlB, causes cytotoxicity in eukaryotic cells (Mahlen, 2011). In *S. marcescens,* s*hlA* encodes the hemolysin and *shlB* encodes an outer membrane protein essential for the secretion of ShlA protein (Poole & Braun, 1988; Poole *et al.*, 1988). Without the presence of ShlB, the hemolysin ShlA is inactive (Poole & Braun, 1988; Poole *et al.*, 1988).

Recently, a type VI secretion system has been described in *S. marcescens* (Murdoch *et al.*, 2011). This secretion system is encoded in a highly conserved gene cluster (Murdoch *et al.*, 2011). The proteins Hcp and VgrG in this system have structural homology with the bacteriophage tail proteins gp27-gp5 (Leiman *et al.*, 2009; Pell *et al.*, 2009). In general, the type VI secretion system has an important role in the virulence of human pathogens and also it seems to have a role in bacterium-bacterium interactions (Filloux, 2013; Iguchi *et al.*, 2014; Records, 2011). This secretion system in *S. marcescens* may enhance the interaction of this particular pathogen in a competitive environment, facilitating the secretion of toxins, during the initial interaction with the host (Murdoch *et al.*, 2011).

1.1.3. *S. marcescens* during infections.

Various quorum sensing systems have been described in this microorganism, such as the SwrI/SwrR and the SmaI/SmaR systems. In *S. marcescens* strain MG1, an strain isolated from rotting cucumber (Labbate *et al.*, 2007), the SwrI/SwrR quorum sensing system controls motility, biofilm formation and production of serrawettin among other factors (Labbate *et al.*, 2007). The SmaI/SmaR quorum sensing system in *S. marcescens* is closely related by sequence identity to the SwrI/SwrR system and also regulates prodigiosin production and carbapenem biosynthesis (Slater *et al.*, 2003).

During host colonization, adhesion is considered the first step of bacterial infection. Multiple factors affect the physicochemical interactions between the bacteria and surfaces. The adherence of *S. marcescens* to solid surfaces is proposed to protect cells from antibiotics and the host immune system response, enhancing bacterial pathogenesis (Shanks *et al.*, 2007). For attachment to abiotic surfaces such as medical devices, it has been reported the *luxI* quorum sensing homolog *swrI*, the two component regulatory genes (*rrsA, lipB*) and quorum-sensing controlled genes (*bsmA, bsmB*) mediate exopolysaccharide production during *S. marcescens* biofilm formation (Shanks *et al.*, 2007).

In *S. marcescens*, the *fimABCD* is a type I fimbrial operon and the expression of the *bsmA* and *bsmB* genes have been reported to act as a system of genes involved in adhesion and biofilm formation (Labbate *et al.*, 2007). The *fimABCD* expression is regulated by cyclic-AMP (cAMP) and sugar availability in the environment. Reduction in cAMP production or AMP-mediated responses (i.e. CRP) stimulates attachment phenotypes. Flagella production is positively regulated through cAMP–CRP control of *flhDC*. Contrary to what has been reported for *E. coli*, glucose stimulates *S. marcescens* biofilm and in the presence of cAMP, decreased biofilm formation (Kalivoda *et al.*, 2008; Labbate *et al.*, 2007; Pozo & Patel, 2007; Shanks *et al.*, 2007; Stella *et al.*, 2008).

OxyR, involved in the oxidative stress response, also promotes biofilm formation in *S. marcescens* (Chiang & Schellhorn, 2012). OxyR is a transcription factor involved in the oxidative stress response and pathogenicity in bacteria. This protein in *E. coli*, controls a group of almost 40 genes, that protect the cell from hydrogen peroxide toxicity, heat stress and neutrophil-mediated killing (Chiang & Schellhorn, 2012; Staudinger *et al.*, 2002). In contrast to the role described for *S. marcescens* OxyR, *E. coli* OxyR inhibits production of biofilm by

affecting fimbria assembly-associated disulfide bridge formation (Schembri & Klemm, 2001; Shanks *et al.*, 2007).

S. marcescens, is known for the production of two secondary metabolites, prodigiosin and carbapenem (Coulthurst *et al.*, 2005; Thomson *et al.*, 2000; Williamson *et al.*, 2006). Prodigiosin is a red pigment that gives dark red to pale pink color to environmental isolates of *S. marcescens*. (Williamson *et al.*, 2006). Non-pigmented isolates are more resistant to antimicrobials than pigment producers. Of the two secondary metabolites, prodigiosin has been the most studied; the metabolic pathway is characterized and was determined to be regulated by different ecological conditions such as nutrient deprivation, temperature, and pH (Grimont & Grimont, 1978; Harris *et al.*, 2004; Hejazi & Falkiner, 1997; Slater *et al.*, 2003; Williamson *et al.*, 2006). Prodigiosin has antimicrobial and antiprotozoan activities that in addition to other mechanisms, constitutes a competitive advantage when bacteria are grown in the presence of others organisms in the environment. Clinical isolates usually do not produce prodigiosin and the cause is still unknown (Carbonell *et al.*, 2000; Harris *et al.*, 2004; Hejazi & Falkiner, 1997; Thomson *et al.*, 2000;Williamson *et al.*, 2006). Carbapenem produced by *S. marcescens* ATCC39006, is a β-lactam antibiotic 1-carbapen-2-em-3-carboxylic acid molecule with antimicrobial properties, produced as a secondary metabolite mediated by quorum sensing (Coulthurst *et al.*, 2005; Slater *et al.*, 2003; Thomson *et al.*, 2000).

1.2. PORINS.

The first interaction of Gram-negative bacteria with the environment is through the cell envelope, where a group of biomolecules mediate the communication and the transport into and out of the cytoplasm. Outer membrane proteins play a determinant role in the cell's interaction with the environment and other systems. The research described in this thesis is focussed on the

role of trimeric porins on bacterial pathogenicity and antimicrobial resistance. Porins are a group of proteins in the outer membrane involved in the movement of solute through the outer membrane, providing a selective barrier to different molecules and they represent a point of resistance to antimicrobials.

1.2.1. Structure and function of porins.

In Gram-negative bacteria, the outer membrane possesses β-barrel forming proteins. They form a group of outer membrane proteins (OMP) with high diversification in functions. As OMPs they can act as porins, receptors, enzymes, transporters, and attachment structures. β-barrel forming proteins also span the outer membrane contributing to the structural stability of the membrane (Bos *et al* 2007; Fairman *et al* 2011).

The outer membrane in Gram-negative organisms is asymmetric where it is formed by an inner leaflet containing phospholipids and proteins as well as an outer cell envelope that contains LPS and proteins. The composition of the outer membrane allows it to be impermeable to many substrates causing most of the transport across this membrane to be achieved by proteins with water-filled channels which are referred as porins.

The name porin was used for the first time to define a group of outer membrane proteins during a study on membrane permeability (Nakae, 1976). These proteins are open channels that permit the uptake of nutrients. The molecules that pass through porins are restricted by the size of the porin's internal channel (cut-off 600 Da) (Achouak *et al.*, 2011; Davin-Regli *et al.*, 2008; Delcour, 2003). Porins can be non-specific or substrate-specific and represent important structures in the chemical communication of the cell with the external milieu (Delcour, 2003; Mark *et al.*, 2011; Zeth & Thein, 2010).

Bacterial porins have common characteristic features (Schulz, 2002). They are formed by antiparallel β-strands with hydrophobic amino acids interacting with the lipid layer (outwards) and hydrophilic amino acids oriented inwards. This distribution makes the porin adopt a β-barrel conformation in contrast to inner membrane proteins that are rich in α-helices (Fairman *et al*, 2011; Koebnik *et al*, 2000). Transmembrane β-barrels proteins consist of between 8 and 22 β-strands and have a simple topology that is probably enforced by the folding process (Schulz, 2002). All β-strands are antiparallel and locally connected to their neighbors. The C- and N-terminal amino acid residues are at the periplasmic face. Many bacterial porins form trimers. The central part of the trimer resembles a water-soluble protein and the external strand connections are long loops while the periplasmic strand connections are small-length turns. In all porins, the constriction at the barrel center is formed by a long loop, Loop 3, that bends inside the channel restricting the movement of solutes. Porin channels exist in two different states, where they can fluctuate between the ion-conducting open state and the non-ion-conducting closed state. The open state is typical of neutral pH and low membrane potentials. It is also modulated by voltage-dependent function of the pore (Delcour, 2003). Loop 3 and amino acids in the internal face of the pore are directly involved in the abrupt closure of the pore when a drastic current (~ 100 pA), is applied (Delcour, 2003; Wager *et al*. , 2010).The sequence variability in transmembrane β-barrels is higher than in water-soluble proteins and exceptionally high in the external loops (Schulz, 2002). These common characteristics have been confirmed in multiple studies about the molecular function of β-barrels proteins (Cowan *et al*. 1992; Fairman *et al*. 2011; Koebnik *et al*. 2000; Naveed *et al*. 2012; Nikaido, 2003) .

Porins can form either monomeric or trimeric conformations in the membrane. *E. coli* OmpA and *P. aeruginosa* OprF are examples of monomeric proteins. *E. coli* OmpF, OmpC,

PhoE and LamB are examples of trimeric porins in *Enterobacteriaceae*. OmpF, OmpC and PhoE are also referred as the classical porins because these porin structures were the first types characterized forming the basis of the current knowledge about porins (Fairman *et al*. 2011; Nikaido, 2003).

E. coli OmpF and OmpC were the first porins to have the structures determined on a molecular level (Nikaido & Vaara, 1985; Nikaido, 1992, 2003). The X-ray crystallographic structures for OmpF and OmpC have confirmed the structure of 16 stranded sheets forming a β-barrel (Cowan *et al.*, 1992). One of the loops, loop 2, bends over the wall of the other β-barrel to stabilize the structure (Figure 1.1). In the internal face of the barrel, Loop 3 controls the size of the pore channel forming a constriction region that limits the entrance of specific solutes according the molecular size (Nikaido, 2003). Loop 3 in non-specific porins, creates a constriction zone with an electrostatic field, created by acidic residues in the conserved -PEFGGD- motif (Cowan *et al.*, 1992) and basic residues in the opposite wall. This arrangement creates the selectivity of the size and charge of permeating molecules that will be allowed to move inside the channel (Koebnik *et al*. 2000).

Due to the position of porins in the cell's surface, they have exposed loops (L1, L4, L5, L6, L7, L8), that interact with the environment. These segments are driven by evolutionary changes to perform different roles such as receptors for bacteriophages, bacteriocins, nutrient-uptake sites, and attachment molecules (Fairman *et al.*, 2011). The domains located at the cell surface show high variability from strain to strain in homologous porins, a feature that has been hypothesized to constitute an evolutionary mechanism to evade adaptive immune responses (Pagès *et al*. 2008).

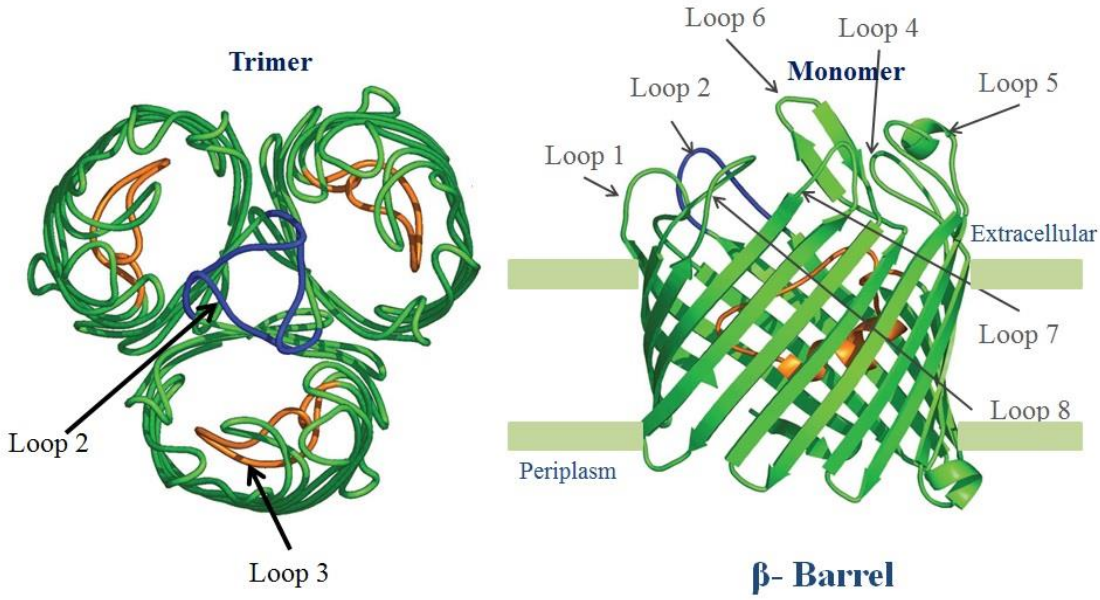

Figure 1.1. Representation of the tertiary structure of OmpF porin in *E.coli*. OmpF monomer on the right is formed by 16 anti-parallel β-strands forming a β-barrel conformation. Long loops are in the external face of the molecule and short turn are characteristic of the periplasmic face. The trimeric structure is stabilized by loop 2 (blue) and the presence of conserved amino acid motive present in the protein-protein interaction inside the trimer (Naveed *et al.*, 2012). The loop 3 bend inside the channel restrict the size of the pore which interacts with amino acid residues inside the channel wall. The structure of the OmpF porin of *E. coli* presented is a modification of a model described in a review article of Hiroshi Nikaido (Nikaido, 2003). Figure was used with permission of the copyright holder, the American Society for Microbiology (ASM).

1.2.2. Genetic regulation of porins.

In Gram-negative bacteria, porins are important checkpoints for the entrance of a wide variety of molecules that move into and out of the cell according to physiochemical and metabolic requirements. Molecular studies led to the proposal that the classical porins OmpF and OmpC allow the diffusion of hydrophilic molecules (<600 Da) (Nakae, 1975). The synthesis of porins in *E. coli* is regulated by availability of nutrients and environmental factors such as osmotic pressure, pH and temperature (Pratt *et al.*, 1996).

The most extensively studied mechanism of porin regulation in *E. coli* has been a two-component regulatory system (Figure 1.2), composed of the inner membrane sensor kinase protein EnvZ and the response regulator OmpR (Walthers *et al.*, 2004). EnvZ is an inner membrane protein of 450 amino acids with a short N- terminal in the cytoplasm, two transmembrane regions, a periplasmic loop and a large C-terminal domain in the cytoplasm (Figure 1.2). The EnvZ C-terminal domain contains the kinase/phosphatase catalytic domain (Walthers *et al.*, 2004). In the C-terminal domain of EnvZ, the His243 residue can be phosphorylated by ATP (Yoshida *et al.*, 2007). OmpR is a DNA binding protein of 239 amino acids that regulates *ompF* and *ompC* transcription. In this mechanism OmpR is phosphorylated by EnvZ histidine kinase to form OmpR-P (Nikaido, 2003; Walthers *et al.*, 2004). The phosphorylation of OmpR increases the affinity of OmpR-P for binding the promoter regions of *ompF* and *ompC* (Quinn *et al.*, 2014).

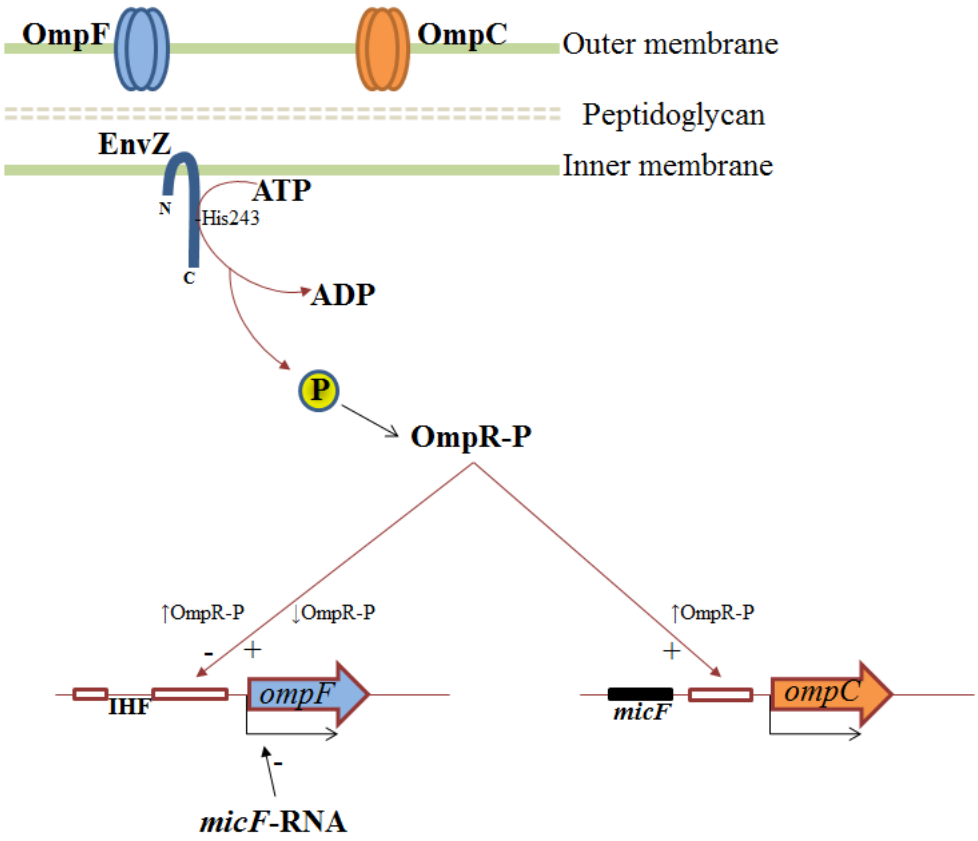

Figure 1.2. Representation of the OmpR/EnvZ two-component system. EnvZ is a sensor kinase that phosphorylates OmpR. OmpR-P is a response regulator that regulates the expression of *ompF* and *ompC* in response to osmolarity fluxes. High osmolarity induces a high concentration of OmpR-P that bind to the regulatory regions of *ompF* and *ompC*. Low concentrations of OmpR-P (↓)activate *ompF*(+) transcription and high concentrations of OmpR-P (↑) repress *ompF*(-) and activate *ompC*(+). IHF in the diagram represents the region where a loop formed facilitated by the integration host factor, that represses *ompF* expression by restricting access to RNA polymerase. Additional repression of *ompF* occurs on a post-transcriptional level with *micF* binding sense RNA of the *ompF* transcript preventing translation. White rectangles represent promoter region with the consensus sequences OmpR-P binding sites for regulation.

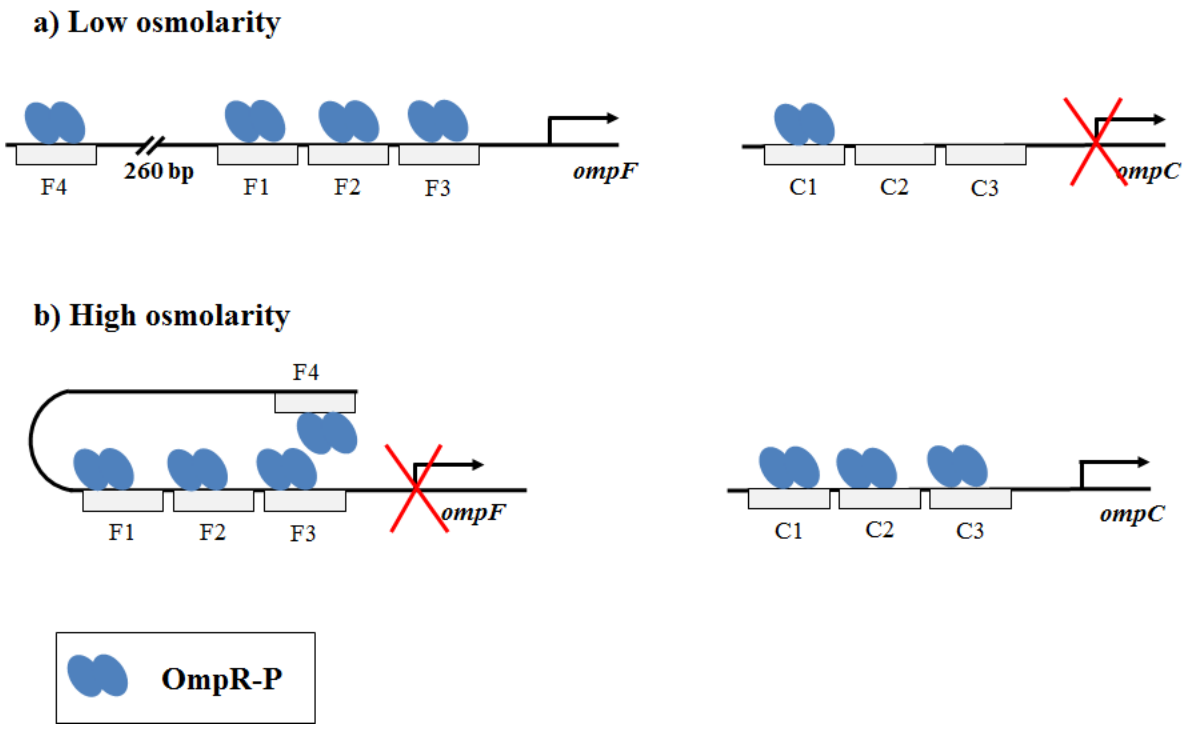

Figure 1.3. Osmoregulation of *ompF* and *ompC* by OmpR. OmpR-P binds to three sites between –100 and –40 upstream from the transcriptional start site of *ompF* and *ompC* and an upstream site between –380 and –350 at *ompF* . At low osmolarity, OmpR–P binds F4, F1, F2, F3 and C1, and OmpF is expressed. At high osmolarity, OmpR-P interactions stimulate formation of a loop that represses *ompF*. OmpR–P binds to C2 and C3, leading to activation of *ompC*. Diagram with some modifications was based on (Kenney, 2002)

EnvZ monitors the external osmolarity, and communicates this information to OmpR (Quinn *et al.,* 2014). Under conditions of low osmolarity, OmpR-P activates transcription of *ompF* in higher levels than *ompC,* whereas under conditions of high osmolarity, OmpR-P represses transcription of *ompF* and activates *ompC* transcription (Figure 1.3). OmpR-P binds to consensus sequences upstream *ompF* (F1, F2, F3 and F4), and *ompC* (C1, C2, C3) to facilitate the binding of RNA polymerase. The F4 consensus sequence is a weak OmpR-P binding site approximately 260 bp upstream of the F1 consensus sequence. When the concentration of OmpR-P increases in high osmolarity, the OmpR-P binding to the F4 site forms a loop that interacts with OmpR-P molecules in F1, F2 and F3 DNA sequences blocking *ompF* transcription (Kenney, 2002; Yoshida *et al.,* 2006). The integrator host factor (IHF) is the DNA binding protein that binds to the *ompF* promoter regulatory region and bends the DNA; facilitating the formation of a DNA loop that repress *ompF* transcription (Huang *et al.*, 1994; Ramani *et al.*, 1992). As a result, when external osmolarity increases, the levels of OmpC increase, while those of OmpF decrease (Forst *et al.*, 1989; Kenney *et al.*, 1995).

Another level of regulation of porin production is the effect of *micF,* an antisense RNA gene involved in stress response (Delihas & Forst, 2001). The *micF* gene encodes a non-translated antisense small RNA that binds to *ompF* mRNA and influences OmpF translation and downregulates the transcription of *ompF* (Delihas, 2012; Holmqvist *et al.*, 2012; Nikaido, 2003; Pratt *et al.*, 1996).

The levels of OmpR-P under nutrient limitation can be also influenced by the activity of other factors such as acetyl phosphate (AcP), and cAMP. AcP can acts as a phosphoryl donor to OmpR in nitrogen limitation (Liu & Ferenci, 2001a). Porin expression is also regulated by the expression of *rpoS, lrp, soxS,* Rob and MarA protein in *E. coli* (Pratt *et al.*, 1996). In *E. coli,* the

levels of the stationary sigma factor RpoS are considered important when the cell is faced with nitrogen or glucose limitation (Liu & Ferenci, 2001a). This sigma factor downregulates the expression of *ompF* in the stationary phase (Pratt *et al.*, 1996). The expression of *rpoS* has a greater effect on *ompF* expression than on *ompC* (Liu & Ferenci, 2001a). The leucine-responsive regulatory protein (Lrp) is a global regulator that responds to nutrient availability (Delihas, 2012; Holmqvist *et al.*, 2012). This protein regulates approximately 10 % of genes in *E. coli* and it is also regulated by the expression of *micF* (Delihas, 2012). The activity of Lrp increases in low nutrients availability and is repressed at high nutrient levels. Lrp also inhibits the expression of *ompC* and positively regulates *ompF* transcription (De la Cruz & Calva, 2010).

According to Holmqvist *et al.* (2012), *micF* can act as a repressor of *lrp* in nutrient-rich media, and Lrp also, can autoregulate *lrp* and *micF* in low nutrient media. They propose a model of Lrp regulation based on MicF-Lrp negative feedback loop that ensures cell adaptation in accordance to the availability of nutrients. In low levels of nutrients, Lrp is able to downregulate *micF*. Low expression of *micF* increases the expression of *ompF* which facilitates the entry of more nutrients through OmpF (Holmqvist *et al.*, 2012). SoxS is a DNA-binding protein defined as an oxidative stress regulator that activates the transcription of *micF*, which then results in decreased *ompF* expression (De la Cruz & Calva, 2010; Pratt *et al.*, 1996). SoxS, MarA and Rob belong to the AraC/XylS family of transcriptional regulators (Gallegos *et al.*, 1997). These three proteins reduce *ompF* expression by activating *micF* transcription in response to environmental changes (Delihas & Forst, 2001). SoxS is the effector of the *soxRS* global superoxide response regulon. *SoxS* has about 50% homology with MarA and is also able to activate MarA expression. SoxS is involved in the generation of multi-drug resistant phenotypes. SoxR is a cytoplasmic sensor protein activated by oxidative stress that activates the SoxS regulator. When oxidising

agents are present (e.g. H_2O_2 and NO), SoxR triggers the transcription of the *soxS* (Davin-Regli *et al.*, 2008; Delihas & Forst, 2001). MarA and Rob can independently activate *micF* transcription to reduce OmpF expression. Both MarA and Rob-dependent pathways are involved in OmpF reduction in the presence of toxic chemicals (Chubiz & Rao, 2011)

Two other trimer-forming porins have been described in *Enterobacteriaceae*. These are LamB and PhoE (Van der Ley *et al.*, 1987; Schirmer *et al.*, 1995), that provide hydrophilic channels from the environment to the periplasm. The presence of maltose, as the only sugar source, induces the expression of LamB in *E. coli* (Figure 1.4), that is controlled by the *mal* operon (Figure 1.4) (Koebnik *et al* 2000; Liu and Ferenci 1998).

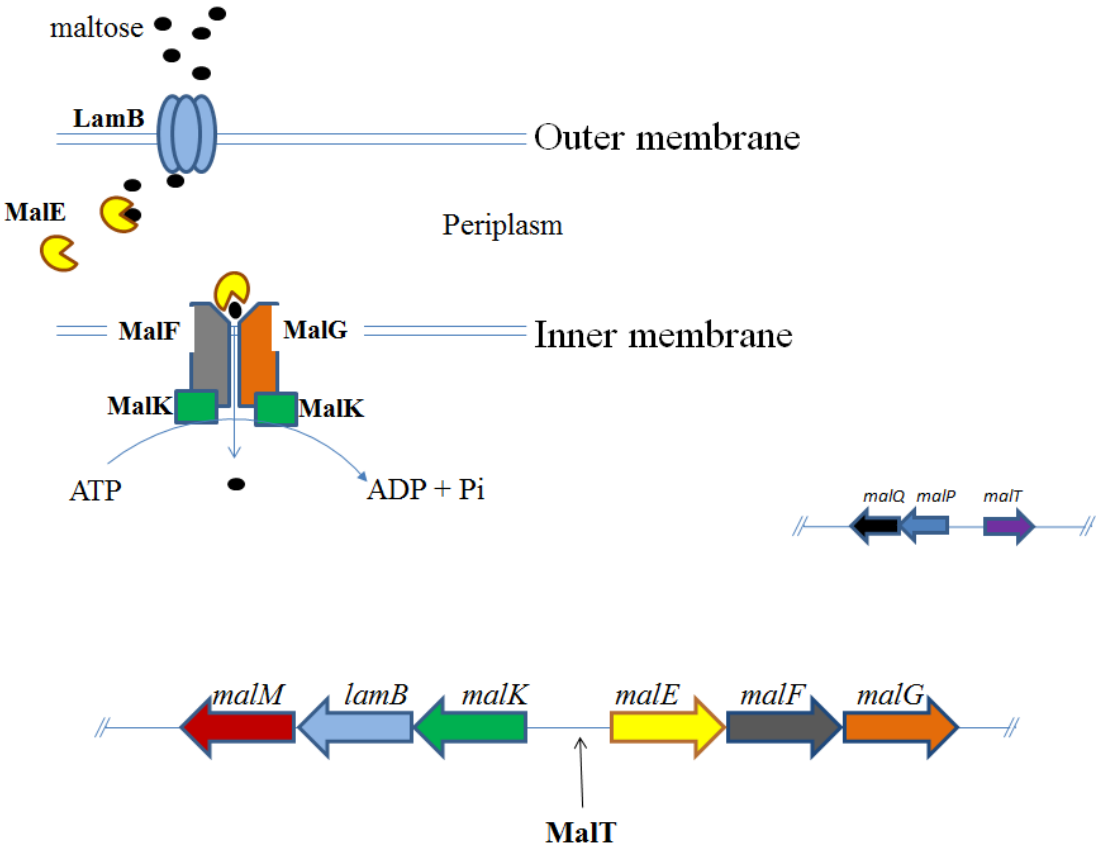

Figure 1.4. The *malMlamBmalK* and *malEFG* operons in the transport of maltodextrins. MalT, encoded by *malT*, is the positive regulator that promotes the transcription of maltose operons. *malQ* encodes amylomaltase and *malP* encodes the maltodextrin phsphorylase, both are essential enzymes for maltose and maltodextrin metabolism (Boos & Shuman, 1998). The cAMP/CAP is required for expression of *malT*. MalM: periplasmic protein, LamB: porin protein, MalK: transport ATPase, MalE: Periplasmic maltose binding protein, MalF: transporter membrane protein, MalG: transporter membrane protein.

In *E. coli*, phosphate starvation induces the expression of PhoE (Figure 1.5), controlled by the two component system *phoB/phoR* (Korteland *et al.*, 1982). PhoB activates the transcription of *phoE* by binding the Pho-box in the promoter region (Lamarche *et al.*, 2008). It has been seen in *E. coli* that *micF* can downregulate the expression of *phoE* mRNA, when *micF* expression is activated under various stress conditions such as nutrient limitation (Delihas, 2012; Holmqvist *et al.*, 2012). In the case of *lamB,* Lrp has been reported to be involved in the transcription regulation of genes associated with maltose metabolism such as *malT, malE,*and *malK* (Tchetina & Newman, 1995). The homologue of *E. coli* PhoBR system in *V. cholerae*, can also affect the production of OmpT, OmpU and OmpA major porins (von Krüger *et al.*, 2006; De la Cruz & Calva, 2010).

1.2.3. Porin assembly.

In Gram-negative bacteria, OMPs after being synthesized in the cytoplasm, need to be transported through the inner membrane, the periplasm and be recognized by the assembly machinery that inserts them in their final location, the outer membrane. The β-barrel assembly machinery (BAM) inserts β-barrel forming proteins in the OM (Albrecht & Zeth, 2011). Once OMP precursors are synthesized in the cytoplasm, the Sec system translocates the unfolded proteins across the inner membrane. In *E. coli*, the Sec system (SecYEG, SecA and SecB) transports and delivers the unfolded protein from the cytoplasm into the periplasm (Götzke *et al.*, 2014; Wickner *et al.*, 1991). Then the unfolded pre-proteins in the periplasm, as soon as they emerge from the Sec channel, are protected by chaperone proteins (Bos *et al.*, 2007; Driessen & Nouwen, 2008). Chaperones proteins such as SurA, Skp and DegP deliver OMP precursors to the Bam machinery for assembly in the OM (Bennion *et al.*, 2010; Fairman *et al.*, 2011; Kim *et al.*, 2012; Noinaj & Buchanan, 2014; Noinaj *et al.*, 2011; Silhavy *et al.*, 2010; Sklar *et al.*, 2007).

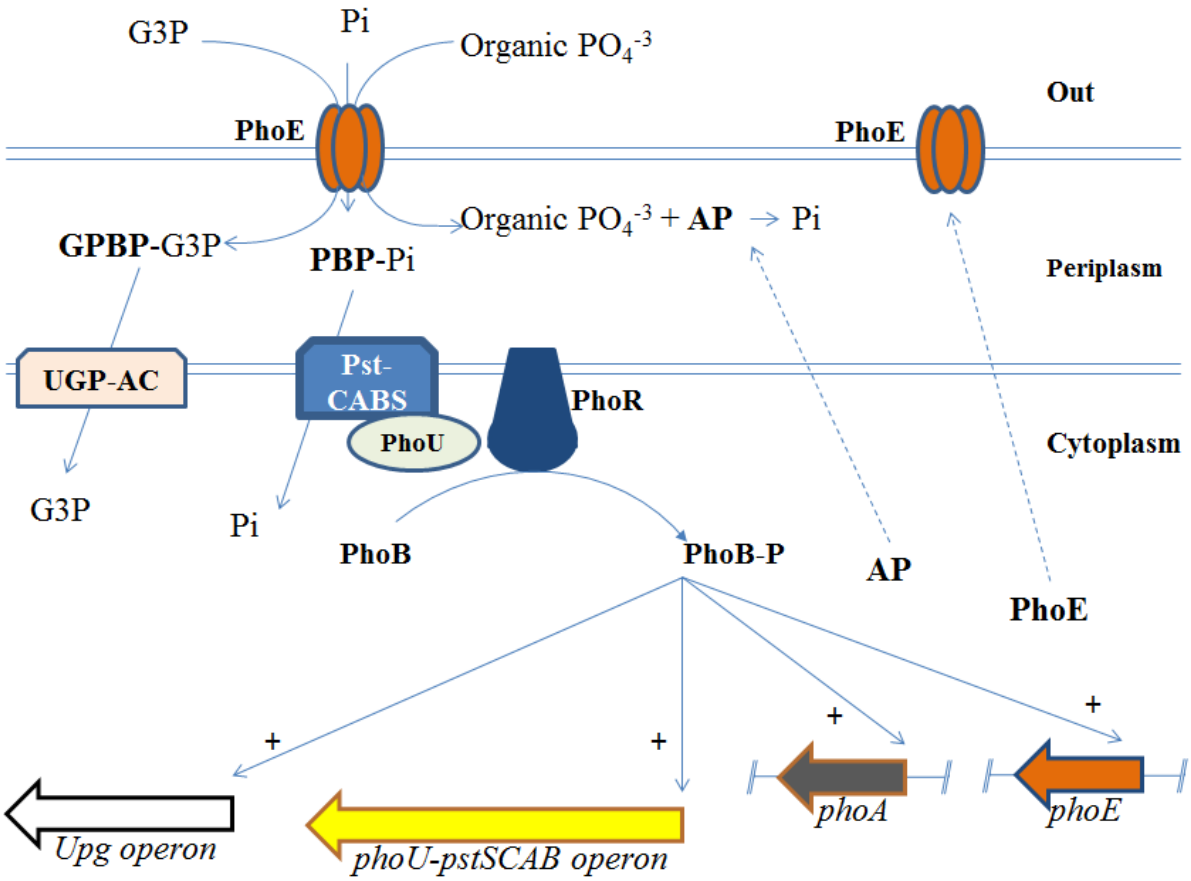

Figure 1.5. Diagram for the regulation of phosphate transport in *E. coli*. Based on the phosphate transport system presented by Rao *et al.* (1990). *phoA*: encodes alkaline phosphatase (AP), *phoE*: encodes PhoE porin, *upg* operon: encodes glycerol-3-phosphate transporters and *pst* operon: encodes the Pi transporter Pst. The Pi produced is captured by the (PBP) Pi-binding protein and transported to the cytoplasm via the phosphate-specific transport (Pst) formed by PstC, PstA and PstB. The PhoU during Pi starvation signal interacts with PhoR that activates PhoB. The PhoB-P protein will recognize the 'Pho box', a consensus sequence presents in the genes of the Pho regulon. The regulon consists of about 20 genes induced by PhoB-P. (+) refers to the activation of operons and genes members of the Pho-box.

Several studies describe the structural and molecular function of the BAM complex, formed by the five protein complex BamABCDE (Albrecht & Zeth, 2011; Fairman *et al.*, 2011; Walther *et al.*, 2009; Zeth & Thein, 2010). BamA (88 kDa), is an outer membrane protein that interacts with the rest of the BAM complex (Noinaj & Buchanan, 2014). The periplasmic domain of BamA has five polypeptide translocation-associated (POTRA) domains that assist the β-barrel OMP assembly (Bennion *et al.*, 2010; Knowles *et al.*, 2008; Silhavy *et al.*, 2010). Once the nascent porin amino acid chain reaches the assembly machinery, BamA β-barrel acts to facilitate the formation of the β-strands that will be inserted in the outer membrane to form the porin (Noinaj & Buchanan, 2014). BamB (40 kDa), BamC (34 kDa), BamD (26 kDa), and BamE (10 kDa) are lipoproteins anchored to the periplasmic surface of the outer membrane in association with BamA. BamA and BamD are essential for cell viability and porin assembly, whereas BamB, BamC, and BamE, are non-essential. BamB has been proposed to facilitate the efficient interaction of PORTA regions of BamA with the other Bam components, chaperones and new outer membrane proteins (Noinaj *et al.*, 2011). BamE stabilizes the interaction of BamA and BamD. The specific role of BamC in the complex is still unknown (Albrecht & Zeth, 2011).

The precise number of molecules that can be translocated by the BAM complex and how trimeric porins are assembled has not yet been completely elucidated (Naveed *et al.*, 2012). Different studies defined the presence of one amino acid residue, phenylalanine, in the C terminal position of porin monomers that is essential for porin insertion and oligomerization of porins in the outer membrane (de Cock *et al.*, 1997; Struyvé *et al.*, 1991).

Another feature is the monomer-monomer interaction necessary to stabilize the trimer. Oligomerization of porins to form the trimer needs the presence of Gly and Asn in the internal face of each OmpF monomer, to stabilize the trimeric structure (Naveed *et al.*, 2012). Hydrogen-

bond network between β- strands (Haltia & Freire, 1995) and the Loop 2 (Phale *et al.*, 1998) also contribute to the stability and physical resistance of trimeric structures such as OmpF, OmpC, PhoE and LamB.

1.2.4. Porins in *S. marcescens*.

OmpF and OmpC

In 1990, the first systematic functional characterization of *S. marcescens* outer membrane porins was published (Malouin *et al.*, 1990). They defined the existence of at least one porin of about 40 to 41 kDa, based in previous studies on the evaluation of membrane permeability in *S. marcescens* (Gutmann *et al.*, 1985). In the following years, other authors described the presence of three porins (Omp1, Omp2, Omp3) that were present in the same size range, with similarity to OmpF and OmpC porins already described in *E. coli* (Puig *et al.*, 1993). Hutsul and Worobec (1994, 1997) published the first DNA sequences for two porins in *S. marcescens* UOC-51, a clinical isolate. They found only two porins instead of three and named them orthologs of OmpF and OmpC in *E. coli*, and determined their molecular weight to be 41 kDa for OmpF, and 40 kDa for OmpC. OmpF and OmpC in *S. marcescens* share approximately 70% DNA sequence homology with orthologs within the *Enterobacteriaceae* family. Analysis of the promoter regions upstream of *ompF* and *ompC* identified OmpR-binding sites that are analogous to the *E. coli* osmoregulator OmpR-binding regions, implying a similar mechanism of control (Hutsul & Worobec, 1994, 1997; Begic & Worobec, 2006). In *S. marcescens*, the expression of OmpF over OmpC depends on physiochemical conditions such as pH, osmolarity, temperature (Begic & Worobec, 2006) as has been reported previously for *E. coli* reviewed by Nikaido (Nikaido, 2003). Studies of the regulation of these two porins by factors such as OmpR, *micF, rpoS, lrp*

and SoxS need to be performed in *Serratia* to confirm the presence of the same regulatory mechanisms that have been described in *E. coli* (Pratt *et al.*, 1996)

S. marcescens OmpF and OmpC classical porins are predicted to be similar in structure and function to the orthologous porins in *Enterobacteriaceae*. The porins all contain the conserved -PEFGGD- amino acid motif in Loop 3. In the particular case of the OmpF porin, there is a two Cys residue in Loop 6 amino acid sequence (-CAATENC-) that is unique in comparison to orthologous porins in *Enterobacteriaceae* (Hutsul & Worobec, 1997). It is possible that this two Cys residues are involved in other functions in addition to the normal interactions with solutes before moving across the channel. The precise function of these two Cys residues is unclear. In *E. coli*, site-directed mutagenesis to incorporate two Cys residues in different loops of OmpF was carried out to study the contribution of the Cys group in porin behavior at different pHs (Wager *et al*. 2010). None of the modifications affected the voltage-dependent function of the pore when subjected to pH changes (Wager *et al*. 2010).

PhoE

In *E. coli*, the PhoE is a porin with structural homology to OmpF and OmpC with higher specificity to phosphate (Cowan *et al.*, 1992). PhoE is considered to be a classical porin that allows the entrance of multiple solutes with specificity for phosphorus or molecules that contain this molecular group (Korteland *et al*. 1982). In *E. coli*, the expression of *phoE* increases the ability of the cell to use sources of phosphate including inorganic phosphates. The main function of PhoE is to enhance the uptake of phosphorus-containing compounds under phosphorus starvation (Korteland *et al*. 1982). Phosphate starvation induces the expression of a system of genes (i.e. the Pho regulon) whose products increase the transport of inorganic phosphorous (Pi) and phosphorus-containing compounds (Korteland *et al*. 1982). The Pho regulon is regulated by

the two component signal transduction PhoB-PhoR system (Crépin *et al.*, 2011; Vershinina & Znamenskaia, 2002). PhoR is a transmembrane histidine protein kinase that detects levels of Pi in the medium and phosphorylates/dephosphorylates the transcriptional regulator PhoB (Figure 1.5) (Crépin *et al.*, 2011; Lamarche *et al.*, 2008; Vershinina & Znamenskaia, 2002). The regulation of PhoE expression, could also facilitates the diffusion of other molecules with similar characteristics and provide the cell with more potential channels for nutrient intake (Kaczmarek *et al.*, 2006; Rao *et al.*, 1990).

The Pho regulon and the two component system is considered to be a universal system as orthologous systems have been found in many Gram-negative bacterial species (Vershinina & Znamenskaia, 2002). In addition to controlling the availability of phosphate in the cell, the PhoE regulon also contributes to bacterial virulence by modifying the expression of virulence factors such as LPS in *E. coli* (Lamarche *et al.*, 2008). In pigmented *S. marcescens* strains, the Pho regulon is also directly involved in the expression of the pigment prodigiosin (Crépin *et al.*, 2011; Lamarche *et al.*, 2008; Slater *et al.*, 2003). The *pstSCAB-phoU* genes, controlled by PhoP/PhoB, are also involved in the production of quorum sensing mediators in conditions of low Pi concentrations in *S. marcescens* (Gristwood *et al.*, 2009; Harris *et al.*, 2004; Slater *et al.*, 2003).

LamB

In *E. coli*, LamB forms trimers in the outer membrane of a larger size than the rest of the other trimeric porins described herein. Each monomer contains a β-barrel consisting of 18 transmembrane β-strands (Koebnik *et al.*, 2000) that differs from the 16 stranded β-barrel described for OmpF, OmpC and PhoE monomers. Likewise for other porins described herein, Loop 3 bends into the channel and reduces the internal pore size (Gelder *et al.*, 2002). The

internal channel contains a succession of six aromatic amino acid residues, important in sugar transport (Gelder *et al.*, 2002). The pore function of the LamB channel is not restricted to maltose, and it can facilitate the movement of different kinds of carbohydrates when they are required for bacterial metabolism (Nikaido, 2003). The presence of LamB channels in the outer membrane has been shown to give a competitive advantage to *Enterobacteriaceae* in the uptake of larger molecules of sugar such as maltodextrins (Boos & Shuman, 1998; Ferenci, 1996; Gelder *et al.*, 2002; Schirmer *et al.*, 1995). When *E. coli* grows in low concentration of sugars, the activity of LamB increases to facilitate the influx of carbohydrates molecules (Ferenci, 1996; Liu & Ferenci, 1998). OmpF and OmpC as general porins permit the entrance of sugars to the cell as well (Ferenci, 1996). Levels of cAMP and maltotriose induces expression of LamB when glucose concentration decrease in the medium (Boos & Shuman, 1998). The regulation of *lamB* is controlled by the maltose regulon, a group of genes responsible for maltose transport and described in a review article by Boos and Shuman (1998). The maltose regulon contains two divergently transcribed operons: the *malEFG* operon in the counter-clockwise orientation and *malK lamB malM* operon in the clockwise orientation. The products of all of these genes enable the transport of maltodextrins using porin channels and periplasmic binding protein-dependent ABC transporters (Boos & Shuman, 1998). The overexpression of the LamB porin depends on the level of specific sugar in the medium (Boos & Shuman, 1998; Schirmer *et al.*, 1995). It should be noted that the function and regulation of the maltoporin LamB porin has not been studied in *S. marcescens* to the same extent as in *E. coli* (Boos & Shuman, 1998).

LamB porin is also a well-known receptor for phage λ (Boos & Shuman, 1998). In coevolution studies with λ virus, it has been discovered that under certain growth conditions the OmpF porin in *E. coli*, can also act as a λ receptor to initiate infection (Meyer *et al.*, 2012;

Thompson, 2012). It is noteworthy that LamB contains two Cys residues as observed for OmpF but in different position in the amino acid chain.

OmpA

OmpA is another porin that contributes to maintenance of normal outer membrane permeability, and in some bacteria such as *E. coli*, it is highly expressed (Henning *et al*. 1978). OmpA forms two separate domains: one domain integrated into the membrane forming a small β -barrel of eight antiparallel β–strands and a C-terminal globular domain of approximately 150 residues in the periplasmic space (Ringler & Schulz, 2002). OmpA preferentially exists as a monomer in the outer membrane (Smith *et al.*, 2007). The OmpA barrel is very tight, and one of its functions is to provide structural stability to the cell by connecting the C-terminal domain of the protein with the peptidoglycan and is described in a minireview by Smith (Smith *et al.*, 2007). OmpA can also act as a receptor for bacteriophages and bacteriocins (Arora *et al.*, 2000; Smith *et al.*, 2007).

OmpA in *S. marcescens* has 79% homology with OmpA in *E. coli*. In *Enterobacteriaceae*, OmpA can be present at a level of about 100,000 molecules/cell (Koebnik *et al.*, 2000). Its function as a pore is still a subject of study (Bartra *et al*. 2012; Henning *et al*. 1978; Smith *et al*. 2007). Since it is a β-barrel protein, it is usually considered a porin that plays a role in host-pathogen interactions, bacterial adhesion and invasion of epithelial cells. It has also been shown to be a target for the immune system (Smith *et al*. 2007). In *A. baumannii*, OmpA has been reported as the major outer membrane protein that provides a low-permeability channel similar to that of OprF in *P. aeruginosa* (Sugawara & Nikaido, 2012).

OmpX

In *E. coli*, the OmpX porin is a conserved outer membrane protein with a molecular weight of 18 kDa, that forms an eight-stranded antiparallel β-barrel (Arnold *et al.*, 2007; Dupont *et al.*, 2007; Vogt & Schulz, 1999), and has been described in various enterobacterial species including *S. marcescens* (Guasch *et al.*, 1995). This porin has a characteristic four-stranded β-sheet exposed on the extracellular face that is involved in cell adhesion and bacterial pathogenicity (Dupont *et al.*, 2004; Lolicato *et al.*, 2011). OmpX is a cation-selective channel that is upregulated by MarA and high osmolarity (Dupont *et al.*, 2004, 2007). MarA is one of the chromosomally-encoded transcriptional regulators that control efflux and influx in enterobacterial species (Davin-Regli *et al.*, 2008; De la Cruz & Calva, 2010; Li & Nikaido, 2009). High expression of OmpX has been observed when the expression of OmpK36 (an OmpC orthologue in *Klebsiella pneumoniae*) is low (Dupont *et al.*, 2004). In an study evaluating a cosmid-based genomic library of *S. marcescens* N28 in *E. coli*, it was found that the expression of a 17 kDa Omp4 porin conferred resistance to bacteriocin N28, Omp4 was then determined to be an orthologue of OmpX in *E. coli* (Guasch *et al.*, 1995). The role of OmpX within *Enterobacteriaceae* is linked with its overexpression in antibiotic resistant strains (Dupont *et al.*, 2004) and its function in neutralizing host defence mechanisms (Dupont *et al*. 2007; Vogt and Schulz 1999). In the plant-derived *S. marcescens* MG1 isolate, it was found that OmpX is dependent on the presence of C_4-HSL, the quorum sensing molecule produced by SwrI (Labbate *et al.*, 2004). In this study, mutants for *swrI* did not show production of OmpX (Labbate *et al.*, 2007). The *swrI* is also known as the swarming initiation gene (Eberl *et al.*, 1996).

1.3. ANTIBIOTICS.

Porins allow hydrophilic antibiotics to enter the cell. Antibiotics are molecules that kill or interrupt the normal growth of microorganisms, usually by blocking specific functions that are required for bacterial development (Sheldon, 2005). Among all microbial targets, the biosynthesis of the cell wall and the recycling of its components are the best studied (Green, 2002; Johnson *et al.*, 2013). The cell envelope of Gram-negative bacteria is structured in three different layers. The cytoplasmic membrane is the most internal and maintains a dynamic interaction of the cytoplasm with the periplasm; a second layer is the peptidoglycan that confers resistance to changes in osmolarity (Park & Uehara, 2008). This layer in Gram-positive organisms is thicker than in Gram-negative ones and is the main target for antimicrobials such as β-lactams (Ehrmann, 2007; Green, 2002). The unit structure of the peptidoglycan (PG) consists of alternating residues of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) (Figure 1.6), that form a long helical structure, and units of four alternated D- and L- amino acid residues connected to the carboxyl group of NAM (Green, 2002; Park & Uehara, 2008; Silhavy *et al.*, 2010). Crosslinking of PG between PG-strands is achieved by pentapeptide bridges or connecting adjacent strands by the amino acid in position 3 to the of the amino acid in position 4 of the other peptide chain (Schleifer & Kandler, 1972). When PG forms the cross-linkage, PG resembles a 3-D meshlike structure.

Disruption of PG synthesis, by β-lactams, leads to cell lysis by interfering with peptidoglycan synthesis and disrupting the formation of a normal growing cell wall. This group of antibiotics bind to penicillin binding proteins (PBP) that are found in the periplasmic space and form a stable acyl complex with PBP transpeptidase (Kong *et al.*, 2010). PBP are a group of proteins responsible for transglycosylation and transpeptidation reactions that are necessary for

peptidoglycan elongation (Park & Uehara, 2008). PBP transpeptidase during peptidoglycan elongation binds to a donor peptidoglycan strand and helps to establish a covalent bond with the amine group of the acceptor peptidoglycan strand (Green, 2002).

The β-lactam ring is a cyclic dipeptide structure, present in antibiotics such as penicillins, cephalosporins, carbapenems and monobactams (Figure 1.7), that is an analogue of the normal substrate for the transpeptidation reaction that occurs during the cross-link. Its covalent binding to the active site of PBP inactivates transpeptidation and interfere with the normal PG polymerization (Green, 2002). β-lactams are probably the most utilized and studied antimicrobial. They can be produced naturally, semi- or synthetically and are grouped in 6 different structural subtypes: penams (e.g. ampicillin), cephems (e.g. cefotiam, cefuroxime, ceftazidime), cephamycins (e.g. cefoxitin), monobactams (e.g. aztreonam), penems (e.g. faropenem), and carbapenems (e.g. imipenem) (Pfeifer *et al.*, 2010).

Glycopeptide antimicrobials such as vancomycin, are another group of molecules that interfere with PG biosynthesis. Vancomycin binds to the D-Ala-D-Ala terminal sequence forming a complex that due to steric hindrance generated by this complex prevent the reaction of transpeptidation and glycosylation in the PG biosynthesis (Sheldon, 2005).

There are different antimicrobials currently available to control bacterial infections. In addition to those antibiotics that target PG biosynthesis, there are other groups that interfere with bacterial metabolism and growth. Antibiotics that target metabolism can interfere with the production of precursors for DNA synthesis such as trimethoprim. The antimicrobial trimethoprim blocks the formation of tetrahydrofolate. This antimicrobial when bound to dihydrofolate reductase inhibits the reduction of dihydrofolic acid, essential in bacterial DNA synthesis (Huovinen, 2001).

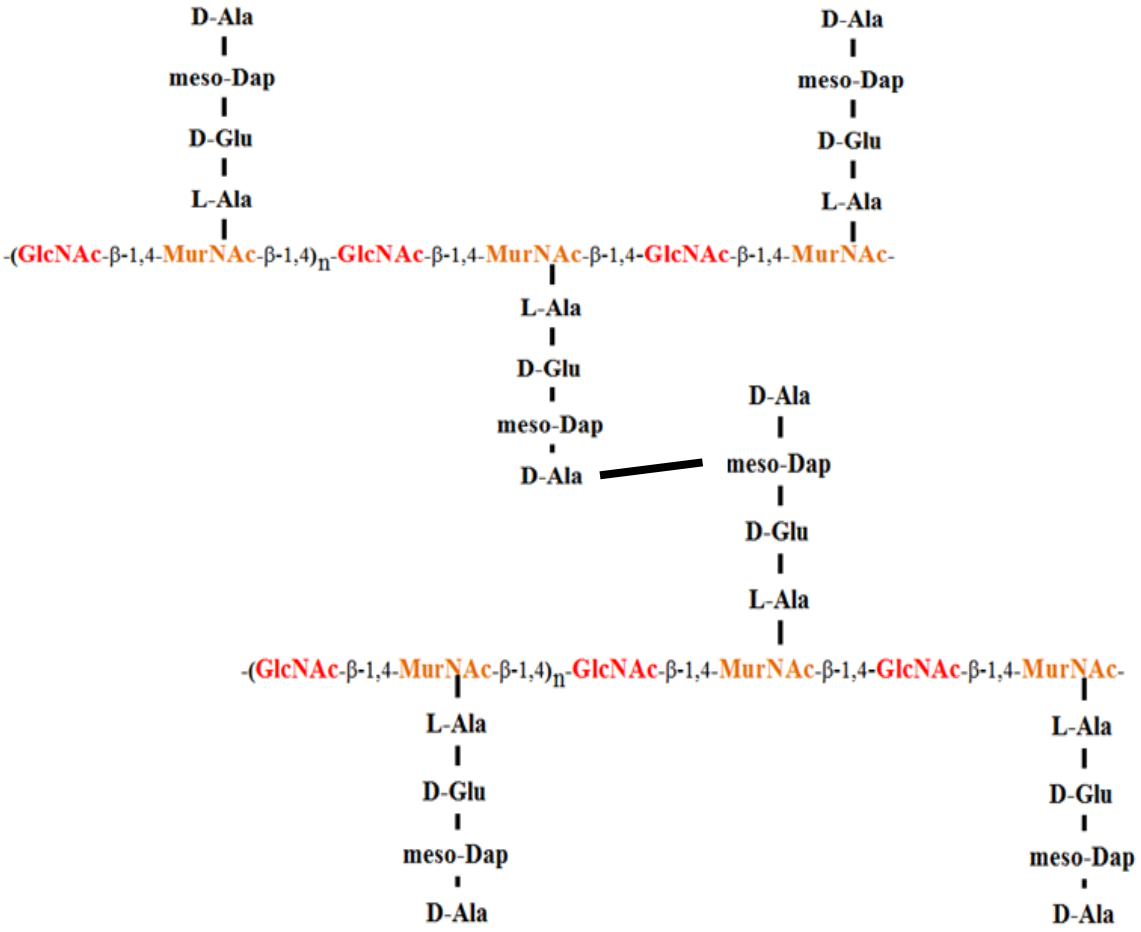

Figure 1.6. Representation of peptidoglycan cross-links in *E. coli*. The unit structure of the PG consists of alternating residues of N-acetylglucosamine (in red) and N-acetylmuramic acid (orange) that form a long helical structure, and units of four alternated D- and L- amino acid residues connected to the carboxyl group of NAM. Four amino acids form the tetrapeptide: L-alanyl, D-glutamyl, L-lysyn and D-alanine.

Quinolones are another group that have a broad spectrum of antimicrobial activity; they affect DNA replication by targeting enzymes necessary for DNA synthesis. Fluoroquinolones, for example, interact with DNA gyrase and block the normal DNA replication that is important for bacterial cell growth (Lister *et al.*, 2009; Xiao *et al.*, 2014).

Protein biosynthesis is an essential central cellular function that can be inhibited by drugs that target mRNA translation by interacting with rRNA subunits (Wilson 2014). There are multiple examples of commonly used antimicrobials that target ribosomal function; those for the 30S subunit include aminoglycosides, tetracyclines, and others, and for the 50S subunit, macrolides and streptogramins are known (Wilson 2014).

Another group of molecules with antimicrobial properties is the antimicrobial peptides (AMPs); they are small molecules with a broad spectrum of antimicrobial activity against microorganisms (Fjell *et al.*, 2012; Hancock & Chapple, 1999; Haney & Hancock, 2013). These molecules can be produced by vertebrates, invertebrates and plants as important components of the natural defense against pathogens (Hancock & Chapple, 1999). AMPs are evolutionarily conserved, predominantly positively charged with hydrophobic and hydrophilic residues and they are soluble in multiple environments (Jenssen *et al*. 2006). AMPs generally cause destabilization of lipid bi-layer membranes that can lead to cell death (Hancock & Chapple, 1999; Jenssen *et al.*, 2006). AMPs interact with lipids in cytoplasmic membranes forming transient channel and membrane damages or they can mediate bacterial cell death by targeting essential cellular processes such as inhibition of nucleic acid and protein synthesis and cell wall synthesis (Brogden, 2005; Jenssen *et al.*, 2006).

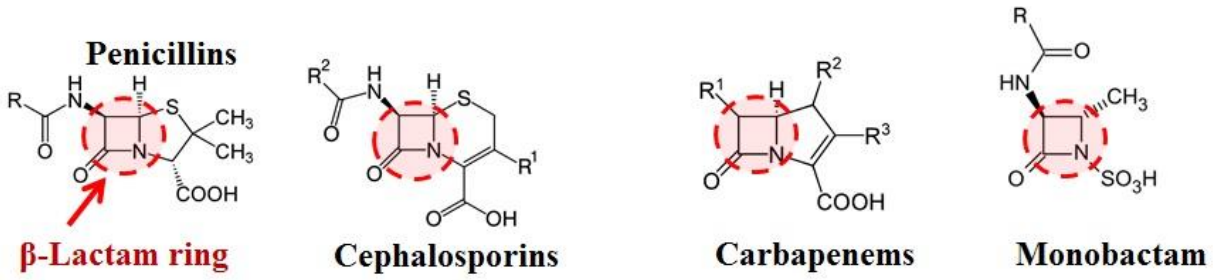

Figure 1.7. ß-lactam antibiotics. The ß-lactam antibiotics have in common the ß-lactam ring (in circle) that is inactivated when ß-lactamase enzymes react with this structure. The figure shows examples of the four groups of antimicrobials: penicillins, cephalosporins, carbapenems and monobactams antibiotics. The backbone structures used in the figure were obtained from (Konaklieva, 2014) and modified to show similarities among structures.

1.3.1. Mechanisms of antibiotic resistance.

The increase of bacterial resistance to antimicrobials constitutes a serious problem to the control of bacterial infections (Laxminarayan *et al.*, 2013). Antimicrobials force bacteria to select for new efficient mechanisms to maintain cell growth in adverse conditions (Figure 1.8). Microorganisms become resistant to antimicrobial agents by alterations in cell envelope permeability, modification of drug receptor, inactivation of antimicrobials and modification of binding sites for specific molecules among other mechanisms (Wilson, 2014). In addition, microorganisms can acquire and disseminate antimicrobial resistance by DNA mobile elements that are moved from one cell to another and enhance inherent mechanisms of antimicrobial resistance (Chancey *et al*. 2012; Tenover 2006).

In order for antibiotics to affect cellular processes, they must penetrate the cell wall and membranes. The entrance of a particular antibiotic depends on intrinsic characteristics of the drug and the nature of the cell envelope. Components in the cell envelope can restrict the uptake of a particular group of antimicrobials. One example is, the β-lactam antimicrobials that require the presence of hydrophilic channels (porins) to penetrate the outer membrane in Gram-negative bacteria (Bos *et al.*, 2007). The amount of porins present in the outer membrane contributes to decreasing the levels of antimicrobials that can reach the periplasm; low porin expression correlates with increases in bacterial resistance to hydrophilic antimicrobials (Pagès 2009).

1.3.2. Antibiotic resistance in *S. marcescens*.

S. marcescens is resistant to penicillin, oxacillin, cefazolin, macrolides, streptogramins, and rifampicin (Stock *et al,* 2003). This bacterium frequently shows susceptibility to most aminoglycosides, third generation cephalosporins, carbapenems and quinolones (Stock *et al,* 2003). *S. marcescens*, as an opportunistic pathogen, infects immunocompromised patients

producing high persistence infections. *S. marcescens* strains are characterized for high resistance to antimicrobials due to modifications in bacterial permeability and acquisition of mobile genetic elements or plasmids that carry antibiotic resistance genes (Mahlen, 2011; Rieber *et al.*, 2012; Suh *et al.*, 2010).

Alteration of two of the most important antimicrobial mechanisms, porins and efflux pumps, have been studied in *S. marcescens* (Berlanga *et al.*, 2000; Kumar & Worobec, 2005; Suh *et al.*, 2010). These two mechanisms can act synergistically and decrease the concentration of antimicrobials in the periplasm. In addition, *S. marcescens* produces chromosomally-encoded AmpC β-lactamase enzymes that constitute to heightened antimicrobial resistance when overproduced. In *S. marcescens* clinical isolates, the presence of plasmid mediated metallo β-lactamases and extended spectrum β-lactamases (ESBL) add new challenges to control outbreaks of this bacterium in hospital (Laupland *et al.*, 2008; Mahlen, 2011; Rieber *et al.*, 2012; Yatsuyanagi *et al.*, 2006). ESBL are enzymes commonly found in *Enterobacteriaceae* clinical isolates and are able to hydrolyse different class of β-lactams such as penicillins and cephalosporins. ESBL are mutated versions of the native β-lactamase gene (TEM-1, TEM-2, SHV-1) (Pfaller & Segreti, 2006). In contrast, metallo β-lactamases need divalent cations, as metal cofactors for enzyme activity. Metallo β-lactamases can be normally chromosomally mediated or encoded by transferable genes in plasmids (Walsh *et al.* 2005).

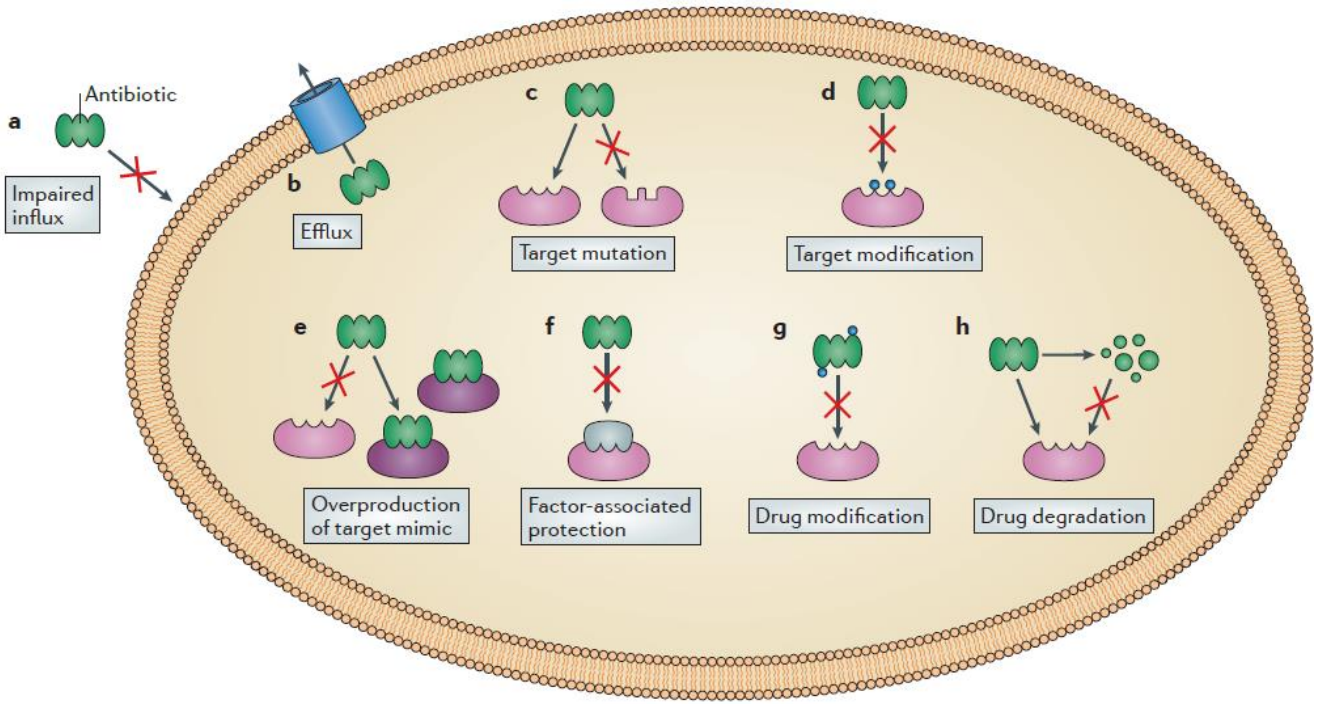

Figure 1.8. Antimicrobial resistance mechanisms. a) Low membrane permeability; b) Active efflux of the drug; c) Mutation of the target; d) Target modification (ribosomal RNA or ribosomal proteins), which reduces drug affinity for the target; e) Reduction of the antimicrobial concentration by the overproduction of a molecules that mimics the target; f) Production of molecules that remove the drug from the target; g) Antimicrobial modification and h) Antimicrobial degradation. The legend and the diagram were obtained from Wilson (2014), with permission of the copyright holder.

1.3.3. Porins and antimicrobial resistance in *S. marcescens*.

Studies on *S. marcescens* multi-drug resistant clinical isolates have shown mutations in the OmpF and OmpC porins suggesting a link between porins and antibiotic resistance (Begic, 2009; Ruiz *et al.*, 2003; Suh *et al.*, 2010). Porins are integral to the stability of the cell envelope and have a contributing role to the rapid adaptation of bacterial cells to survive in the presence of harmful compounds such as antimicrobial molecules and toxic components (Achouak *et al.*, 2011; Fernández & Hancock, 2012; Nikaido & Rosenberg, 1983; Pagès *et al.*, 2008). In the *Enterobacteriaceae* family, mutations that modify either the expression, the structure or the function of OmpF and OmpC porins have been shown to alter the susceptibility of bacteria to antimicrobials (Pagès *et al.*, 2008). In clinical isolates that show high resistance to antimicrobials, it is not uncommon to find mutations that alter porin expression (Fernández & Hancock, 2012; Suh *et al.*, 2010). In *E. coli*, mutations in Loop3 result in modification of charge distribution in the constriction zone that can reduce the passage of β-lactams (Nestorovich *et al.*, 2002; Ziervogel & Roux, 2013). Other examples are mutations affecting the regulatory proteins that control the expression of porin genes such as mutations in the *ompB* operon, that contains the *ompR/envZ* regulator genes (Hall & Silhavy, 1981).

Alteration in the expression of other porins has been observed in clinical isolates (García-Sureda *et al.*, 2011a). Different studies suggest that the overexpression of LamB may compensate for the loss of the major porins OmpF and OmpC with respect to membrane permeability (García-Sureda *et al.*, 2011a, b). In *Klebsiella pneumonia*, the presence of an antimicrobial such as cefoxitin brings a selective pressure for the expression of LamB in OmpK36 deficient strains (OmpK36 is an OmpC orthologue) (García-Sureda *et al.*, 2011a). In *S.*

marcescens, the compensation of OmpF and OmpC porin deficiency by PhoE and/or LamB has not been demonstrated.

1.3.4. Efflux pumps and antibiotic resistance in *S. marcescens*.

Efflux pumps are considered cell defense tools that actively extrude noxious compounds such as detergents, heavy metals, antibiotics and toxins from the cell, thus decreasing the intracellular concentration (Li & Nikaido, 2004, 2009). They have been divided into five groups (Figure 1.9), based on their characteristics: the ATP-Binding Cassette (ABC) superfamily, the Major Facilitator Superfamily (MFS), the Resistance Nodulation cell Division (RND) family, the Small Multidrug Resistance (SMR) family and the Multi-drug and Toxic compound Extrusion (MATE) family (Fernández & Hancock, 2012; Hinchliffe *et al.*, 2013; Kumar & Schweizer, 2005; Li & Nikaido, 2009).

All efflux pumps are present in the inner membrane and only the RND family contain a periplasmic and an outer membrane component. The AcrAB-TolC in *E. coli* is one example of this tripartite multidrug efflux machinery: AcrB, an inner membrane protein that functions as the transporter component of the pump; TolC, the protein that traverses the cell envelope and provides a conduit to the exterior, and AcrA, two periplasmic embedded membrane proteins that anchor TolC to the plasma membrane (Hinchliffe *et al.*, 2013). Active efflux systems in bacteria are also responsible for resistance to fluoroquinolones (Fernández & Hancock, 2012).

In a *S. marcescens* clinical isolate, the activity of different efflux pumps in the presence of harmful molecules has been reported. SmfY is proposed to belong to the MFS (Shahcheraghi *et al.*, 2007). *E. coli* expression of SmfY is linked to an increase in minimum inhibitory concentration (MIC) values for norfloxacin, acriflavine and ethidium bromide (Shahcheraghi *et al.*, 2007). SmdA and SmdB are members of the ABC superfamily in *S. marcescens* (Matsuo *et*

al., 2008), and SsmD and SsmJK belong to the SMR-type pump system, but they do not change MIC values (Minato *et al.*, 2008). In *S. marcescens*, a proton-dependent RND SdeAB efflux pump has been described as the factor responsible for resistance to fluoroquinolones, chloramphenicol, SDS and ethidium bromide (Kumar & Worobec, 2005). Recently, overexpression of the tripartite AcrAB-TolC system was found to be a major cause of the multidrug resistant phenotypes in clinical isolates (Hinchliffe *et al.*, 2013).

1.3.5. AmpC β-lactamase production in *S. marcescens*.

AmpC β-lactamases hydrolyze the β-lactam ring in antimicrobials and have been the subject of multiple studies and reviews (Hanson, 2003; Jacoby, 2009; Mahlen *et al.*, 2003). AmpC enzymes belong to the class C according the Ambler classification, and to group I of the Bush classification scheme (Jacoby, 2009; Joris *et al.*, 1986). Bush scheme classifies β-lactamases by (phenotype 1, 2, 3, 4) their substrate preferences and by their susceptibility to inhibition. Ambler classify β-lactamases by their sequences recognizing four classes, Class A, B, C and D (Livermore, 1995).

AmpC β-lactamases can be found in both Gram-negative and Gram-positive microorganisms (Jacoby, 2009; Livermore, 1995). The basal levels of AmpC in the cell are low in wild-type strains, and can be induced in the presence of cefoxitin and imipenen (Jacoby, 2009; Tracz *et al.*, 2005, 2007).

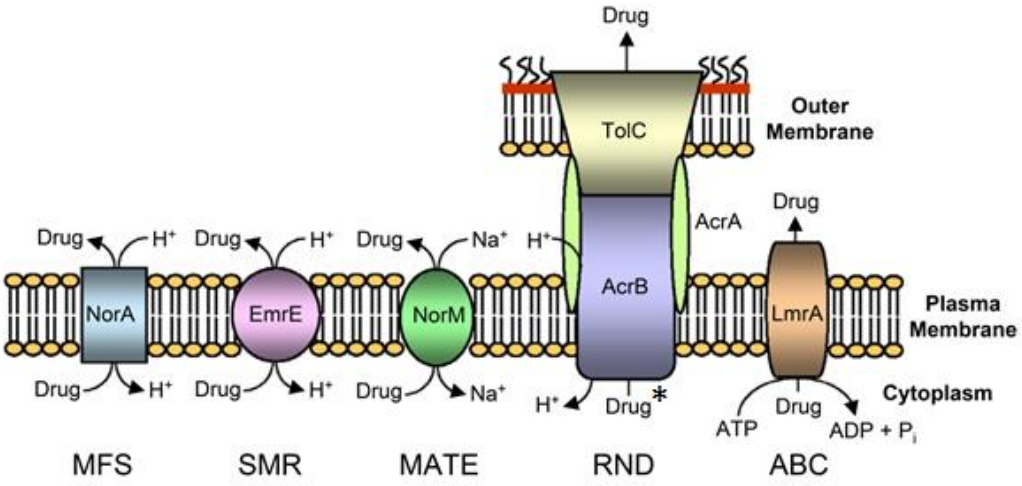

Figure 1.9. The main five groups of efflux pump systems (Kumar & Schweizer, 2005). NorA is a member of the MFS in *Staphylococcus aureus*; EmrE is a member of SMR superfamily in *E. coli*, NorM belongs to the MATE superfamily in *Vibrio parahaemolyticus*, the AcrAB-TolC is an example of RND superfamily in *E. coli*, and LmrA is an example of ABC efflux pump family in *Lactobacillus lactis.* All efflux pump systems are present in the inner membrane and only the RND efflux pump system has a more complex functional structure. RND efflux pump system contain the outer membrane protein TolC that interact with the periplasmic component of the system AcrB and the inner membrane protein AcrA to export harmful compounds such as fluoroquinolones, from the cytoplasm and periplasm. The function of all efflux pumps is energy-dependent and requires a proton ion gradient or ATP. In the figure are represented the ATP-Binding Cassette (ABC) superfamily, the Major Facilitator Superfamily (MFS), the Resistance Nodulation cell Division (RND) family, the Small Multidrug Resistance (SMR) family and the Multi-drug and Toxic compound Extrusion (MATE) family. (*) Recent studies have indicated that drugs are taken up from the vestibules on the side of AcrB and not from the cytoplasm and then released from AcrB trimer into the TolC channel. New models support that drugs are not taken by AcrB from the cytoplasm (Nakashima *et al.*, 2011).

The regulation of *ampC* expression is actively linked to the peptidoglycan recycling machinery (Park & Uehara, 2008). In the presence of β-lactams, cell wall fragmentation increases and the resulting peptidoglycan fragments are transported to the periplasm through AmpG permease. During the recycling of the cell wall-degradation products in the cytosol NagZ, a β-N-Acetylglucosaminidase, hydrolyzes them into smaller fragments (stem peptides). The increase of stem peptides in the cytosol induces the production of AmpC β-lactamase (Zamorano *et al.*, 2010). The amidase AmpD in the cytosol (Höltje *et al.*, 1994) reduces the concentration of stem peptide, degrading them to small peptides and as a consequence the *ampC* induction signal decreases (Figure 1.10) (Park & Uehara, 2008).

The *ampC* and *ampR* genes are transcribed in opposite directions with the AmpR-binding site located between both genes in an intergenic region. The binding of AmpR in the absence of induction signal represses *ampR* expression and the levels of AmpC are low (Lister *et al*. 2009; Mark *et al*. 2011). If AmpC production is constitutive as a result of mutations, high levels of resistance are observed (Moya *et al.*, 2008). An increase in the concentration of stem peptides in the cytosol, induces the expression of AmpR. AmpR up-regulates the expression of *ampC*. When the regulation of *ampC* transcription by AmpR was studied in *S. marcescens,* it was found that the intergenic region between *ampR* and *ampC* genes was longer than those of most of Enterobacteria. The increased 5' UTR of the *ampC* mRNA is proposed confer more stability to the mRNA molecule formed during transcription leading to enhanced expression (Mahlen *et al.*, 2003).

S. marcescens encodes an inducible chromosomal *ampC* gene that is overexpressed under certain conditions (Hanson, 2003; Mahlen *et al.*, 2003). Recently it was reported that a strain isolated from a hospital outbreak featured abnormal overproduction of AmpC and loss of OmpF

porin due to interruption of the OmpF coding sequence (Suh *et al.*, 2010). Another study revealed that when carbapenem is used against AmpC-producing bacteria, resistance to carbapenems may occur by both reduction in the porin expression and increase in efflux pumps (Fernández & Hancock, 2012).The presence of hydrophilic antimicrobials can induce expression of AmpC β-lactamase. It was recently shown that cefoxitin which is taken up by OmpF porin (Vidal *et al.*, 2005) can activate the expression of AmpC β-lactamase in *S. marcescens* (Mahlen *et al.*, 2003).

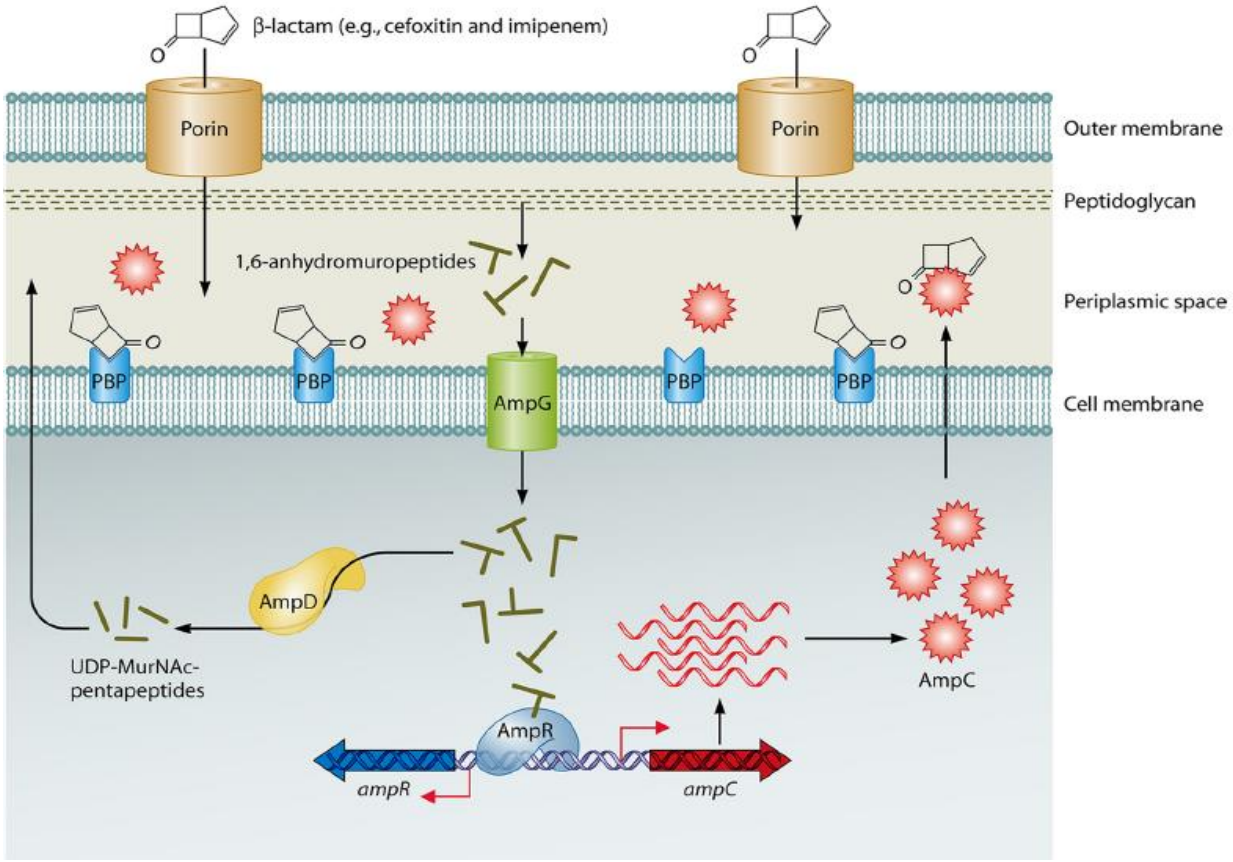

Figure 1.10. AmpC β-lactamase regulation by AmpR and AmpD (Lister *et al*. 2009). Hydrophilic antimicrobials such as cefoxitin and imipenem are inducers of AmpC. They enter the cell using porin channels. Once in the periplasm, they interact with their target molecules increasing the amount of peptidoglycan component fragments. The 1,6-anhydromuropeptides move to the cytoplasm through AmpG and may increase to high enough levels that the amidase AmpD is not able to process all molecules and these stem peptides are able to interact with AmpR protein, that activates AmpC production. The AmpC produced migrates to the periplasm and inactivates the antimicrobial present in the periplasm to re-establish the normal PG recycling pathway.

1.4. ROLE OF CLASSICAL PORINS IN BACTERIAL PATHOGENICITY.

The bacterial surface components (LPS and OMP) are primary factors that interact with a host during infections. These components protect the bacterial cell against toxic molecules as well as promote adherence, motility, and growth in microbial cell communities. As the first physical barrier, bacterial LPS and outer membrane proteins (OMP) have important roles in the physiology and bacterial virulence (Lin *et al.*, 2002). The same mechanisms that control the expression of porins may contribute to activating other systems that allow the bacteria to survive in tough environmental conditions along with controlling the intake of solutes that can be harmful. The contribution of porins to bacterial pathogenicity is an interesting field. To date, only OmpX and OmpA have been studied to some extent. OmpF and OmpC are important porins in the process of nutrient intake, and due to their location represent a point of interaction and communication with the host. The function of these porins during bacterial infection might be altered and their variation regarding protein expression could contribute to bacterial pathogenicity.

1.5. *CAENORHABDITIS ELEGANS*, A MODEL FOR HOST–PATHOGEN INTERACTIONS.

1.5.1. *Caenorhabditis elegans*.

The nematode, *Caenorhabditis elegans* is used to study various biological processes such as aging, reproduction, behavior and defense mechanisms (Ellis & Stanfield, 2014; Greer *et al.*, 2011; Kurz & Ewbank, 2000; Sifri *et al.*, 2005). The invertebrate also offers several advantages with respect to other infection models (Kurz and Ewbank 2000). The genome is available (*C. elegans* sequencing consortium, 1998; Wilson, 1999), the nematode body is small (1 mm adults), easy to maintain in laboratory conditions, has a generation time of 3 days and has a lifespan of 3 weeks with an anatomy and life cycle known in detail (Figure 1.11 and Figure 1.12). *C. elegans*

have a translucent body that enables direct bacterial localization during the observation of infectious process inside the nematode body (Sifri *et al*. 2005). Together, these features make it an attractive model organism to study host-pathogen interactions (Engelmann *et al*. 2011; Kurz *et al*. 2003; Mallo *et al*. 2002).

The life cycle of *C. elegans* involves several different stages. The embryonic stage consists of the first *in uterus* development of 150 minutes after fertilization in which embryonic cells are differentiating, proliferating and undergoing organogenesis/morphogenesis. The eggs are laid and undergo *ex-uterus* development to the 4 larval stages. These four developmental stages (Figure 1.12), precede the final reproductive, gravid, adult nematode that is then able to initiate the cycle again (Brenner, 1974). Nematodes in L4 larvae stage are used to study bacterial virulence when *C. elegans* is used as a host infection model. The following stage is the adult stage where each gravid nematode is able to produce up to 300 progeny during adulthood. There are two different sexes, the self-fertilizing hermaphrodites and males (Brenner, 1974; Haag, 2005). The hermaphroditic nematodes are commonly used to study host pathogen interactions. In a hermaphrodite population, a male is present from a spontaneous (0.1 %) non-disjunction of the X chromosome in the hermaphrodite germ line. With successful mating, male frequency can increase in occurrence to 50% of the population (Brenner, 1974). Male nematodes are used to study other processes such as aging and spermatogenesis (Ellis & Stanfield, 2014; Greer *et al.*, 2011).

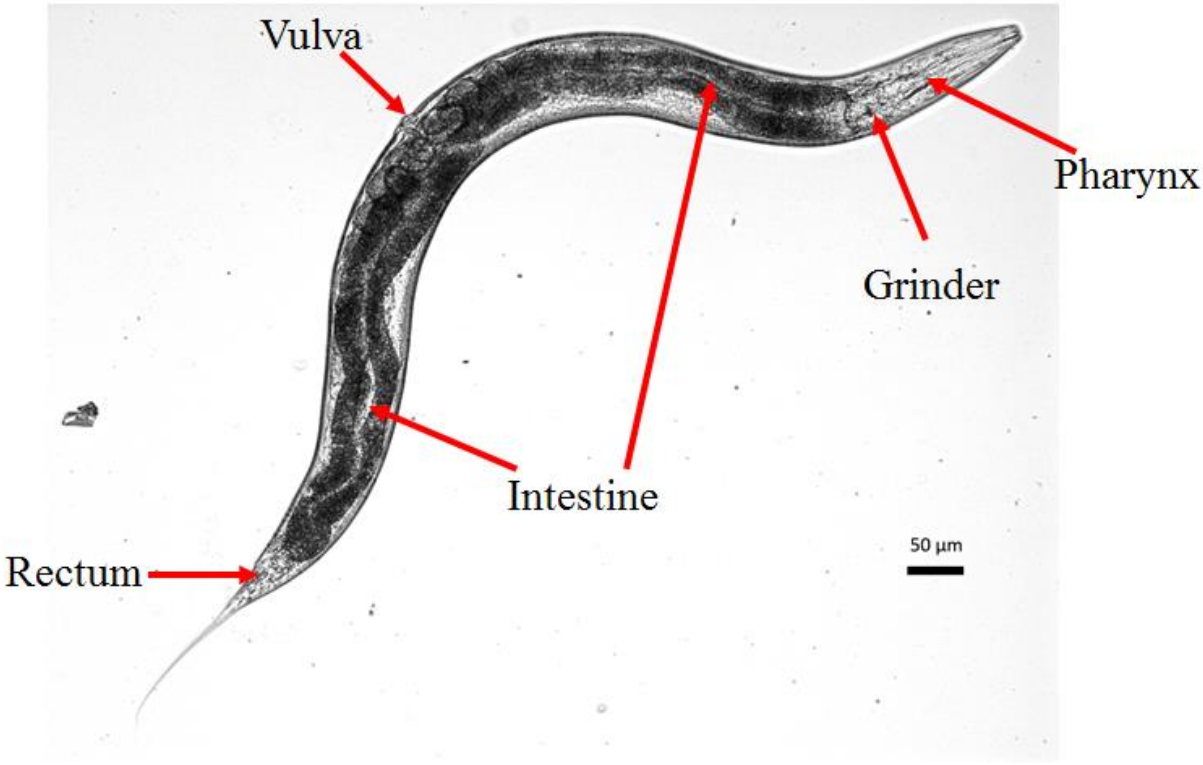

Figure 1.11. Micrograph of the nematode *C. elegans* N2. Arrows show the different anatomical structures in the nematode relevant in this study. The intestinal tract can be visualized as a line that goes from the end of the pharynx and ends in the anus. Photo provided by Hellinga J, 2014. Department of Microbiology, University of Manitoba.

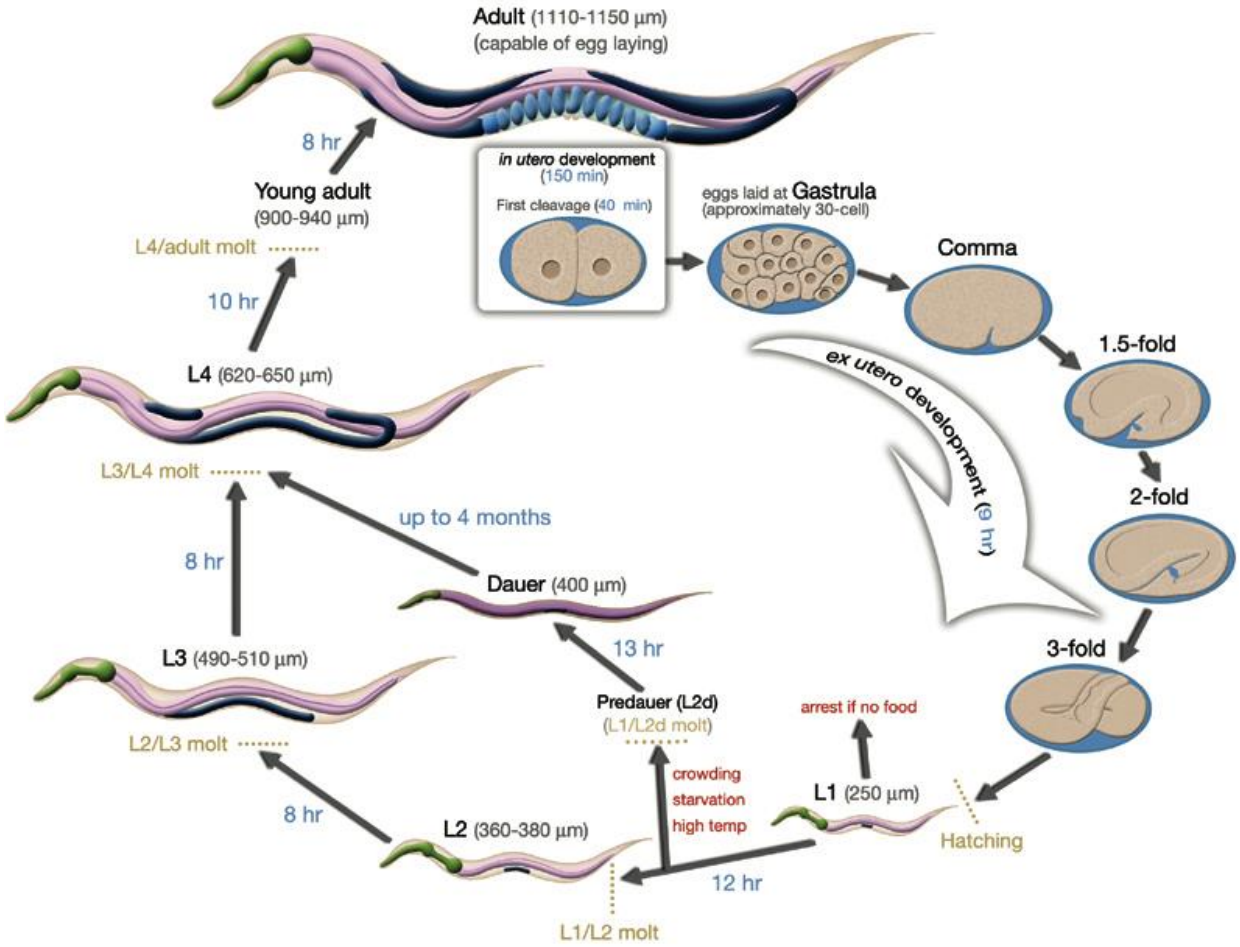

Figure 1.12. The life cycle of *C. elegans* nematode. It is comprised of the embryonic stage, four larval stages (L1 to L4) and adulthood. Diagram was obtained from Wormatlas: http://www.wormatlas.org, following the copy policy of the website: http://www.wormatlas.org/copyrightanduse.htm

1.5.2. The *C. elegans* intestinal tract.

C. elegans has a cylindrical non-segmented body (Figure 1.13) enveloped by an exterior cuticle that protects the nematode from the environment. The nematode is described as a concentric, cylindrical tubed organism. The external tube contains structures such as the hypodermis, musculature, and the secretory-excretory system. The internal tube comprises structures of the alimentary tract and the gonads (Borgonie *et al*. 1995; Sifri *et al*. 2005). The exterior cuticle of the nematode body protects against harmful factors encountered in the environment. As a microvore, the nematodes have mechanisms of defences that include physical barriers and innate immunity mediators that prevent harmful microorganisms from affecting the worm if ingested (Ewbank, 2002). The pharynx, intestine, and the rectum form the nematode gut. The pharynx is a muscular organ that contracts rhythmically to move food into the grinder. The grinder, an organ in the terminal bulb, mechanically breaks down the ingested material before passing into the intestinal lumen (Straud *et al.*, 2013). Between the pharynx and the intestine, is the pharyngeal-intestinal valve characterized as a narrow channel opening to allow the movement of food into the intestinal lumen. The ingested materials are chemically degraded into smaller fragments by the intestinal digestive enzymes (Chauhan *et al.*, 2013). The digestive and metabolic activities are central to the growth and development of the nematode. Within the intestinal lumen, bacteria are physically damaged and transported as mixture of processed food to the intestine for active degradation and adsorption of nutrients. During digestion, the lysozymes, saposins and other hydrolytic enzymes generate nutrients in the intestine to be transported by the vesicular into the intestinal cells (Hermann *et al.*, 2005; McGhee, 2007; Schroeder *et al.*, 2007; Tarr, 2012). In this process, digestive molecules are secreted into the intestinal lumen. The intestine is composed of 20 large cells (McGhee, 2007), that have

microvilli extending on the apical face forming a brush border providing a larger area of absorption (Figure 1.13). Nutrients are taken up by endocytosis and waste products are secreted in the opposite direction through the intestinal epithelia. In the intestine, in addition to food digestion, active processes of synthesis and storage of nutrients occur (McGhee, 2007; Schroeder *et al.*, 2007). The processed food is then moved through the nematode intestinal tract by peristaltic motion to the anus where the nematode excretes wastes every 50 seconds (Altun & Hall, 2009; Brenner, 1974).

In the environment, *C. elegans* is constantly in contact with microorganisms that, in some cases, in addition to being a food source, can be pathogenic and cause severe damage. Disruption in the function of the grinder in the second pharynx bulb (Figure 1.14) facilitates the entrance of intact bacteria into the intestinal lumen that can potentially colonize and proliferate in the intestine. In the event that an infection occurs once the bacterial pathogen is ingested and able to bypass the grinder, different mechanisms seems to be activated to protect the nematode from the effects of the pathogenic bacteria (Powell *et al*. 2009). The digestive system (Figure 1.13), is the major immune organ in direct contact with pathogens (Ewbank & Zugasti, 2011). While the primary function of the intestinal cells is to secrete digestive enzymes and take up nutrients, the intestinal cells also contribute to the defense system in the intestine by secreting antimicrobial effectors such as lectins, antimicrobial peptides and lysozymes. In addition, the intestinal cells themselves have recycling endosomes, autophagic vacuoles and autofluorescent granules that enhance the immune response (Ewbank & Zugasti, 2011; Kurz & Tan, 2004; McGhee, 2007).

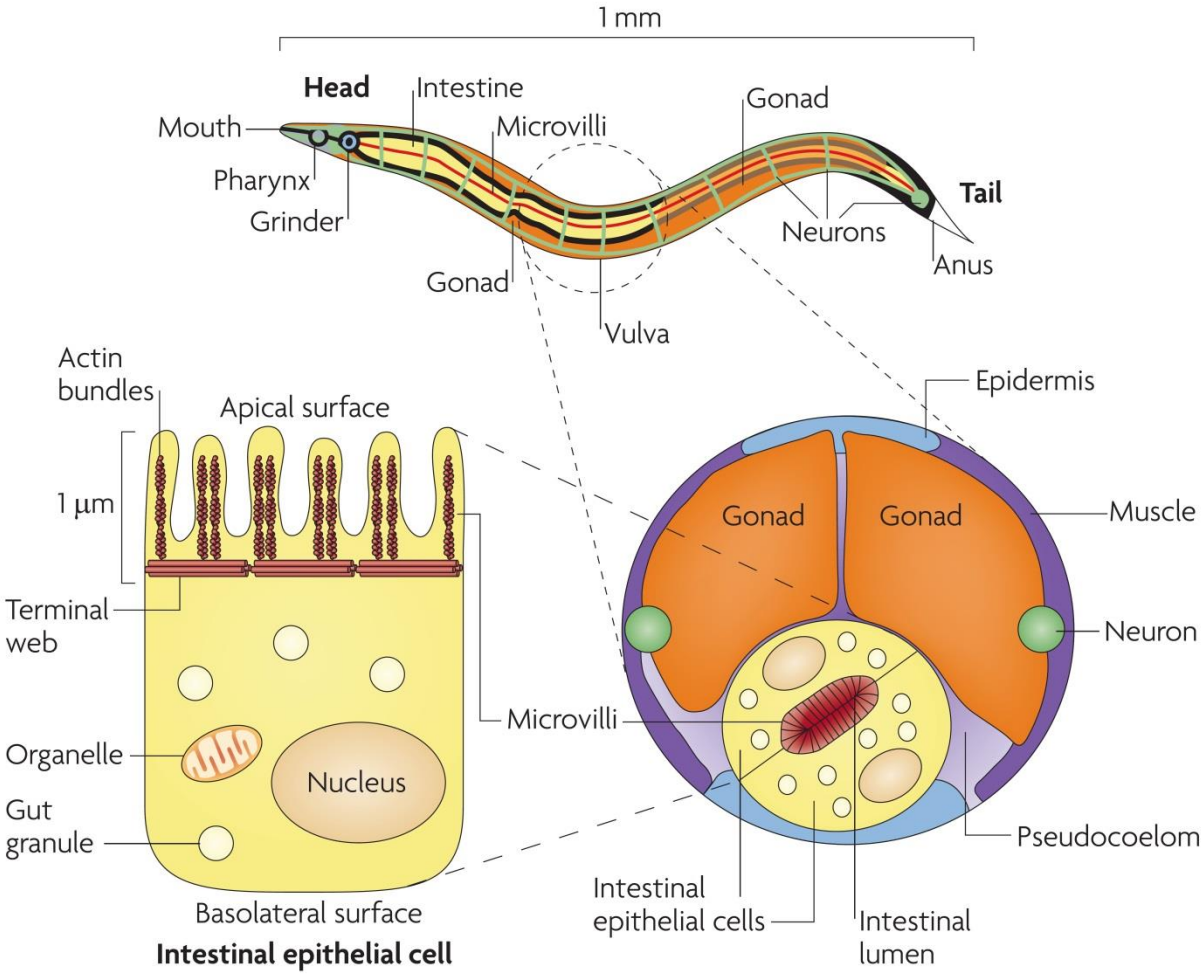

Figure 1.13. Schematic representation of the *C. elegans* intestine The apical surface of the intestine forms microvilli presenting a brush border toward the intestinal lumen (Irazoqui *et al*. 2010).

The *C. elegans* innate immunity system comprises pathways of which components are functionally similar to those that comprise the mammalian innate immune response (Alegado *et al.*, 2003; Irazoqui *et al.*, 2010a; Kurz & Ewbank, 2003; Schulenburg *et al.*, 2004). They are the DBL-1, the p38-like mitogen-activated protein kinase (MAPK) and the DAF-2/DAF-16 pathways as well as the toll-like receptor (TLR) homologue Tol-1 (Kurz & Ewbank, 2003). DBL-1 in *C. elegans* is part of the TGF-β family and it is involved in resistance to pathogen regulating the production of antimicrobial proteins (Ewbank, 2006; Gumienny & Savage-Dunn, 2013; Mallo *et al.*, 2002; Mochii *et al.*, 1999). The p38 MAPK pathway, present in both animals and plants, is important in immune response and mediates stress responses (Kyriakis & Avruch, 2001). In the nematode, the p38 cascade is involved in the protection against bacteria and fungi (Schulenburg *et al.*, 2004; Troemel *et al.*, 2006). The receptors for pathogen-associated molecular patterns (PAMPs) that activate immune response in the presence of bacterial antigens (e.g. LPS) have not been identified in *C. elegans* (Engelmann & Pujol, 2010); however, only one TLR homologue, the Tol-1, has been reported. In humans, the TLRs are involved in the response to bacterial outer membrane components, such as LPS; however, in *C. elegans* the Tol-1 appear to be involved in olfactory avoidance to potential pathogens (Pujol *et al.*, 2001). These described pathways activate innate immune response mechanisms that control the production of antimicrobial molecules (Engelmann & Pujol, 2010; Mallo *et al.*, 2002). These pathways have been extensively studied in other organisms and are for the most part, conserved in *C. elegans*, (Ewbank, 2006; Kurz & Tan, 2004; Tenor & Aballay, 2008).

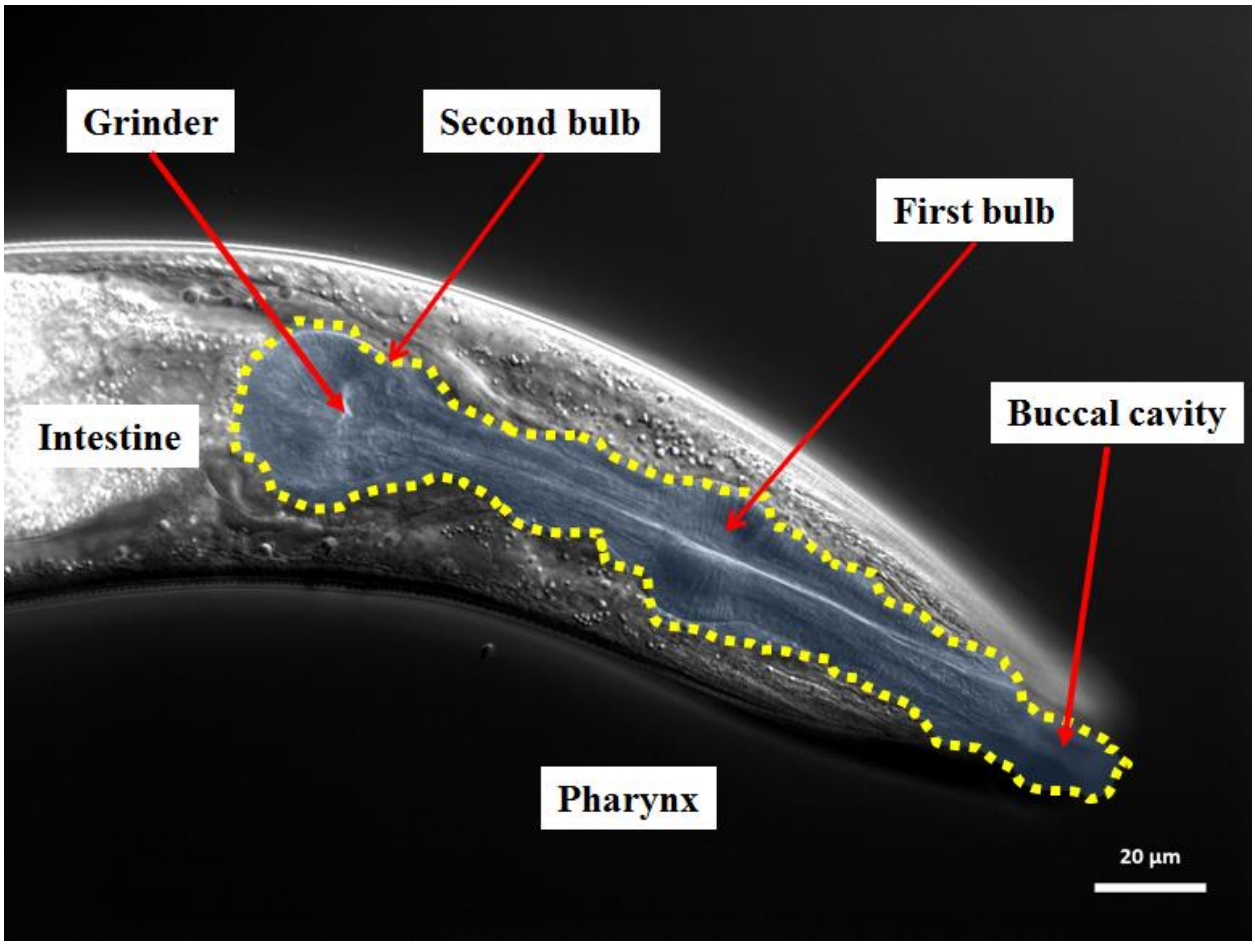

Figure 1.14. Pharyngeal region of *C. elegans* nematode. The structure of the pharynx is surrounded by dashed line. The anatomical aspects of pharynx structure are indicated. Photo provided by Hellinga J, 2014. Department of Microbiology, University of Manitoba.

1.5.3. *Caenorhabditis elegans* as an infection model for bacterial pathogens.

Bacterial pathogenicity can be studied using the *C. elegans* host-model by feeding the bacterial pathogen in question to nematodes as a food source and following the physiological changes that occur within the nematode over the course of the infection (Engelmann *et al*. 2011; Kesika *et al*. 2011; Kurz & Ewbank 2000; Kurz *et al*. 2003). In most cases, the bacterial pathogen establishes intestinal colonization in *C. elegans*. For this reason, the digestive system of the nematode can be used as a model to study the process of bacterial colonization and to discover bacterial virulence factors relevant to the mammalian systems (Kurz *et al.*, 2003).

In order to understand how the nematode digestive system fights the pathogen, it is important to understand how a pathogen is able to colonize and establish a lethal infection in the nematode. When *C. elegans* feeds on non-pathogenic bacteria such as its laboratory food source *E. coli* OP50, the *C. elegans* life span averages two weeks. However, when *C. elegans* feeds on a pathogenic microorganism, the life spans of the nematodes are significantly reduced. The *C. elegans* mouth is the primary contact of the gut with the pathogen. Once the bacteria enter, the pharynx rhythmically moves the bacteria from the anterior to the posterior pharynx bulb where the grinder presumably destroys bacteria before they enter the intestine (Figure 1.14). However, it has been seen that after the L4 developmental stage has been reached, the efficiency of the grinder decreases allowing the passage of intact bacteria such that they are able to colonize the intestinal lumen (Kurz & Tan, 2004). Reduction in the longevity of *C. elegans* is an indication of an infection. To date, five different nematode-killing mechanisms have been described: intestinal colonization, persistent infection, invasion, biofilm formation, and toxin-mediated killing (Sifri *et al.*, 2005). Other ways of microorganism infections reported for *C. elegans,* in addition to intestinal colonization, include intracellular invasion, destruction of the epidermis, uterus

infection and inflammation in the rectum (Engelmann & Pujol, 2010). However, the study of host-pathogen interaction of those bacteria that colonize the intestine have rendered information about the mechanisms of innate immunity involved in host defense (Irazoqui *et al.*, 2010a; Kurz *et al.*, 2003; Powell & Ausubel, 2008). The *C. elegans* innate immune system is centred on three pathways [p38 mitogen activated protein kinase (MAPK), DAF-2 insulin signaling, and transforming growth factor (TGF-β)], that when triggered, activate genes encoding antimicrobial products for subsequent secretion into the intestinal lumen. Successful colonization of bacteria, particularly pathogens, within the intestinal lumen is achieved by resistance to the effects of secreted antimicrobial products (Kurz & Ewbank, 2003). The high degree of conserved function between the *C. elegans* and the human innate immune systems makes the use of *C. elegans* to model the infectious process of human bacterial pathogens relevant (Kurz *et al*., 2003).

1.5.4. Study of *S. marcescens* pathogenicity in *C. elegans*.

S. marcescens infects and kills *C. elegans* by producing a persistent intestinal colonization (Mallo *et al.*, 2002). Among *S. marcescens* strains used to study host-pathogen interactions (Schulenburg & Ewbank, 2004), the pathogenicity of *S. marcescens* Db11 is the most well characterized in the *C. elegans* host model (Kurz *et al.*, 2003).

Nematodes are infected with *S. marcescens* Db11 at the larval stage 4 (L4), when the efficiency of the grinder is compromised (Kurz *et al.*, 2003). Nematodes infected at earlier larval stages show no presence of *S. marcescens* suggesting bacteria are being destroyed in the grinder (Kurz *et al.*, 2003). At the start of a survival assay, when L4 nematodes have been placed on a *S. marcescens* Db11 lawn, the initial colonization can be detected at 6 hours after the first contact with *S. marcescens*. This colonization leads to a progressive intestinal deterioration that includes reduction in the intestinal cell volume and accumulation of bacteria inside the intestinal lumen.

After three days, an advanced destruction of the intestinal epithelium is observed leading to nematode death (Kurz *et al*., 2003; Mallo *et al*., 2002). There is no description of any *S. marcescens* bacterial invasion of the *C. elegans* tissues.

S. marcescens is able to resist the effectors of the innate immune response (Ewbank & Zugasti, 2011; Irazoqui & Ausubel, 2010; Sifri *et al.*, 2005) and colonize the intestinal tract. The pathogenicity of *S. marcescens* includes the colonization of the gut, the excretion of hydrolytic enzymes like proteases and chitinase and the resistance to the immune defence of the host (Flyg *et al.*, 1980; Grimont & Grimont, 1978). During *S. marcescens* infection of *C. elegans,* the mediators of the innate immune response are activated to control the harmful effects of the pathogen. This immune response involve the activation of different signalling pathways such as DBL-1/TGF-β , DAF-2/DAF-16 pathways, and p38 MAP kinase pathway, which leads to the release of defence molecules into the intestine (Alper *et al.*, 2007; Mallo *et al.*, 2002). A variety of antimicrobial peptides (AMPs), lectins, lysozymes and saposin-like proteins are released into the gut of the nematode in order to contain and kill the invading pathogen (Ewbank & Zugasti, 2011; Kurz & Ewbank, 2003; Miltsch *et al.*, 2014). Despite the production of molecules that control the proliferation of the intestinal infection, *S. marcescens* is able to colonize the organ and reduce the nematode lifespan.

Additional mechanisms of innate immunity in *C. elegans* have been discovered using *S. marcescens* Db11. Pujol *et al.,* searching for inducible innate immune system analogues to *Drosophila melanogaster*, found that the *C. elegans* toll-like receptor homologue (*tol-1*), contributes to olfactory sensing of serrawettin secreted by *S. marcescens* Db11 leading to aversive behaviour (Pujol *et al.,* 2001). Using cDNA microarray they found that *C. elegans*

nematodes infection by *S. marcescens* Db11 provoked an upregulation of genes encoding for lectins and lysozymes, key proteins in innate immunity (Mallo *et al.*, 2002; Miltsch *et al.*, 2014).

The innate immune response to bacterial colonization of *C. elegans* has been intensively studied with other Gram-negative bacteria such as *P. aeruginosa* and *S. enterica*. While similar pathways are triggered in response to *P. aeruginosa, S. enterica* and *S. marcescens*, it has been reported that each of these bacterium elicit a specific immune response with respect to the number and type of antimicrobial genes activated (Irazoqui *et al.*, 2010a; Powell *et al.*, 2009; Tenor & Aballay, 2008). Furthermore, the resultant pathology of the infected nematodes differs with each bacterium supporting the notion of a pathogen specific response. Thus, the use of C. elegans to further investigate the virulence mechanisms of *S. marcescens* can make significant contributions to the understanding of *S. marcescens* pathogenesis.

The bacterial cell envelope is constantly in contact with the environment and therefore alters composition accordingly in response to environmental changes. In the human host, the components that constitute the bacterial cell envelope are the first targets of the host immune defense mechanisms. The *C. elegans* infection model serves as a suitable and convenient model to further define the interactions between the bacterial cell envelope and the host environment thereby expanding our understanding of *S. marcescens* pathogenesis. *S. marcescens* as a model pathogen has contributes to the understanding of bacterial pathogenicity mechanisms in the nematode (Kurz *et al.*, 2003). The generation of *S. marcescens* attenuated mutants to study virulence factors helps elucidate why pathogens can survive the nematode defense mechanism already described. The generation of mutants deficient in OMP or LPS, represents an alternative to study their function during bacterial pathogenic processes.

1.6. HYPOTHESIS.

This work evaluates the effect of loss of porins on the antimicrobial resistance and on bacterial pathogenicity using the *C. elegans* host model. It is hypothesized that porins in *Enterobacteriaceae* are required for antibiotic resistance and successful host-pathogen interaction. Deletion of porins genes should affect bacterial fitness. Classical porins are important components of cell envelope in Gram-negative bacteria, and reduction in their expression could affect bacterial fitness, and in turn, pathogenicity.

1.7. THESIS OBJECTIVES.

Bacterial adaptations to the environment imply strict regulation of cell envelope permeability. During host infection, bacteria modify outer membrane permeability with the consequence of reduced intake of molecules used for bacterial metabolism and virulence. *S. marcescens*, with modified outer membrane permeability, is predicted to have a reduced virulence and bacterial fitness. To gain an understanding of how that lack of OmpF and/or OmpC affects antibiotic susceptibility profiles and virulence in the *C. elegans* host model system, the following aims have been pursued:

1.- Generate null porin mutant strains by adopting a strategy to create unmarked in-frame gene deletion strains.

2.- Assess antibiotic resistance of *S. marcescens* mutants by profiling the strains against antibiotics currently in clinical use.

3.- Assess bacterial fitness of the porin mutant strains in the *C. elegans* infection model system.

CHAPTER 2. MATERIALS AND METHODS.

2.1. BACTERIAL STRAINS AND PLASMIDS.

Bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2. *S. marcescens* Db11 was used as the wild-type strain to generate all mutants described in this thesis. *S. marcescens* Db11 is a non-pigmented strain that was isolated as a spontaneous streptomycin mutant (Flyg *et al.*, 1980). *S. marcescens* strains 20C2 and 21C4 as well as the plasmid pUFR047::*gfpmut3* were a kind gift from Jonathan Ewbank. *E. coli* DH5α and DH5α λpir were strains used for cloning strategies (Table 2.1). *E. coli* OP50 (Hope, 1999), was used as sustenance for *C. elegans* nematodes (Table 2.1).

2.2. MEDIA AND GROWTH CONDITIONS.

S. marcescens and *E. coli* strains were grown in Luria-Bertani (LB) media (Difco) at 28^{o}C, 30 °C or 37 °C as determined by experimental conditions and supplemented where appropriate with 0.2% L-rhamnose (Rhm) and the antibiotics gentamicin (Gm) 20 µg/ml, streptomycin (Sm) 100 µg/ml, ampicillin (Amp) 100 µg/ml, carbenicillin (Carb) 50 µg/ml, and zeocin (Zeo) 25 or 350 µg/ml.

HEPES buffer minimal salts medium contained HEPES 29.75 g/l, NaCl 4.65 g/l, KCl 1.5 g/l, NH_4Cl 1.08 g/l, Na_2SO_4 0.425 g/l, $MgCl_2 \cdot 6H_2O$ 0.2 g/l, $CaCl_2 \cdot 2H_2O$ 29.5 mg/l, $FeCl_3$ 0.54 mg/l, final pH was 7.2.

Nematode growth medium (NGM) agar was used for general growth and maintenance of *C. elegans* nematodes and as the assay medium for *S. marcescens* killing assays as described by Powell & Ausubel (Powell & Ausubel, 2008). NGM contained Bacto-Peptone (Difco) 2.5 g/l, NaCl 3 g/l, and 2% (w/v) Bacto-Agar (BD), 5 mg/L cholesterol (filter sterilized), $MgSO_4$ 1 mM, $CaCl_2$ 1 mM and KH_2PO_4 25 mM.

Table 2.1. Bacterial strains.

Strain	Genotype/Phenotype	Source
S. marcescens		
Db11	Db10 spontaneous Sm^r mutant	(Flyg *et al.*, 1980)
20C2	*wzm:: Tn5Cm transposon.* Cm^r. O-Ag biosynthesis. ABC-2 transporter specialized in the translocation of LPS O-antigen.	(Kurz *et al*. 2003)
21C4	*shlB::Tn5Cm* transposon inserted. Cm^r. Defective in hemolysin production.	(Kurz *et al*. 2003)
Db11-GFP	Db11 with pUFR047::*gfpmut3* (pUFR-GFP)	(Kurz *et al*. 2003)
ATF001	*ompF::Gm* Db11 mutant, Gm^r	This study
ATC001	*ompC::Gm* Db11 mutant, Gm^r	This study
ATC101	*ompC* deletion Db11unmarked mutant	This study
ATF101	*ompF* deletion Db11 unmarked mutant	This study
AMTFC1	*ompF/ompC*::Gm double deletion mutant, Gm^r	This study
ATFC01	*ompF/ompC* double deletion unmarked mutant	This study
ATFC02	Double mutant complemented with pUFRF242	This study
ATFC03	Double mutant complemented with pUFRC50	This study
ATF242	ATF101, complemented with pUFRF242	This study
ATC50	ATC101, complemented with pUFRC50	This study

AMTE01	*phoE* deletion Db11 Gm^r marked mutant	This study
ATE101	*phoE* deletion Db11 unmarked mutant	This study
ATFCE1	*ompF/ompC/phoE* triple deletion unmarked mutant	This study
ATF101-GFP	SmATF101 with PUFR-GFP	This study
ATC101-GFP	SmATC101 with PUFR-GFP	This study
ATFC01-GFP	SmATFC01 with PUFR-GFP	This study
ATE101-GFP	SmATE101 with PUFR-GFP	This study
ATFCE1-GFP	SmATFCE1 with PUFR-GFP	This study
ATD001	*ampD::Gm* marked mutant, Gm^r	This study
ATD101	Δ*ampD* unmarked mutant	This study
ATFD01	Δ*ompF*Δ*ampD* unmarked mutant	This study
ATFCD1	Δ*ompF*Δ*ompC*Δ*ampD* unmarked mutant	This study
Escherichia coli		
DH5 α	*supE44 lacU169*(φ80d*lacZ*ΔM15) *hsdR17 recA1endA1 gyrA96 thi-1 relA*	Invitrogen
DH5 α λpir	*supE44, ΔlcU169 (Φ80lacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen*	Lab strain

Table 2.2. Plasmids and phage.

Plasmid	Genotype/Phenotype	Source
pPS856	Ap^R, Gm^R; source of FRT-Gm^R-FRT	(Hoang *et al.*, 1998)
pKAS32	Positive selection vector for allelic exchange	(Skorupski & Taylor, 1996)
pKASF1	pKAS32 derivative containing *ompF::Gm* fragment	This study
pKASC1	pKAS32 derivative containing *ompC::Gm* fragment	This study
pKASE1	pKAS32 derivative containing *phoE ::Gm* fragment	This study
pKASD1	pKAS32 derivative containing *ampD::Gm* fragment	This study
pFLPe2	Zeo^r; contains *rhaS-rhaR-PrhaBAD-FLPe* from pPS2208, *ble-ori*1600-*rep*(Ts*Bt*)	(Choi *et al.*, 2008)
pUFR047	IncW, Gm^R, Ap^R, Mob^+, *mob*(P), *lacZα*$^+$, Par^+	(De Feyter *et al.*, 1993)
pUFR-GFP	pUFR047::*gfpmut3* (EcoRI/PstI)	(Kurz *et al.*, 2003)
pUFRF242	pUFR047 derivative vector containing OmpF from *S. marcescens* Db11	This study
pUFRC50	pUFR047 derivative vector containing *ompC* from *S. marcescens* Db11	This study
Phage		
Φ IF3	*S. marcescens* generalized transducing phage	(Petty *et al.*, 2006)

The pH of the medium was adjusted to pH 6 prior to autoclaving and was cooled to 55^{o}C prior to pouring in 60 x 15 mm petri plates (Sarstedt). Plates were dried in the biosafety cabinet hood for 10 minutes and spotted with 100 µl aliquots of overnight cultures of *E. coli* OP50.

2.3. CHROMOSOMAL DNA ISOLATION.

Bacteria were grown overnight in 3 ml LB medium and a compact pellet was obtained after microcentrifugation in 1.5 ml microcentrifuge tube at room temperature. The pellet was resuspended in 440 µl Tris-EDTA Buffer (TE), (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). To this, 50 µl of 20 mg/ml of Proteinase K (NEB) was added followed by 10 µl of 10% SDS, and mixed well. After 2 hours incubation at 37 °C, 50 µl of 10 M ammonium acetate was added followed by the addition of 550 µl of phenol: chloroform isoamyl alcohol (25:24:1) (Sigma) and vortexed to emulsify the solution. After centrifugation for 30 min at room temperature at maximum speed, the aqueous phase was collected and an equal volume of phenol:chloroform:isoamyl alcohol was added, vortexed and centrifuged to separate the solvent layers. This step was repeated three times after which the aqueous phase was collected in a new microcentrifuge tube and mixed with two volumes of ice-cold 100% ethanol and centrifuged at maximum speed for 30 min at 4 °C. The supernatant was removed, the pellet washed with 70% ethanol and then air dried. The pellet was dissolved in 100 µl TE buffer containing 1 µl of RNAseA (1 mg/ml) to a final concentration of 0.01 mg/ml of RNAse.

2.4. PREPARATION OF COMPETENT CELLS AND TRANSFORMATION.

2.4.1. Chemical Competent cell preparation using rubidium chloride.

E. coli DH5α competent cells were prepared using rubidium chloride (RbCl) (Hanahan, 1983). A volume of 0.5 ml of overnight culture was used to inoculate 500 ml of LB and grown at 37 °C, shaking at 150 rpm to an OD_{600} of 0.45 – 0.5. Cultures were transferred to 250 ml

centrifuge bottles in sterile conditions and kept on ice for 10 min and centrifuged at 3000 rpm (1500 x g) for 20 min at 4 °C. After centrifugation, the supernatant was discarded, and the pellet was re-suspended in 200 ml of ice-cold 100 mM RbCl and centrifuged at 3000 rpm at 4 °C for 15 min in a centrifuge (Thermo Scientific, Sorvall RC6+ centrifuge). The supernatant was discarded and the pellet was re-suspended in 20 ml of ice-cold 10 mM RbCl solution. Aliquots of 200 µl were prepared in 1.5 ml microcentrifuge tubes, frozen quickly and stored at -70 °C. For transformation, a microcentrifuge tube containing a 200 µl aliquot of competent cells was thawed on ice. Generally 30 to 50 ng of plasmid DNA was added to100 µl of competent cells. The mixture was kept on ice for 1 hour, heat-shocked at 45 °C for 90 seconds, after which 0.5 ml of LB broth was added. This was incubated for 1 hour on a shaker at 150 rpm at 37 ^{o}C. The mixture was centrifuged and the pellet resuspended in 0.1 ml of LB broth was spread onto LB agar plates containing the appropriate antibiotic for selection.

2.4.2. Preparing of *S. marcescens* Db11 electrocompetent cells.

S. marcescens Db11 electrocompetent cells were prepared as follows. A volume of 0.5 ml was used to inoculate 500 ml of LB and grown at 37 °C, shaking at 150 rpm to an OD_{600} of 0.450 – 0.500. Cultures were transferred to 250 ml centrifuge bottles in sterile condition and kept on ice for 10 min and centrifuged at 3000 rpm (1500 x g) for 20 min at 4 °C. After centrifugation, the supernatant was discarded and 250 ml of ice-cold sterile water was added and the cells were gently re-suspended. The cell suspension was centrifuged at 3000 rpm at 4 °C for 20 min. The supernatant was discarded and the pellet was once again resuspended in 250 ml of sterile ice-cold water. The procedure above was repeated two more times. Following the final centrifugation step, the pellet was finally resuspended in 10 ml of ice-cold water containing 10% glycerol (v/v). Aliquots of 100 µl were prepared in 1.5 ml microcentrifuge tubes and stored at -

70 °C. For electroporation, each microcentrifugation tube containing electrocompetent cells was thawed on ice and 30 ng to 50 ng of plasmid DNA sample was added and then mixed gently. The mixture was transferred to a 0.1 cm electrode gap electroporation cuvette and a pulse of 2.5 kV was applied in a Bio-Rad microPulser. The mixture was transferred to 1 ml LB medium in a culture tube and incubated until 2 hours at 37 °C. The cells were then harvested by centrifugation. A volume of 900 µl of the supernatant was discarded and the cell pellet was resuspended in the residual 100 µl. The entire mixture was spread on LB agar containing the appropriate antibiotic. Plates were incubated at 37 °C until colonies appeared.

2.5. TRIPARENTAL MATING CONJUGATION.

Triparental mating of *S. marcescens* strains was used to generate Gm^r marked isogenic mutants and for the introduction of pUFR-GFP vector. Briefly, 3 ml of LB liquid medium culture of the donor, recipient and helper strains were incubated at 28 °C separately, overnight. The next day each culture was centrifuged, washed three times with LB media without antibiotic and then re-suspended in 1 ml of liquid LB. Based on OD_{600} values, equal amounts of helper (*E. coli* MT616), donor (*E. coli* DH5α λpir containing the vector) and recipient (*S. marcescens* Db11 wild type or mutant strains) were added to a sterile microcentrifuge tube. Controls consisted of control 1 (helper + donor), control 2 (helper + recipient), control 3 (donor + recipient). The mixture was centrifuged and the pellet was resuspended in 200 µl of LB. A volume of 100 µl was spotted on the surface of an LB agar plate and incubated overnight at 28 °C. The following day each spot was transferred into 1 ml liquid LB media and a volume of 100 µl was spread on LB agar selective medium to select for transformants.

2.6. TRANSDUCTION.

The transducing phage ϕIF3 was a kind gift from George Salmond (Department of Biochemistry, University of Cambridge), and was used to transduce markers between *S. marcescens* strains and to prepare the cell lysates (Petty *et al.*, 2006). Briefly 100 µl of a high-titre phase lysate, propagated in the donor strain, was added to 3 ml of an overnight culture of recipient strain. The mixture was incubated at room temperature for 30 min without shaking, and then incubated on a tube roller at 30 °C for 20 min. The mixture was centrifuged at 2220 x g for 10 min at 4 °C. The supernatant was discarded and the pellet suspended in 300 µl of LB and 150 µl of this was spread onto each of two LB agar plates containing gentamicin (20 µg/ml). Donor and recipient controls were included to control for spontaneous gentamicin resistance or lysate contamination. Plates were incubated at 30 °C for 24 hours. Transductants appeared as Gm resistant colonies on LB plates containing 20 µg/ml gentamicin following a 24 hour incubation at 30 °C. Transductants were purified by restreaking isolated colonies twice onto fresh plates to eliminate any phage carry-over. Transductants were generally confirmed by PCR and DNA sequencing. To generate double mutants by transduction, phage ΦIF3 lysates were used to introduce new mutations into single and double mutants *S. marcescens* strains.

2.7. CLONING OF PCR PRODUCTS.

Primers were designed containing restriction enzymes sites in the 5' end, plus six nucleotides as mismatched sequences. After amplification of the target DNA sequence, the PCR product was purified using PCR purification kit (Qiagen). All restriction digestions with enzymes were followed by a clean-up step using QIAquick Gel extraction kit. The purified PCR products were cloned in the specific cloning vector that was digested with the same set of restriction enzymes (New England Biolabs (NEB)). For ligations, 5 -10 µg of purified DNA

containing a ratio of 1:3 (vector:insert) were treated with 20 units of T4 DNA ligase (NEB) with 1X T4 DNA ligase reaction b b uffer containing ATP for at least 12 hours at 16 °C. A volume of 10 µl of the mixture diluted 1/5 with sterile H_2O was used for transformation.

2.8. KNOCKOUT STRATEGY.

2.8.1. Construction of *S. marcescens* unmarked deletion mutants.

Generation of *S. marcescens ompF* and *ompC* deletion mutants were in accordance to the methodology outlined in Choi *et al*. (Choi *et al*., 2005; Choi *et al*., 2008) with a few modifications. Briefly, two ~1000 bp regions (5' upstream and 3' downstream), flanking the target gene (*ompF* or *ompC*), were PCR amplified generating the amplicons LF01 and RF01 for *ompF*, and LC01 and RC01 amplicons for *ompC.* The 1,053 bp gentamicin resistant cassette (*aacC1*) fragment flanked by FRT sites (FLP recombinase recognition targets) was PCR-amplified from pPS856 with primers (Table 2.3), designed to include a portion of the 5' upstream and 3' downstream regions, respectively, of the target gene to allow eventual pairing between FRT- Gm^r - FRT DNA, 5' (upstream) and 3' (downstream) fragments. To this end, GMF and GMC amplicons were generated by a two-step PCR as outlined in Figure 3.4. In the first step, 25 ng of LF01, 25 ng GMF and 25 ng of RF01 were added to a 50 µl reaction volume containing Phusion polymerase, and placed in cycling conditions of 98 °C for 30 s, 10 cycles of [98 °C for 5 s, 55 °C for 10 s, 72 °C for 30 s] with an extension at 72°C for 30 s. The reaction was paused, 20 pM each of PFompF and PRompF primers were added, and the second step was performed with 25 cycles of [98 °C for 10 s, 55 °C for 10 s, and 72 °C for 2 min 30 s] with a final extension of 72 °C for 5 min. The resultant 3,068 bp amplicon was cloned into KpnI and SacI sites on pKAS32 creating pKASF1, electroporated into *E. coli* DH5α λ*pir* and plated on LB Amp (100 µg/ml), Gm (20 µg/ml). Similarly, to create pKASC1, amplicons LC01, RC01 and

GMF were paired to create a 3,301 bp fragment for cloning into pKAS32. To generate the isogenic marked *ompF::Gm* (ATF001) or *ompC::Gm* (ATC001) mutant strains, pKASF1 or pKASC1 was purified by Qiagen mini-prep and electroporated into Db11, plated on LB Carb 50 µg/ml, Gm 20 µg/ml and incubated at 30°C to select for the first cross-over. pKAS32 *rpsL* encodes wild-type ribosomal protein S12 conferring a streptomycin-sensitive phenotype upon streptomycin-resistant strains allowing for positive selection with streptomycin to detect loss of the vector (Skorupski & Taylor 1996). Thus, to select for second cross-over event, transformant colonies were patched onto LB Sm and incubated at 30 °C to recover colonies that reverted to the streptomycin resistant phenotype. Isogenic ATF001 (*ompF::Gm*) and ATC001 (*ompC*::*Gm*) marked mutant strains were confirmed by PCR and sequencing of the PCR product.

To generate unmarked ATF101 (Δ*ompF*) and ATC101 (Δ*ompC*) mutant strains, the gentamicin cassette was removed via the Flp-*FRT* system from *ompF::Gm* and *ompC*::*Gm* strains, respectively, as per the protocol outlined by Choi *et al*. with a few modifications (Choi *et al*., 2008). Introduction of pFLPe2 into *ompF::Gm* or *ompC*::*Gm* strains and resultant induction of flipase expression by rhamnose allowed excision of the *FRT*-Gm-*FRT* cassette from the mutant strains leaving behind a 84 bp FRT scar but the upstream and downstream regions for both genes remained intact. Briefly, 100 µl aliquots were separately plated on LB Zeo agar, incubated at 30 °C for 24 h after which Zeo^r transformants were streaked for single colonies onto a LB Zeo/Rhm agar and incubated for 24 h at 30°C. To confirm excision of the *FRT*-Gm-*FRT*, colonies were patched onto LB Gm 20 µg/ml and LB Zeo 350 µg/ml agar, and incubated for 24 h at 30 °C. Gm^s Zeo^r colonies were individually streaked onto a fresh LB agar for single colonies, and incubated at 42 °C for 24 h for rapid curing of pFLPe2. Single colonies were tested at 30 °C for susceptibility to Gm and Zeo by patching them onto LB Gm, LB Zeo, and LB Sm

agar. PCR and DNA sequencing using specific primers (Table 2.4), confirmed absence of the Flp marker in both unmarked Δ*ompF* and Δ*ompC* deletion mutant strains.

2.9. PREPARATION OF RNA.

RNA was purified from mid log-phase cultures of *S. marcescens* grown in LB liquid media at 37 °C. RNAeasy Plus mini kit (Qiagen) was used to purify total RNA. Briefly, 40 µl of an overnight culture was used to inoculate 4 ml of LB media and incubated until an OD_{600} nm of 0.3 was reached. A volume of 500 µl of cell suspension was mixed with 1 mL RNAprotect (Qiagen) to inhibit RNA degradation. The suspension was then centrifuged at 5000 x g for 10 min at room temperature and the pellets were stored at −80 °C until use for RNA isolation. Extraction of total RNA was performed by RNeasy mini kit (Qiagen) protocol. Samples were treated with TURBO DNA-free™ DNase Treatment and Removal Reagents (Ambion) to eliminate residual DNA contamination in total RNA samples. RNA integrity was verified by agarose gel and PCR was performed to confirm elimination of DNA contamination in each purified RNA sample, before cDNA synthesis.

Table 2.3. Oligonucleotides used in this study to PCR amplification of *S. marcescens* Db11 genes.

Primer	Sequence (5' → 3')	Product
Gm-F	CGAATTAGCTTCAAAAGCGCTCTGA	Gentamycin cassette flanked by FRT inside pPS856 vector
Gm-R	CGAATTGGGGATCTTGAAGTTCCT	
PFompF	GCGATAGGTACCGCATGCTGAAGTATGTGTTCCA	Primers for 891 nt sequence upstream of *ompF* in Db11
RBompF01	CATTGGTGTTATTCGGACACC	
PRompF	GCGATAGAGCTCGAGTAGGAGCTGGCGACAATCA	Primers for 1069 nt sequence downstream of *ompF* in S m Db11
LEompF01	ATCAACCTGCTGGATGACAA	
PF-FRTGmFRT (*ompF*)	AGAATTATTGGCGGCAGTGGCAAAGGTGTCCGAATAACA CCAATGAGGGTAATATGTGTAGGCTGGAGCTGCTTC	1104 DNA sequence of Gm resistance cassette flanked by FRT sites plus target gene (*ompF*) complementary region
PR-FRTGmFRT (*ompF*)	CAGGGCCGAAGCCCTGTTTTTTAACTTAGCCGACGGTAAA CCGCCGTTAAGCGAGACAACCATATGAATATCCTCCTTAG TTCC	
PFompC left	GCGATAGGTACCCCTGGGAGAAAATGATGACC	Primers for 1161 nt sequence upstream of *ompC* in Sm Db11
PR(Left)ompC	GCACCTCAGTGTGAACGTC	
PRompC right	GCGATAGAGCTCATAGCTTGGACAATATACTCCTTTTGC	Primers for 1163 nt sequence

PF(Right)ompC	CGTTATTATCCTCGTTAATTATGTCG	downstream of *ompC* in S m Db11
PF-FRTGmFRT (ompC)	CGGCTTACAGGCAAAAAAAACGCGGCCCGAAGGCCGCGC GATGACGTTCACACTGAGGTGCTGTGTAGGCTGGAGCTGC TTC	1104 DNA sequence of Gm resistance cassette flanked by FRT sites plus target gene (*ompC*) complementary region
PR-FRTGmFRT (ompC)	TGCTCGAAAAGGGCAGTGGCCGTAGCTCGACATAATTAA CGAGGATAATAACGCATATGAATATCCTCCTTAGTTCC	
PFphoE Flk(KpnI)	GCGATAGGTACCAACGGGTAGAGTATTCCGAAAGTG	Primers for 1152 nt sequence upstream of *phoE* in Sm Db11
PRphoE (Left)	TTCATTACCTTCATCCTGTGTCG	
PRphoE Flk(SacI)	GCGATAGAGCTCTTGGCAGCAACGATCTACAG	Primers for 885 nt sequence downstream of *phoE* in S m Db11
PFphoE (Right)	ATTAGAACCCTGCCCGTCAC	
PFphoE KO(Gm)	AGGGCTTACGCCCTGCCTGATTACCACTCCCGACACAGGA TGAAGGTAATGAAATTGCCGCTGTGTAGGCTGGAGCTGCT TC	1109 DNA sequence of Gm resistance cassette flanked by FRT sites plus target gene (*phoE*) complementary region
PRphoE KO(Gm)	ATAAATTGGGCGTGACGGGCAGGGTTCTAATTATAATATC TAGGAAAATAAATGCATATGAATATCCTCCTTAGTTCC	
PFampD	GCGATAGGTACCTCCATAAGGGTGTGGAAAACAAC	Primers for 1142 nt sequence

Flk(KpnI)		upstream of *ampD* in Sm Db11
PR(N)ampD Left	TCTGCGGTGGTACTTATTCCTG	
PR ampD Flk SacI	GCGATAGAGCTCTGCTACCAGGACGTGATGC	Primers for 1086 nt sequence downstream of *ampD* in Sm Db11
PF ampD Right	TGTTTACGCTGTTGCTGGTTTT	
PFampD(N)KO (Gm)	GATCGTAGGGGAACATGCTACTCTGTTGCAGGAATAAGT ACCACCGCAGAGAGGTGTCTGTGTGTAGGCTGGAGCTGCT TC	1111 DNA sequence of Gm resistance cassette flanked by FRT sites plus target gene (*ampD*) complementary region
PRampD KO(Gm)	GTTCCCCCAGCTTGAACAGGCGCTCCCAGGCCAAAACCA GCAACAGCGTAAACAGCGTCATCATATGAATATCCTCCTT AGTTCC	
PFompF1 EcoRI	GCGATAGAATTCTATTGACGCACGTCACAAAG	Gene complementation. The 1459 nt includes *ompF* and its native promoter region (~225 nt) with binding sites for OmpR binding
PRompF SphI	GCGATAGCATGCGGGGAAATCTGCAGGATAAA	
PFompC1 EcoRI	GCGATAGAATTCATTTTGTTTGTCTGTAATGTTTTGTG	Gene complementation. The 1362 nt includes the native *ompC*

PRompC SphI	GCGATAGCATGCGACGTTCACACTGAGGTGCTT	promoter region (~212 nt) including binding sites for OmpR

Table 2.4. Primers for DNA sequencing to evaluate *S. marcescens* Db11 knockout mutants.

Name	Primers sequence (5`-3`)	Amplicon
PFompF(Seq)01	AACCTGATTGGGACATTGGT	Flanking primers for *ompF* (Db11)
PRompF(Seq)01	TTTATCCTGCAGATTTCCCC	
PFompF(Seq)02	TGGCGACACCTACACCTAC	Internal primers for *ompF* (Db11)
PRompF(seq)02	GCAGAGCGAAGTTCAGACC	
PFompC(Seq)01	ATAAACCCGGTAGGATAGTGC	Flanking primers for *ompC* (Db11)
PRompC(Seq)01	TGGCTCAATTGTATGGTTGG	
PFompC(Seq)02	TCATGGACATACCGTAGCCTTC	Internal primers for *ompC* (Db11)
PRompC(seq)02	GCGACCTACCGTAACAACG	
PFphoE(Seq)01	CAAAGGCTGAGTTCCCGATA	Flanking primers for *phoE*
PRphoE(Seq)01	AGAGTGCGATGCACCATTTT	
PFphoE(Seq)02	AATCACGGTTACGGTAGG	Internal primers for *phoE*
PRphoE(seq)02	TACGGTGTGGTGTATGAC	
PFampD(Seq)01	TCAACTTCACCTCCCAGGTC	Flanking primers for *ampD*
PRampD(Seq)01	AAAGAATAACCCGTGCGACA	
PFampD(Seq)02	CGGGCGTTTCTCTGTATC	Internal primers for *ampD*
PRampD(seq)02	TCGGTGTAGGCTAACTGG	

2.10. QUANTITATIVE REAL-TIME PCR (qRT-PCR).

Transcript levels of porin genes *ompF, ompC, ompA, ompX, lamB* and *phoE* were determined by qRT-PCR. Primers were designed using Beacon Designer™ 7software. Synthesis of cDNA was accomplished using a SuperScript VILO™ cDNA Synthesis Kit (Invitrogen) using total RNA obtained as described above. qRT-PCR was performed in MicroAmp Fast optical 96-well plate using SYBR Green PCR master mix in an Applied Biosystems 7500 fast Real-Time PCR System. 12 µM of each primer was used per reaction (Table 2.5). Primer efficiency was determined for each primer set used. To accomplish this, 5-fold serial dilutions of *S. marcescens* Db11 cDNA was performed in DEPC-treated sterile water, beginning with 1/30 and carried out until 1/18750 was reached. Diluted cDNA was used as template in qRT-PCR reactions as described above. Cycle threshold (C_t) values were plotted and the slope utilized to calculate efficiency with the following formula: $E = (5^{(-1/slope)} - 1)\times 100$. Primer sequences for housekeeping and target genes used in this study are shown in (Table 2.4).

2.11. DNA SEQUENCING.

DNA samples to be sequenced were amplified with specific primers (Table 2.5) and the corresponding band was purified using QIAquick Gel Extraction Kit (Qiagen). A concentration of 130 ng of purified amplicon was used as template and mixed with the appropritate sequencing primers (Table 2.5) in a 0.2 ml strip PCR tube (VWR). Specific primer was added at a concentration of ~50 ng in 0.7 µl volume, for a total volume of 7.7 µl. DNA sequencing was carried out by the Centre for Applied Genomics (TCAG), at the Hospital for Sick Children (SickKids), Toronto, Ontario, Canada.

Table 2.5. Primers used for qRT-PCR.

Name	Primers sequence (5`-3`)	Gene	Amplicon Length
Sm16SHK(L)1	CACGCTGTAAACGATGTC	*16S(RNA)*	193 nt
Sm16SHK(R)1	GATGTCAAGAGTAGGTAAGG		
Sm gyrA(L)1	GCCGATTTCTTGTATGGTTTATTC	*gyrA*	132 nt
Sm gyrA(R)1	CTATGCGATGTCCGTTATTGTC		
Sm gyrB(L)1	CATTACGAAGGCGGCATC	*gyrB*	169 nt
Sm gyrB(R)1	TGTTGTTGGTGAAGCAGTAG		
Sm rplU(L)1	AACACCGAGTAAGCGAAGG	*rplU*	173 nt
Sm rplU(R)1	ACGACCGTGAGCAACAAC		
Sm rpoB(L)1	TACGCACAGACTAACGAG	*rpoB*	103 nt
Sm rpoB(R)1	CTTCTTCAATAGCAGACAGG		
Sm LamB(L)1	CTCTTGTCGCCTTCTTTC	*lamB*	197 nt
Sm LamB(R)1	GGTGTTCTCTCTACTCAGG		
Sm ompA(L)1	TGGTCAGTGCGTATTCAAC	*ompA*	129 nt
Sm ompA(R)1	CTCGTCTGGGTGGTATGG		
Sm ompC(L)1	TGGACATACCGTAGCCTTC	*ompC*	145 nt
Sm ompC(R) 1	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		

2.12. PREPARATION OF CELL ENVELOPE FOR SDS-PAGE.

An isolated colony was inoculated into a 3 ml of LB liquid media and incubated overnight at 37 °C. A volume of 1.5 ml was centrifuged to remove the supernatant. The pellet was washed three times with TNG buffer (0.15 M NaCl; 0.1 M Tris-HCl, pH 7.5; 10 % Glycerol) and resuspended in 150 µl of TNG. The cell suspension was centrifuged at 13 000 x g for 10 min prior to sonication. Sonication was carried out on ice where 3 intervals of 3 pulses were applied to the sample (Branson Sonifier 450). Between each interval a centrifugation step at 10 000 x g for 20 min at controlled temperature of 4 °C, was performed. After sonication, the supernatant (lysate) was discarded and pellet dissolved in 150 µl of TNG buffer and stored at – 20 °C, until use. For SDS-PAGE, 25 µl of the pellet suspension was diluted with 25 µl of 2X SDS-PAGE loading buffer .

2.13. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method described by Lamneli *et al.* (1970). A precast 10% polyacrylamide gel (Mini-PROTEAN TGX, BIORAD) was used. Mini-PROTEAN TGX gel retains Laemmli-like separation characteristics and uses the same running and sample buffers used for Laemmli system SDS-PAGE. Protein samples were solubilized (1:1) with (2X) loading buffer containing 24 mM Tris-Cl, pH 6.8, 0.8% SDS, 4% glycerol, 0.0125% bromophenol blue, and 2% β-mercaptoethanol. Samples were heated at 95 °C for 5 min prior to loading and a volume of 10 µl of sample was loaded into each well. The precision Plus Protein Dual Color standards (BIO-RAD) was used as a molecular weight ladder. This protein ladder contains a mixture of ten recombinant proteins (10–250 kDa), including eight blue-stained bands and two pink reference bands (25 and 75 kDa). Proteins bands were stained for 30 min using Coomassie Brillant Blue R-

250 staining solution (BIO-RAD). Destaining of gels was performed in two steps using destaining solution 1 (50% ethanol, 10% acetic acid) for 1h and destaining solution 2 (5% ethanol, 7% acetic acid) overnight.

2.14. MICROSCOPY.

To immobilize live bacterial cells, a layer of molten 0.8 % agarose was placed on microscope slides and left to dry overnight at room temperature, resulting in a thin layer of agarose. A 10 µl aliquot of overnight bacterial culture diluted (1:10) in LB was placed on the agarose layer and then covered with a coverslip. Bacteria were imaged using a Zeiss AxioObserver Z1 inverted microscope, equipped with an AxioCam HRm microscope camera. Images were optimized using AxioVision Rel 4.8 software.

2.15. EVALUATION OF THE SENSITIVITY OF *S. marcescens* MUTANTS TO SODIUM DEOXYCHOLATE.

Bacterial cultures grown overnight in LB liquid medium were adjusted to OD_{600} of 1.0 then serially diluted in LB. 10 µl was spotted on LB agar plates containing 10 mM sodium deoxycholate. Plates were then incubated overnight at 28 °C. Sensitivity was quantitated by scoring at which dilutions visible cell growth was observed.

2.16. MOTILITY ASSAY.

Swimming and swarming motility was evaluated by inoculating stationary-phase cells onto the centers of 9-cm-diameter LB agar swimming plates (0.3% agar) or swarming plates (0.5 % agar). Plates were incubated at 25 and 28 °C, and the diameters of halos due to bacterial migration were measured 16 hours post-inoculation (Soo *et al.* 2014).

2.17. ANTIBIOTIC SUSCEPTIBILITY ASSAY.

Antimicrobial susceptibility testing was carried out by the VITEK 2 System (Version: 05.04.) and plates susceptibility system for ESB1F were used. MICs were determined according to the Clinical and Laboratory Standards Institute (CLSI M7-A7 2007). MIC was performed in collaboration with Dr. Mulvey laboratory at National Microbiology Laboratory, Winnipeg, MB.

2.18. β-LACTAMASE ASSAY.

This method is based on the rate of breakdown of nitrocefin when serving as the substrate for AmpC β-lactamase (O'Callaghan *et al.*, 1972). Nitrocefin is a cephalosporin with chromogenic properties that is used to detect β-lactamase enzyme activity. An ultraviolet absorption shift from yellow with intact nitocefin (λmax = 390 nm at pH 7.0) to red (λmax = 486 nm at pH 7.0) when the nitrocefin is hydrolyzed and rendered inactive by β-lactamase. Cell lysates were prepared as follows. 40 µl of an overnight culture were inoculated into 4 ml of LB liquid media. Tubes were incubated in a rotor at 37°C for 3 hours until an OD_{600nm} of approximately 0.5 was reached. Induction of β-lactamase production was performed by the addition of 2 µl of cefoxitin (20 mg/ml) to a final concentration of 10 µg/ml, and incubated for 1 hour at 37 °C. Control strains were prepared without the addition of cefoxitin. All samples were transferred to sterile 1.5 ml microcentrifuge tubes and centrifuged 10 min at 10 000 x g. The pellet was washed with 200 µl of sterile PBS pH 7.2 and resuspended in a final volume of 150 µl of PBS, pH 7.2. The cell suspension was centrifuged at 10 000 x g 10 min before sonication. Sonication was carried out on ice and consisted of 3 intervals of 3 pulses using a Branson Sonifier 450. Centrifugation step in between each sonication was, 20 min at ≥ 10 000 x g at 4 °C. After the final sonication, supernatants (lysates) were transferred to new sterile microcentrifuge tubes and stored at – 20 °C, until use. The β-lactamase activity was assayed

spectrophotometrically using 100 μM nitrocefin in PBS pH 7.2 as substrate. Briefly, 90 μl of cell lysate in PBS pH 7.4, was added to a quartz cuvette and used to the background to zero OD $_{486nm}$. A volume of 10 μl of 1 mM nitrocefin was added to a final concentration of 100 μM and the reading at OD486 nm were taken every 1 min for a period of 6 min. All determinations were carried out in triplicate. One unit of β lactamase was defined as the amount needed to form 1.0 μmol of product per minute.

2.19. *C. elegans* SURVIVAL KILLING ASSAY.

2.19.1. Nematode growth and maintenance.

Wild type N2 Bristol nematodes were maintained as described by Powell & Ausubel (2008). 50 μl of a saturated culture of *E. coli* OP50 grown in LB broth at 37 °C overnight was spotted and spread onto a 6-cm NGM plate to create a bacterial lawn. Plates containing OP50 were incubated overnight at 37 °C and kept at room temperature until use. Nematodes were maintained by transferring wild-type (N2) gravid hermaphrodite nematodes to a fresh OP50 to allow them to lay eggs overnight. 3-5 gravid (egg-laying) nematodes were seeded onto NGM seeded with *E. coli* OP50. The nematodes would lay between 30 -300 eggs overnight. Gravid nematodes were then removed from the plate. Nematode populations were maintained on NGM plates at 16 °C, until they reached the L4 stage, approximately 4 days after hatching.

2.19.2. Nematode synchronization.

Propagation plates with gravid nematodes with sufficient eggs were used. A 5 ml volume of M9 medium was poured onto each plate and then gently swirled to dislodge eggs from the agar. A glass Pasteur pipette was then used to transfer all eggs and gravid nematodes to a 15 ml capped conical Falcon tube. The suspension was centrifuged at a low speed of 1000 rpm for 1

minute to pellet the nematodes, and the supernatant was aspirated avoiding disruption of the nematode pellet. A 15 ml volume of the 20% alkaline hypochlorite solution (8.25 ml of ddH_2O, 3.75 ml of 1 M NaOH, 3.0 ml of bleach with 6% sodium hypochlorite active agent) was added to the tube containing the nematode pellet. The tube was inverted gently until the gravid nematodes were dissolved (approximately 5 minutes). The egg pellet was washed twice with M9 buffer (22mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$). A final centrifugation step and the addition of a fresh sterile M9 buffer was performed and the tube was kept at room temperature, gently mixed in a rotator overnight to allow the eggs to hatch into L1 nematodes. Since the medium did not contain food, nematode growth would halt at L1 stage. The following day, the L1 nematodes were centrifuged at 1000 rpm for 1 minute. The M9 solution was aspirated off leaving approximately 1 ml of solution remaining. The L1 nematode stage contained in a pellet was remixed into M9 buffer using a glass pipette. One drop of solution was placed onto a new NGM plate, and the nematodes were allowed to grow until L4 stage at 16 °C.

2.19.3. Preparation of *S. marcescens*-killing plates.

S. marcescens bacterial lawns were prepared as described by Powell and Ausubel (2008). Briefly, 3 ml of LB broth was inoculated with a single colony and grown with shaking at 37°C for 8 h. 50 µl of this was spotted onto a 6-cm NGM plate and spread such that, the culture did not touch the edges of plate. Plates were incubated at 37°C overnight.

2.19.4. Survival killing assay in *C. elegans.*

Following an overnight incubation at 37^{o}C, the plates containing *S. marcescens* lawns were cooled to room temperature and seeded with 30 L4 stage hermaphrodite nematodes. Plates were then incubated at 25 °C and the nematodes were scored (live vs dead) every 24 h.

Nematodes were transferred to fresh *S. marcescens* assay plates during the first three days to separate subjects from progeny, and then transferred every other day. Dead nematodes were removed when plates were scored. Nematodes that crawled off the agar plate surface were censored from data analysis. Kaplan-Meier survival curves were performed and the log-rank (Mantel-Cox) statistic test was used to determine significance (Bewick *et al.*, 2004). All killing assays were performed in triplicate.

2.19.5. Quantification of bacteria within the *C. elegans* digestive tract.

Bacterial colonization of the *C. elegans* intestine was quantified in accordance to Moy *et al*. (2006). L4 nematodes were seeded onto prepared *S. marcescens* assay plates and allowed to feed for up to 72 hours. At 24, 48 and 72 hours post-seeding, 30 *C. elegans* nematodes were selected and bacteria attached to the nematode surface were removed by washing the nematodes once with M9 medium on a NGM 6 cm plate. Nematodes were transferred with a nematode-pick to a new 3.5 cm NGM agar plate containing 1 ml of M9 medium plus 50 mM of NaN_3. The NaN_3 solution was used to prevent expulsion of *S. marcescens* from the *C. elegans* gut. Then 30 nematodes were transferred manually to a sterile 1.5-ml microcentrifuge tube containing 1 ml of M9 liquid medium 50 mM NaN_3. Nematodes were washed three times to eliminate bacteria from the surface, and centrifuged at 500 rpm. A volume of 50 µl was serially diluted in M9 liquid media without NaN_3 and transferred to a LBA (strep 100 µg/ml) to determine bacterial count remaining outside the nematode as expressed as CFU/ml. Tubes were centrifuged at 500 rpm, liquid was carefully removed and replaced by 200 µl of M9 liquid medium without NaN_3. An amount of 0.4 gram of sterile silicon carbide (1.0 mm Ø, BioSpec) was added to the tube. The tube containing the nematodes and silicon carbide was vortexed for 20 seconds at max speed to accomplish nematode body disruption leaving bacterial cells intact. A volume of 50 µl of

supernatant was serial diluted with M9 liquid medium in a 96 well plate (Flat bottom tissue culture plate, SARSTEDT). A volume of 100 µl of each selected dilution was plated in an 100 µg/ml Strep-LBA plate, incubated overnight at 30 °C and CFU/ml was determined. Significant plate count values were used to determine the CFU/nematode taking into consideration the number of nematodes present in 0.2 ml of M9 and the background bacterial CFU/ml before adding the silicon carbide particles.

2.19.6. Bacterial lawn avoidance assay.

On NGM plates (100 mm x 15 mm Petri Dish, Fisher), bacterial lawns of approximately 7 mm were prepared by adding 10 µL of bacterial cultures grown in LB liquid for 8 hours. Bacterial lawns were distributed 3 cm from the center of the plate and 2.5 cm equidistant from each other. Plates were incubated overnight at 37°C. L4 stage synchronized nematodes were collected from three NGM plates and washed three times with M9 buffer followed by centrifugation at 500 rpm. A volume of 1 ml of M9 buffer was added to the pellet and the nematodes were left without bacterial food for 30 min before centrifugation for 1 min at 500 rpm. A volume of 900 µl of buffer was removed leaving a highly concentrated suspension of nematodes in 100 µl, and 5 µl of the nematode suspension containing ~150 to 250 nematodes and was spotted in the center of the plate. The plates were incubated at 25 °C, and at 1 and 4 h time points, the nematodes in each bacterial lawn were counted. Three technical replicates were included. The procedure was repeated three times to obtain biological replicates. *E. coli* OP50 was used as a control.

2.19.7. Microscopy to study bacterial colonization of *C. elegans.*

After initial seeding of L4 nematodes on prepared *S. marcescens* assay plates, 10-20 nematodes were collected on a daily basis for five days. Collected nematodes were washed in 1 ml of M9 buffer on a NGM plate. Nematodes were paralyzed by 10 µl of 10 mM levamisole and placed on a microscope slide with a 2% agarose pad. Levamisole causes spastic paralysis in the nematode (Martin *et al.*, 2005). A coverslip sealed with silicon grease around the agarose pad was used, and imaged using DIC and/or fluorescence by Zeiss AxioObserver Z1 inverted microscope or Zeiss LSM 700 confocal microscope.

2.20. PHYLOGENETIC ANALYSIS

BLASTP (protein-protein BLAST) was used to identify similar sequences in protein databases and sequences with high homology were selected for this preliminary study (Table 2.6). *S. marcescens* Db11 OmpF (GenBank:CDG11589), OmpC (GenBank:CDG13231) and PhoE Db11 (GenBank:CDG12689) amino acid sequences were used as query sequences. The alignment of three classical porins was performed using PRALINE multiple sequence alignment (Simossis & Heringa, 2005) and saved as (.txt) format. The PRALINE alignment result was transformed into NEXUS format (.nex) using DAMBE software (Xia, 2013). The *Markov chain Monte Carlo* (MCMC) sampler for 100,000 generations was performed using the mixed amino acid model in the program MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003).

Table 2.6. Information on protein sequences used in phylogenetic analyses organized by source database.

	Name of protein	Name of organism	GenBank Identifier (GI)
1	OmpC	*S. marcescens* Db11	gi|560174497
2	OmpC	*E. coli str.* K-12	gi|16130152
3	OmpK36	*K. pneumonia*	gi|295881596
4	OmpC	*Y. enterocolitica* (type O:5)	gi|571261665
5	OmpC	*S. enterica subsp. enterica serovar Typhi*	gi|390989030
6	OmpC	*C. freundii*	gi|517906587
7	OmpC	*E. aerogenes* EA1509E	gi|444350376
8	OmpC	*P. mirabilis*	gi|516224454
9	OmpF	*S. marcescens* Db11	gi|560172860
10	OmpF	*E. coli str.* K-12	gi|16128896
11	OmpK35	*K. pneumonia*	gi|529123336
12	OmpF	*Y. enterocolitica*	gi|300680056
13	OmpF	*S. enterica subsp. enterica serovar Typhi*	gi|468741
14	OmpF	*C. freundii*	gi|517906874
15	OmpF	*Enterobacter aerogenes* EA1509E	gi|443903138
16	OmpF	*P.mirabilis*	gi|406365507
17	PhoE	*S. marcescens* Db11	gi|560173958
18	PhoE	*Escherichia coli str.* K-12	gi|4902976

19	PhoE	*Y. enterocolitica*	gi|517910869
20	PhoE	*S. enterica subsp. enterica serovar Typhi*	gi|47822
21	PhoE	*C. freundii*	gi|517906196
22	PhoE	*E. aerogenes* EA1509E	gi|443903785
23	OmpU	*V. cholera*	gi|557362325

* Sequences downloaded from GenBank:

http://www.ncbi.nlm.nih.gov/GenBank/index.html

CHAPTER 3. GENERATION AND CHARACTERIZATION OF PORIN MUTANTS IN *S. marcescens* Db11.

This chapter contains the preliminary material related to the identification of the *ompF, ompC and phoE* genes in *S. marcescens* Db11, a discussion of the disruption strategy, and the generation of double and triple mutants in *S. marcescens* Db11. The strategy presented was used to create unmarked deletion mutants for three porin genes *(*ompF, ompC and *phoE)* and other (*ampD*), involved in the regulation of AmpC β-lactamase production.

3.1. INTRODUCTION TO OmpF, OmpC AND PhoE PORINS.

The bacterial outer membrane is a dynamic structure that limits the passage of certain molecules into and out of the cell. As the most external cell structure in Gram-negative microorganisms, this membrane provides permeability and protection during cell interactions with the external environment. Porins, the subject of this study, refer to a group of proteins that form channels for the entrance and exit of hydrophilic solutes, and in conjunction with other proteins and lipopolysaccharide form an structured matrix, known as outer membrane.

OmpF and OmpC are well conserved porins in the *Enterobacteriaceae* family. These non-specific porins have been classified as classical porins because they share structural homology in that they form 16-stranded β-barrels that are associated in tightly packed trimers (Nguyen *et al.*, 2006; Nikaido & Rosenberg, 1983). PhoE is also considered to be a classical porin with specificity for phosphate (Korteland *et al.*, 1982). For the classical porins, the β-barrel structure formed by each monomer is necessary for pore function (Balasubramaniam *et al.*, 2012; Baslé *et al.*, 2006; Lauman *et al.*, 2008). While no crystal structures are published for *S. marcescens* OmpF, OmpC and PhoE porins, the structural model solved by X-ray

crystallography studies for homologous proteins in *E. coli*, can be used to predict *S. marcescens* porin structures. While the regulation and function of OmpF, OmpC and PhoE porins have been well-characterized in *E. coli* (Cowan *et al.,* 1992), the role of the orthologous porins in *S. marcescens* in bacterial fitness and antibiotic resistance is not as well defined. Thus, to ascertain these functions, a gene deletion strategy was adopted to generate gene-knockout strains for further investigations in this study.

3.1.1. Identification and comparison of *S. marcescens* OmpF, OmpC and PhoE porins.

In previous studies, the expression of *ompF* and *ompC* in *S. marcescens* was found to alter with environmental conditions (Begic & Worobec, 2006). However, the expression of *phoE* or it function in *S. marcescens* was not previously investigated. For this study, the insect pathogen *S. marcescens* Db11 was selected because the genome sequence is available at the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/resources/downloads/bacteria/). The genome of *S. marcescens* strain Db11 is contained on a single circular chromosome of 5,113,802 bp with a G+C content of 59.51%. *S. marcescens* Db11 is a spontaneous streptomycin resistant strain, a derivative from the *Drosophila melanogaster* pathogen *S. marcescens* Db10 strain (Flyg *et al.*, 1980). *S. marcescens* Db11 has also been proposed to be the reference strain for *S. marcescens* research (Kurz *et al.*, 2003; Murdoch *et al.*, 2011; Petty *et al.*, 2006) .

Previous work with *S. marcescens* OmpF and OmpC porins was based on the OmpF (GenBank:U81967) and OmpC (GenBank:L24960) porin sequences reported for the *S. marcescens* human-clinical isolate UOC51 (non-pigmented) (Hutsul & Worobec, 1994, 1997). In this study, these porin amino acid sequences were used as query sequences for BLAST analysis in the *S. marcescens* Db11 genome where the sequence for porins OmpF (GenBank: CDG11589) and OmpC (GenBank:CDG13231) were found. The third classical porin, PhoE in *S. marcescens*

Db11 (GenBank: CDG12689) was found after using the *E. coli* K-12 PhoE sequence (GenBank: P02932). At the protein level, it was found that each amino acid sequence analyzed shared high percent of homology (90 to 100 %) with the same porins, previously reported for *Serratia* species and other members of the *Enterobacteriaceae* family.

Figure 3.1 shows the comparison of the OmpF, OmpC, and PhoE porins in *S. marcescens* Db11, using PRALINE multiple sequence alignment (Bawono *et al.*, 2014; Pirovano *et al.*, 2008; Simossis & Heringa, 2005). PRALINE is a sequence alignment toolkit that allows the comparison of amino acid sequences based on the conservation of amino acids, hydrophobicity, secondary structure and residue type. The colors show the conservation, scored from 0 to 10, where consistency refers to the consensus sequence (*= score of 10). The higher variability was found in the predicted variable loop regions, that are known to experience relatively rapid evolutionary changes (Nikaido 2003). The higher homology of porins was observed in regions that are normally in contact with the lipid bilayer membrane. Porin β-sheets maintain a conserved amino acid composition and are immersed in the outer membrane; however, loops are more surface exposed and their amino acids reflect the history of multiple interactions with the external milieu.

As seen with *S. marcescens* UOC51, *S. marcescens* Db11 OmpF features the characteristic two -Cys group (Figure 3.1). In addition, OmpF, OmpC and PhoE also have on the carboxy-terminus the conserved phenylalanine (F390) residue which has been shown previously to be necessary for porin assembly in the outer membrane (de Cock *et al.*, 1997; Struyvé *et al.*, 1991)

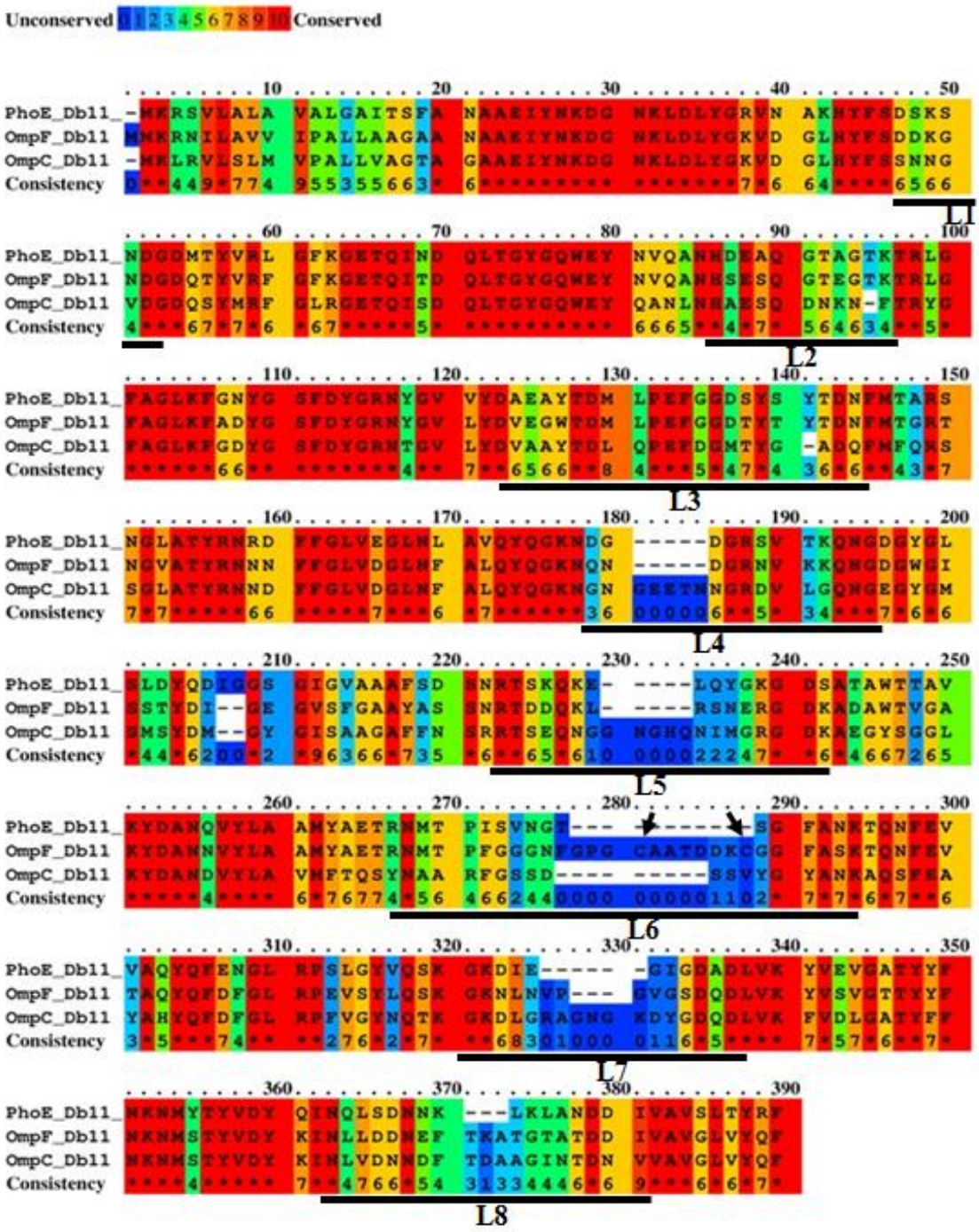

Figure 3.1. Alignment of three classical porins. Comparison of OmpF, OmpC and PhoE porins in *S. marcescens* Db11 using PRALINE multiple sequence alignment. The colors show the amino acid conservations scored from 0 to 10 where consistency refers to the consensus sequence. (*= score of 10). A solid line indicate the putative loops (L1, L2, L3, L4 L5, L6, L7, L8). In loop 6, two arrows indicate the position of the two –Cys groups in OmpF amino acid sequence.

A phylogenetic analysis of the three porin types in different Enterobacteria was also performed to determine their phylogenetic relationships (Figure 3.2). The phylogenetic analysis shows the evaluation of 23 porin amino acid sequences of *S. marcescens* Db11, *E. coli* K-12, *K. pneumonia, Y. enetrocolitica, S. enteric, C. freundii, E. aerogenes and P. mirabilis* bacterial species (Table 2.6). The OmpU porin from *V. cholera* was used as an out-group. Both *ompF* and *ompC* are classified as paralogous genes and express OmpF and OmpC respectively. Genes *ompF* and *ompC* are homolgous genes that code for proteins with similar function in the same organism. OmpC in *S. marcescens* Db11, *Y. enterocolitica, E. aerogenes, K. pneumoniae, E. coli* K-12 and *S. enterica* sequences have strong support to be considered members of a sister-group. After building the phylogenetic tree OmpF amino acid sequences were split into three distinct groups indicating higher variability in amino acid composition. Similar results were reported elsewhere suggesting that OmpF may have a complex evolutionary history at the DNA sequence level (Nguyen *et al.*, 2006). A far more extensive analysis is required to resolve the relatedness of this group of proteins, but the preliminary analysis is presented here, supports the identification of OmpF, OmpC and PhoE in *S. marcescens* Db11.

Porins are part of the first line of bacterial defense and are constantly interacting with the environment. This interaction enhances the variation of amino acid contents in the most external porin structures such as loop structures. A recent study with the *Yersinia* family was performed to demonstrate that positive selection drives the variability in *Yersinia* OmpF. In this study, high variability was found in the amino acid sequence of OmpF most likely due to recombination events and positive selection (Stenkova *et al.*, 2011).

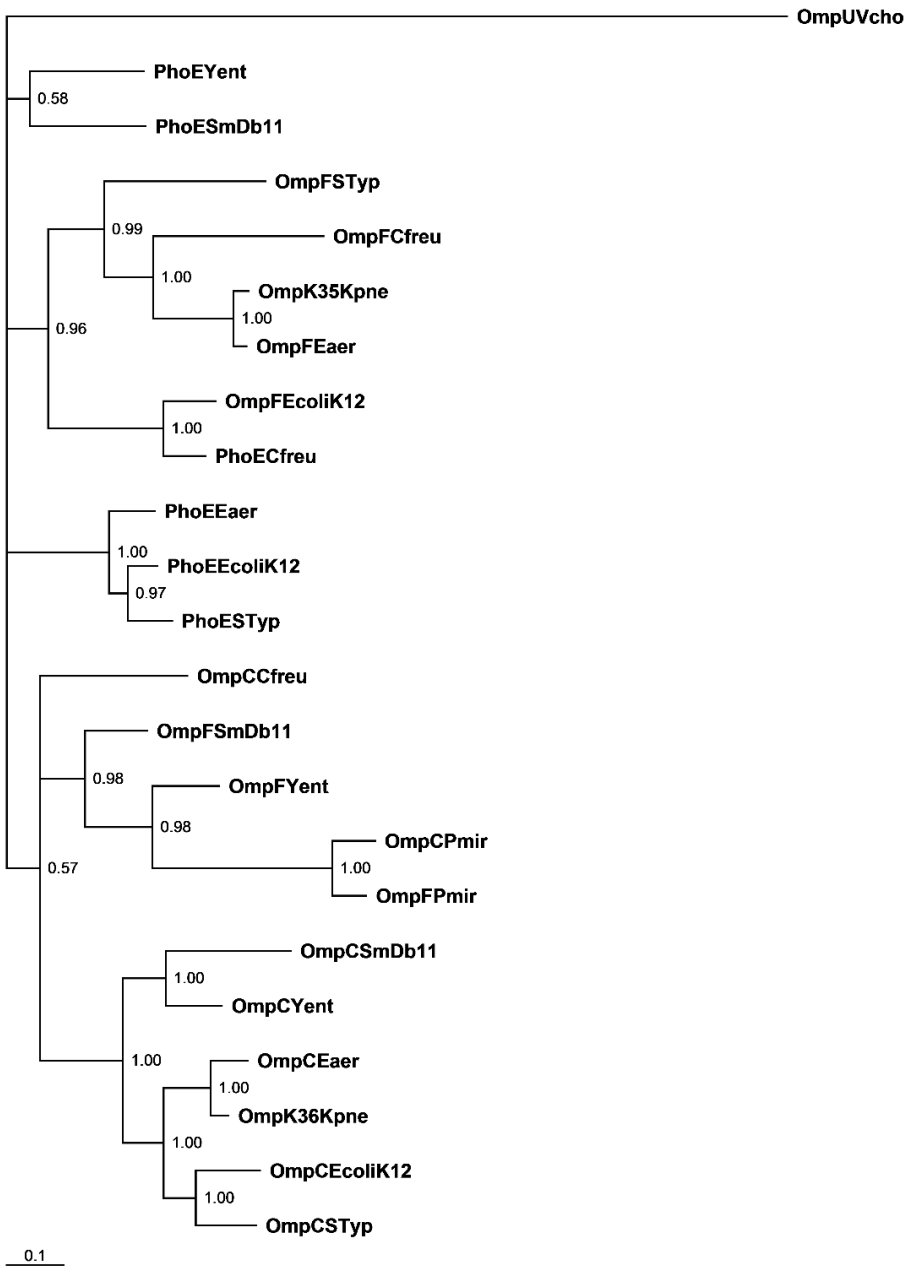

Figure 3.2. Phylogenetic analyses using MrBayes version 3.1.2. MrBayes estimates the model that best fits the amino acid sequences data and determines the highest likelihood model of protein evolution. The *Markov chain Monte Carlo* (MCMC) sampler for 100,000 generations using the mixed amino acid model was used. BLASTP (protein-protein BLAST) was used to find similar sequences in protein databases. *S. marcescens* Db11 OmpF (GenBank:CDG11589), OmpC (GenBank:CDG13231) and PhoE Db11 (GenBank:CDG12689) were used as query sequences. OmpU in *Vibrio cholera* was designated as the outgroup. Phylogenetic trees were drawn with the TreeView program. Values in the nodes indicate posterior probabilities (1.0 = maximum probability). The scale bar represents the number of substitution or changes.

The phylogenetic analysis (Figure 3.2), showed that *S. marcescens* Db11 PhoE and OmpF are not closely related sequences suggesting similar results as obtained for *Yersinia.* These two porins showed higher evidence of change and evolution inter species probably as result of environmental pressure.

3.1.2 Generation of *S. marcescens* Db11 *ompF* and *ompC* deletion mutant strains.

Porin gene deletion mutants represent an essential tool for bacterial physiology and molecular structural studies. The generation of a rapid and efficient protocol that permits the analysis and deletion of particular genes without the introduction of antibiotic resistance genes in the chromosome or without the generation of polar effect is important. This work details the creation of *S. marcescens* porin knockout mutants to evaluate their effect on bacterial outer membrane permeability as well as bacterial pathogenicity.

3.1.3 Generation of unmarked knockout mutants

The method for rapid porin gene deletion adopted in this study avoids the incorporation of antibiotic resistance genes. The method was applied to systematically delete genes encoding three porins in *S. marcescens* Db11 (OmpF, OmpC and PhoE) and to delete the *ampD* amidase gene, a regulator of AmpC β-lactamase expression.

In previous studies using *S. marcescens* UOC-67, knockout mutants were generated after disrupting *ompF* or *ompC* by insertion of kanamycin and gentamicin antibiotic resistant genes (Begic & Worobec, 2007). In those works the suicide vector pKNG101was used as a gene replacement vector. pKNG101 is based on the *pir*-minus origin of replication (*oriR6K*) and the counter-selection marker *sacB* of *Bacillus subtilis* which confers sucrose sensitivity (Kaniga *et al.,* 1991). The pKNG101 plasmid containing the disrupted gene was integrated into the *S. marcescens* UOC-67 chromosome after a first cross over. The merodiploids generated after chromosomal integration of the suicide plasmid were resolved by the counter-

selection marker *sacB* (Begic & Worobec, 2007). However, the *sacB* counter-selection marker was not always efficient in *S. marcescens* UOC-67 and it was decided to take the advantage of the streptomycin resistance phenotype in *S. marcescens* Db11. For that reason, the suicide vector pKAS32 that contains *rpsL* (streptomycin counter-selection) was implemented. In this strategy, the suicide vector pKAS32, derived from pGP704 (Miller & Mekalanos, 1988; Skorupski & Taylor, 1996) contains the π-dependent R6K replicon (Figure 3.3), and only can be maintained in the cell if it integrates in the chromosome.

The strategy for the construction of gene replacement fragments before cloning them into the suicide vector pKAS32 includes the generation of three partially overlapping DNA fragments. The resistance cassette for gentamicin (*aacC*$_1$) (in pPS856 vector), the 5` and 3` regions flanking the target gene were PCR amplified, gel purified and fused by PCR. The PCR-fused product was cloned into the pKAS32 suicide vector to generate the construction for gene replacement (Figure 3.4), that was introduced by tri-parental mating conjugation into *S. marcescens* Db11.

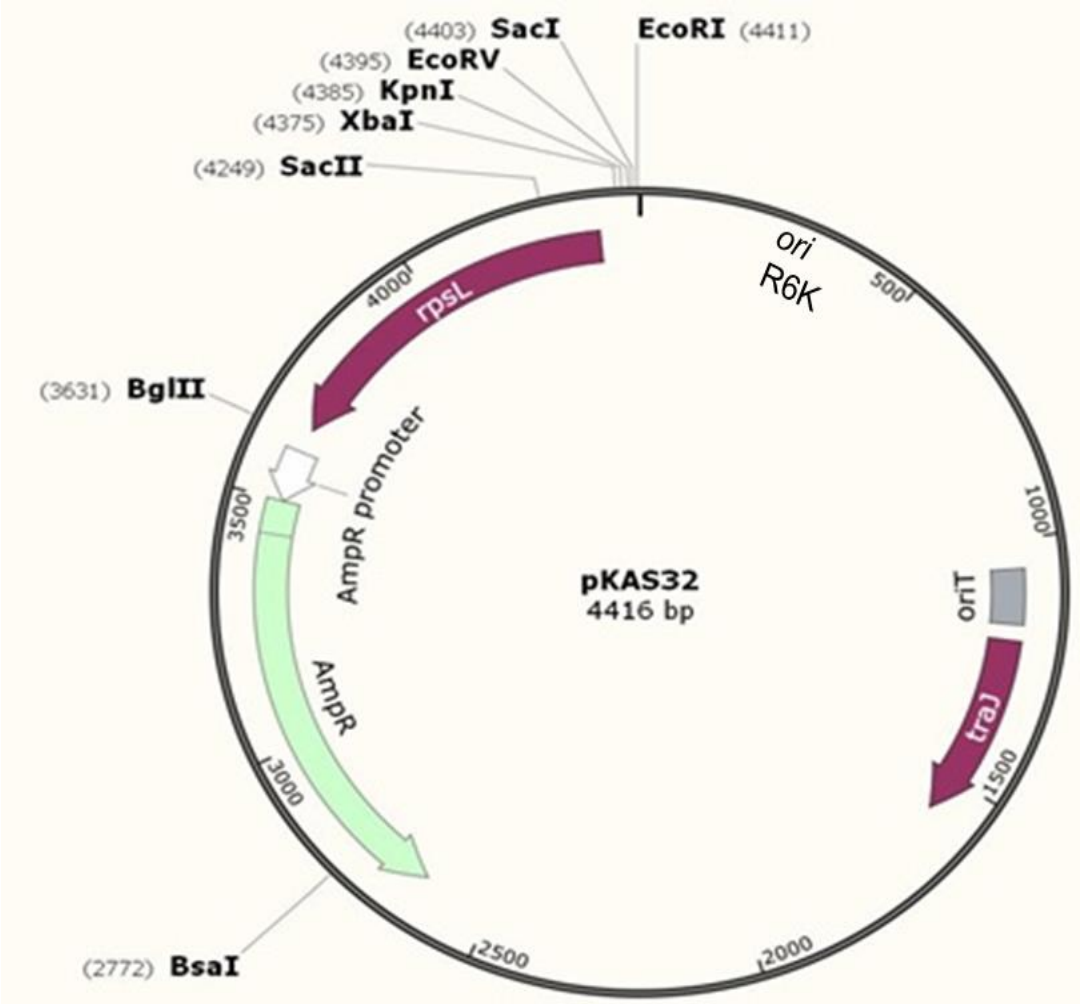

Figure 3.3. Positive selection vector pKAS32 map. Plasmid map adapted from (Skorupski & Taylor, 1996), with some modifications to show the regions relevant for this study. AmpR: ampicillin resistant gene; *ori*R6K: origin of replication that is dependent on the pir gene-encoded π protein.; *ori*T: origin of replication; *rpsL*: gene encoding ribosomal protein S12.

Once the suicide vector with the gene replacement construct was introduced into *S. marcescens*, the plasmid was integrated into the chromosome forming the merodiploid state. The integration of the pKAS32 into *S. marcescens* hosts lacking π function generated the transconjugants which contained a gentamicin resistant marker flanked by two FRT sequences. In a later recombination event, the excision of the plasmid occurred and the target gene was replaced by the desired mutation in the chromosome. With the loss of pKAS32 the cell recovered its resistance to streptomycin while acquiring resistance to gentamicin (Figure 3.5). The selection for streptomycin resistance permitted the identification of transconjugants that had excised the plasmid with the *rpsL* DNA sequence. Colonies that were resistant to streptomycin and gentamicin were selected for further screening to confirm gene replacement.

After the excision of the plasmid, the pFLPe2 vector is introduced into the *S. marcescens* mutant strain to eliminate the resistance cassette from the chromosome and to obtain the unmarked deletion mutant. The use of this suicide vector in the spontaneous streptomycin resistant *S. marcescens* Db11 was advantageous in generating knockout strains. Another benefit in this strategy was the use of pFLPe2 plasmid containing the flipase enzyme coded by *flpe*, which allows the successful deletion of the temporary resistance marker (Gm^r), from the chromosome to generate gene deletion mutants (Choi *et al.*, 2008). The Flp-FRT system is a handy tool for genetic engineering manipulation due to the high specificity of the recombinase Flp. The Flp recombinase promotes recombination at a specific 13-bp site inside the 65-nt FRT sequence. This versatile system can also be used in yeast and mammalian cells in addition to other bacteria (Choi *et al.*, 2008; Hoang *et al.*, 1998). Primers designed to build the allelic exchange fragments, were based on the genome sequence of *S. marcescens* Db11. The steps used in the generation of *S. marcescens ompF*, *ompC, phoE,* and *ampD* deletion

mutants are described in Materials and Methods and represented in the diagrams presented in Figures 3.4 and 3.5.

3.1.4. *S. marcescens* double and triple mutant strains.

The following diagram outlines the generation of double and triple mutants using generalized transduction (Figure 3.6). The bacteriophage ΦIF3 was used to transduce the marked gene replacement construct (FRT-Gm^r-FRT) from the donor strain into the unmarked recipient strain. ΦIF3 is able to do generalized transduction in *S. marcescens* Db11 and Db10 (English *et al.*, 2012; Petty *et al.*, 2006).

A phage ΦIF3 lysate was prepared from *S. marcescens* Gm^r strains containing the mutation that needed to be introduced into the Gm^s recipient strains. Figure 3.6, shows an example of the generation of *ompC/ompF* double mutant. In this example the ATF001 (Δ*ompF*::Gm^R) was infected by the bacteriophage ΦIF3 to obtain the phage lysate used to introduce the *ompF*::FRT-Gm^r-FRT DNA fragment into Gm^s single or double mutants. The gene replacement construct prepared to transduce the FRT-Gm^r-FRT DNA sequence into the recipient Gm^s single unmarked strain ATC101 (Δ*ompC*) allowed to generate double and triple unmarked mutant presented in this study (Table 2.1) The unmarked deletion mutant was obtained with the use of the pFLPe2. All mutants generated were confirmed by PCR and DNA sequencing.

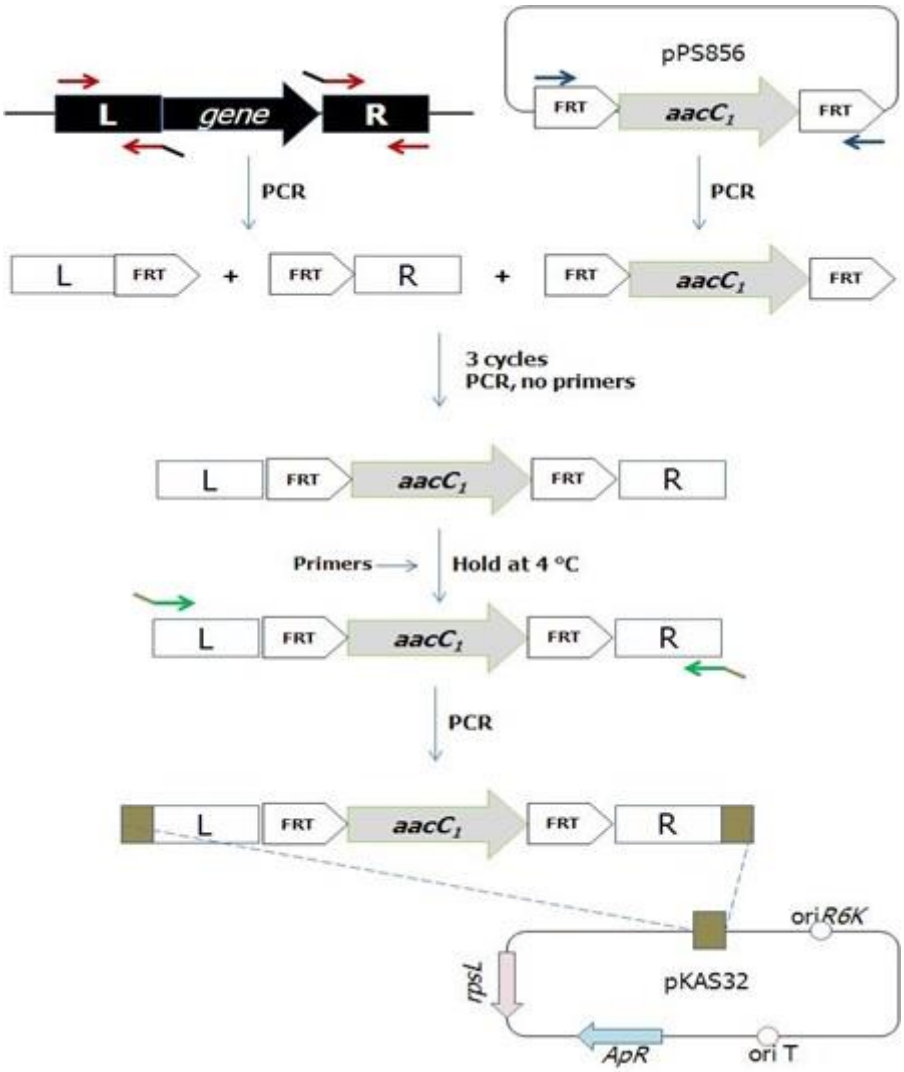

Figure 3.4. Generation of gene replacement construct. In the diagram L and R represent the 5' and 3'end of the gene respectively; *aaC1*: Aminoglycoside N(3')-acetyltransferase I (Gm^R); FRT: Flp-FRT recombination cassette.

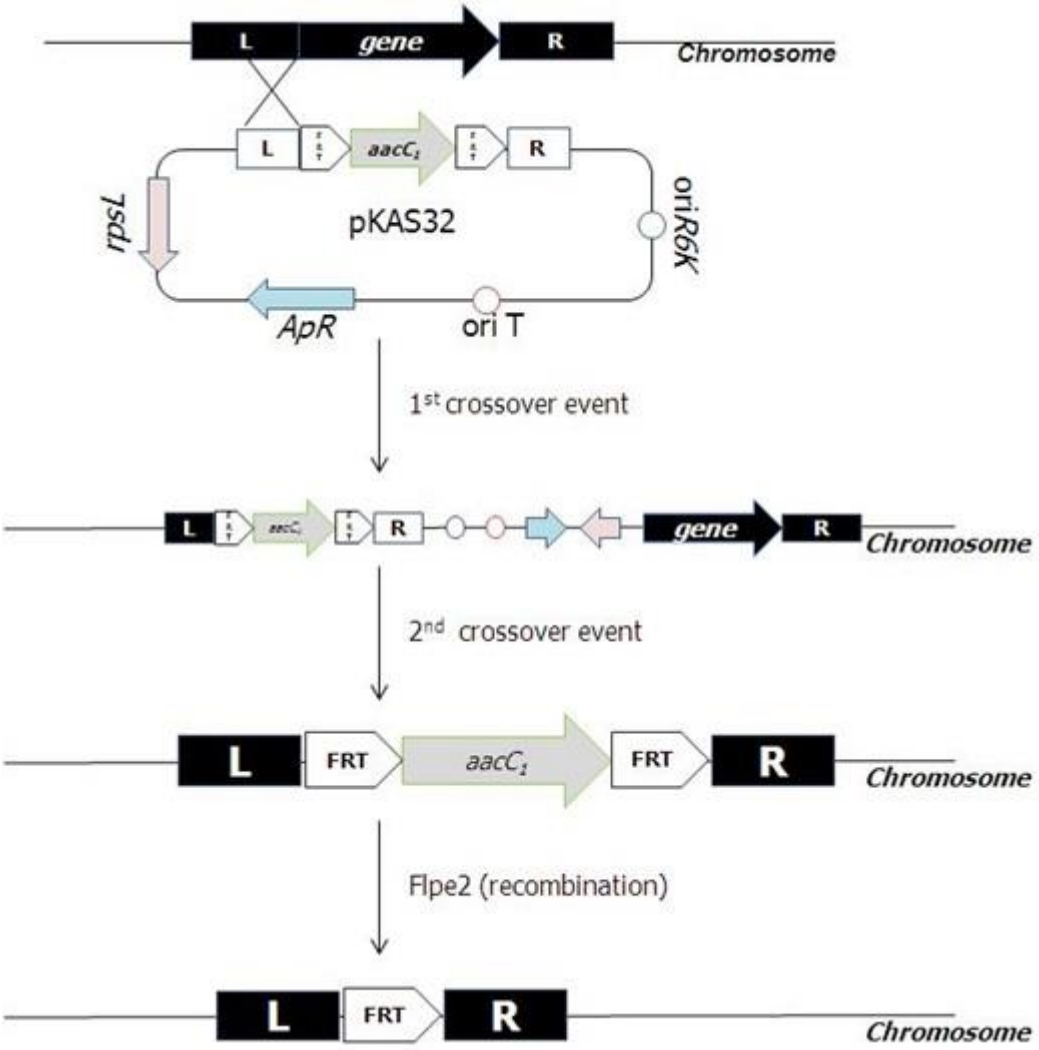

Figure 3.5. Elimination of resistance marker from the knockout mutant. Vector pFLPe2 expresses Flp recombinase and mediates the recombination between FRT sites to excise the gentamicin resistant marker. Elimination of pFLPe2 from the mutant is achieved after growing the gentamicin susceptible strains on LB agar at 42 °C.

3.1.5. Unmarked deletion mutant and -*Gm*^{r} excision.

A first attempt using Flp-FRT system from pFLP2 vector (Hoang *et al.*, 1998) did not allow the elimination of the merodiploid state. The plasmid-borne *sacB* as a counter-selection marker did not function properly in *S. marcescens* Db11. After few tries, it was decided to change to a different vector pFLPe2, with improved capacity to remove the Gm-FRT cassette (Choi *et al.*, 2008). The pFLPe2 vector (Figure 3.7), allowed the excision of FRT-Gm-FRT gene cassettes from the mutant strains with high efficiency by the activity of the *FLPe* gene that express a more efficient and stable flipase. The vector pFLPe2 in addition, contains a Zeo selection marker and a temperature sensitive Ts*Bt* replicon for selection that allows plasmid curing at 42 °C (Choi *et al.*, 2008).

In other studies, the mini-*Tn5* Cm has been used to create chloramphenicol marked insertion mutants in *S. marcescens* Db11 (Kurz *et al.*, 2003). However, that strategy focused on the disruption of gene function by the insertion of a mobile element which may also cause polar effects. In this study the unmarked gene deletion mutants generated by FLP-FRT system allowed the inclusion of additional mutations in the same strain.

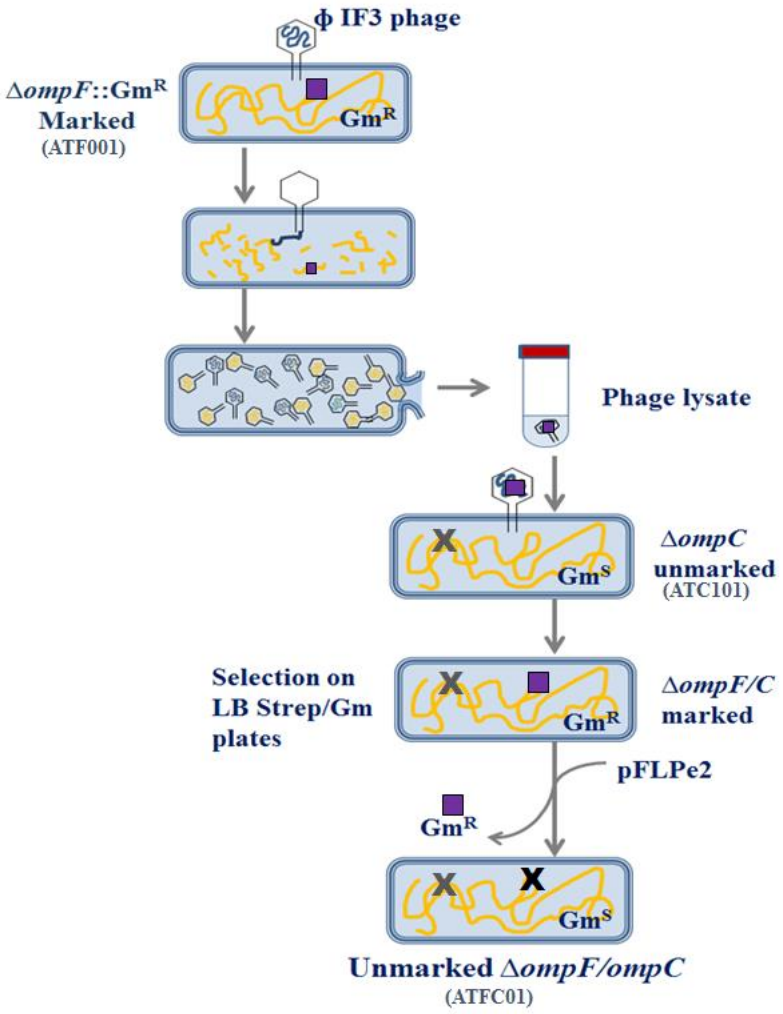

Figure 3.6. Generation of double knockout mutants. Phage ΦIF3 lysate was prepared from *S. marcescens* Gm^r strain ATF001 (Δ*ompF*::Gm^R), containing the gene replacement construct to transduce the FRT-Gm^r-FRT DNA sequence into the recipient Gm^S single unmarked strain ATC101 (Δ*ompC*) to produce the double porin mutant Gm^r *S. marcescens* AMTFC1 (Δ*ompC/ompF::*Gm^R). The pFLPe2 plasmid was used to obtain the unmarked deletion double mutant ATFC01 (Δ*ompFompC*) Triple mutants (ATFCE1 and ATFCD1) were obtained using the same methodology infecting the Gm^S double mutant ATFC01 (Δ*ompFompC*) with the lysate of Gm^r strain of the third target gene to be deleted. In the figure a purple square (■) represents the FRT-Gm^r-FRT fragment and the deletion are represented with an (X).

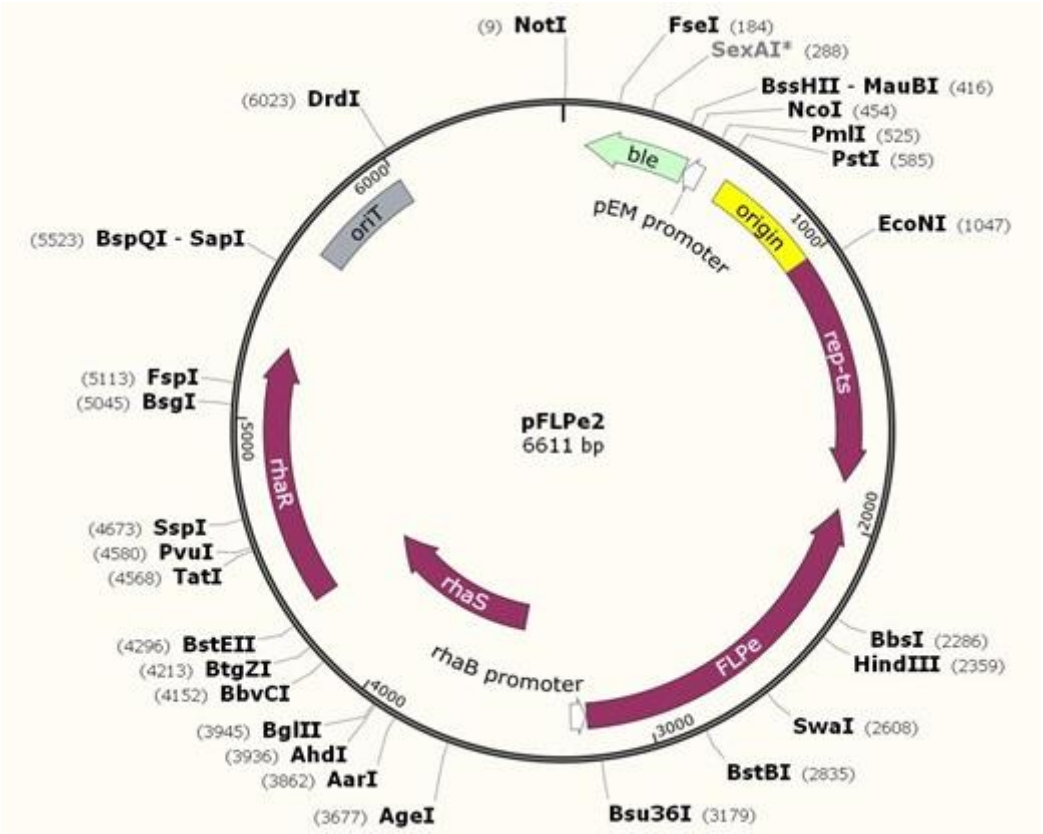

Figure 3.7. Vector pFLPe2, a curable Flp recombinase-expressing plasmids. pFLPe2 contains: *ori*, the *E. coli* pMB9 origin of replication; *ori*1600, the pRO1600 origin of replication, requiring the *rep*(TsBt)-encoded replication protein, which confers a TS phenotype at temperatures above 37°C; *oriT*,an RK2-derived origin for conjugal plasmid transfer; and P*rhaBAD*, the rhamnose-inducible *E. coli rhaBAD* operon promoter controlled by the *rha*R– and *rha*S- encoded regulatory proteins. The *ble*, the bleomycin resistance gene, which confers Zeo resistance; *FLPe*, a gene encoding an enhanced Flp recombinase which is active over a larger temperature range than the wild-type enzyme. Adapted from (Choi *et al.*, 2008).

3.1.6. Complementation *in trans* of *ompF* and *ompC*.

The pUFR-GFP vector was used to complement *in trans* the *ompF* and *ompC* genes in single and double mutant strain (Figure 3.8). Vector pUFR-GFP is a low copy number vector (IncW replicon) and can replicate in *S. marcescens* Db11. A derivative of the pUFR047, pUFR-GFP features *gfpmut3* inserted into the EcoRI-PstI restriction sites under transcriptional control of the kanamycin resistance gene promoter P_{aphA3} (De Feyter *et al.*, 1993). Replacement of P*aphA3-gfpmut3* by either *ompF* or *ompC* under control of their native promoters allowed restoration of *ompF* or *ompC in trans* in the porin mutant ATFC01 (Figure 3.9). The corresponding gene with its native promoter was inserted in this vector in two possible orientations after a blunt-end ligation. The orientation of the genes was confirmed by restriction digestion and DNA sequencing.

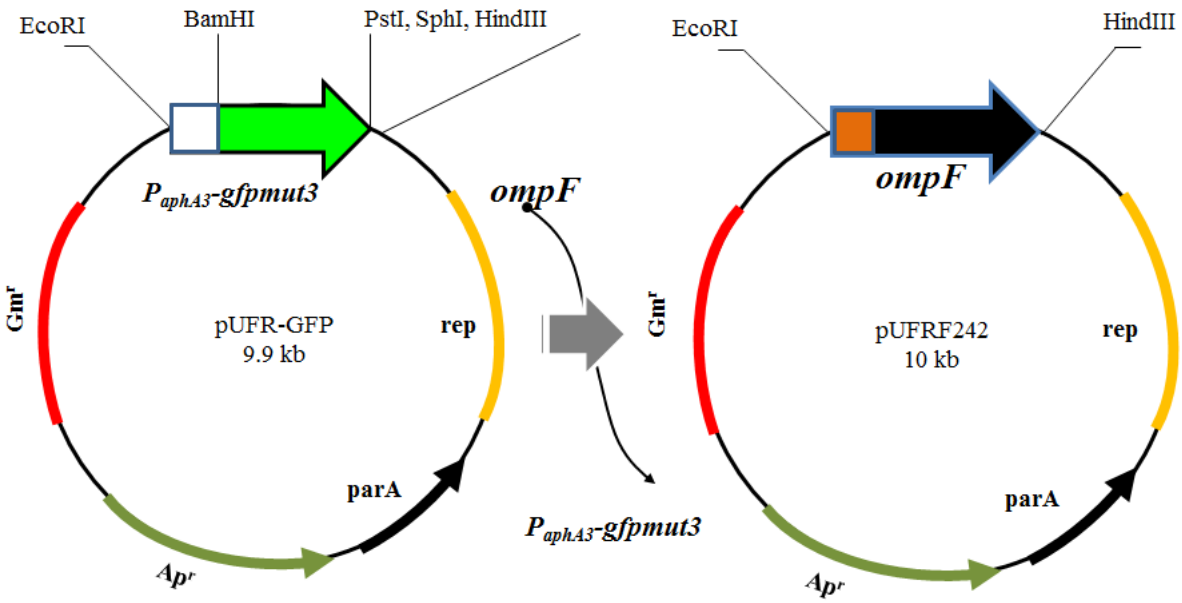

Figure 3.8. Construction of pUFRF242 vector. The figure represent the replacement of the P_{aphA3}-*gfpmut3* (GFP gene) by the porin gene and its native promoter (black arrow). The pUFR-GFP vector is a derivative of pUFR047 by insertion of *gfpmut3* driven by the kanamycin promoter P_{aphA3} into EcoRI-PstI of the pUFR47 multiclonal site (De Feyter *et al.*, 1993). Vector pUFR-GFP was double digested with EcoRI and PstI to replace the P*aphA3-gfpmut3* DNA fragment by *ompF* under the control of corresponding native promoter.Vector pUFRC50 for *ompC* complementation was constructed using the same procedure. Abbreviations: *rep*, replication origin (IncW replicon); *parA*, partition locus, Ap^r, resistance to ampicillin; Gm^r, resistance to gentamycin.

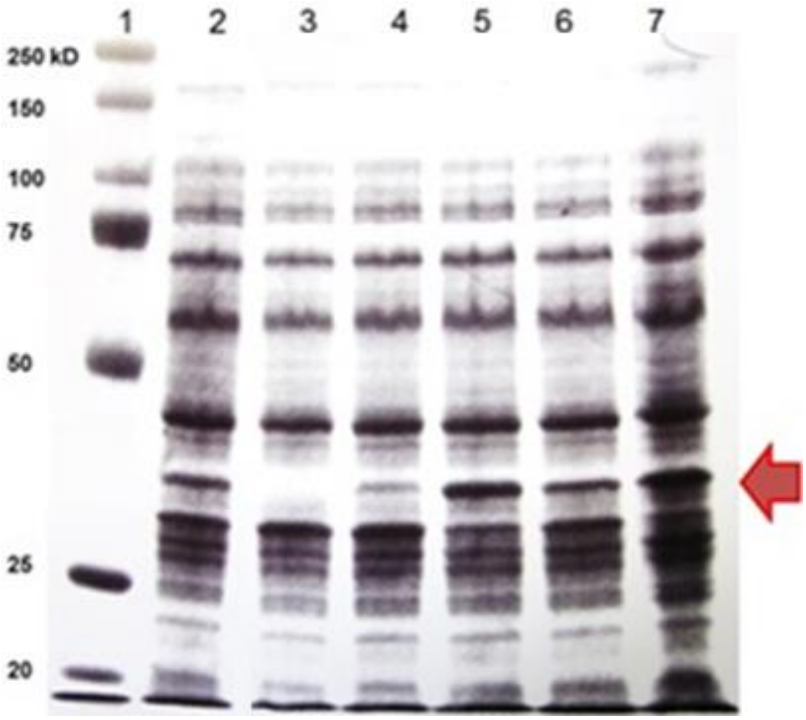

Figure 3.9. SDS-PAGE of *S. marcescens* mutant strains. OMP profiles from *S. marcescens* Db11 wild type and double mutant ATFC01(Δ*ompFompC*) complemented, grown in LB liquid medium at 37 °C. Lane 1: Protein MW Ladder (BIO-RAD), lane 2: Db11, lane 3: ATFC01 (Δ*ompFompC*), lane 4: ATF242 (+), lane 5: ATF10 (-), lane 6: ATC50 (+), lane 7: ATC45 (-). An arrow indicates complemented porin bands in the region of 40 kDa. The signs (+) and (–) refer to the two different orientations of the porin genes in the pUFR vector.

3.1.7. Porin expression is affected by nutrient availability.

To further investigate if the control of porin expression is linked to nutritional requirements rather than structural compensatory purposes, the effect of nutrient limitations on porin expression in the double porin mutant ATFC01 (Δ*ompFompC*) strain was assessed. The outer-membrane protein fractions of ATFC01 (Δ*ompFompC*) cells grown in different concentrations and type of carbohydrates, phosphate and/or amino acids in HEPES-buffered minimal salt medium were evaluated using SDS-PAGE (Figure 3.10). As expected, different banding patterns of outer membrane protein profiles were observed when ATFC01 (Δ*ompFompC*) cells were grown in varying nutrient conditions. Specifically, alterations in the intensity of bands corresponding to the molecular sizes of LamB (48 kDa), PhoE (39 kDa) and OmpX (18 kDa) porins were noted (Figure 3.10). Increased intensity of the band corresponding to LamB was observed when cells were grown in medium containing maltose (lane 4). This upregulation is not unexpected considering that expression of LamB is controlled by the *mal* operon that is activated when maltose is present (Boos & Shuman, 1998). Increased intensity of the band corresponding to PhoE was observed when ATFC01 (Δ*ompFompC*) cells were grown in medium lacking phosphate (lanes 2-4). Expression of PhoE is controlled by the Pho regulon and expression of PhoE is upregulated when phosphate is limited (Barchiesi *et al.*, 2012; Crépin *et al.*, 2011; Hsieh & Wanner, 2010). Availability of phosphate causes downregulation of PhoE as indicated by the decreased intensity of the band associated with PhoE when the medium was supplemented with $KHPO_4$.

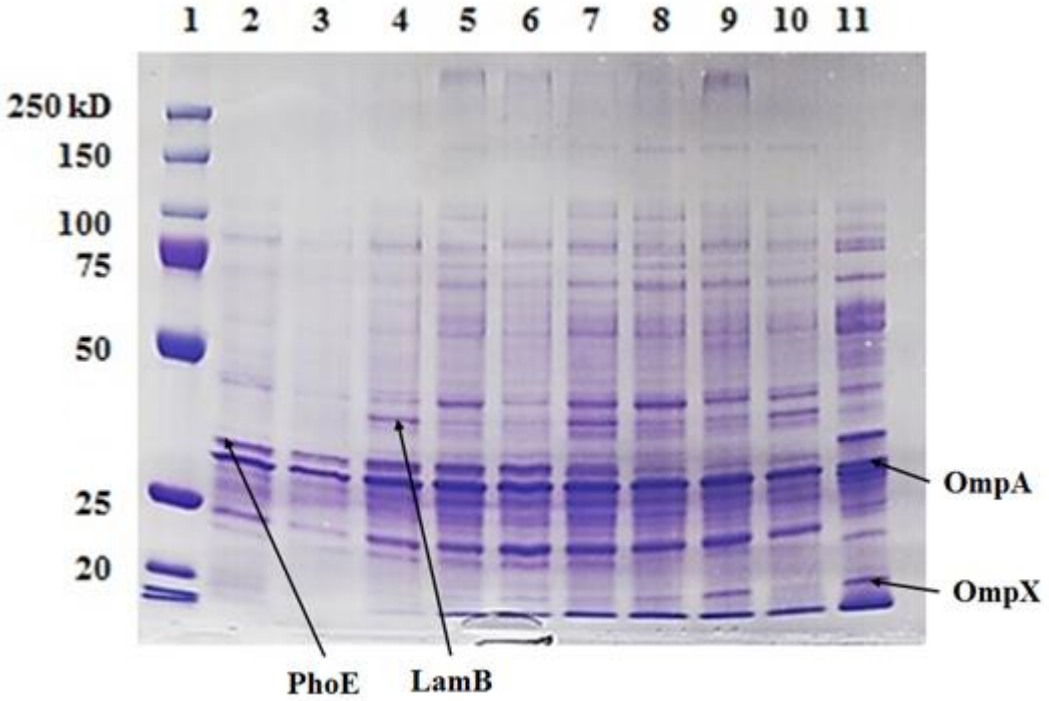

Figure 3.10. SDS-PAGE of the outer membrane fraction of *S. marcescens* strains grown in different conditions. Strain ATFC01 (Δ*ompFompC*) after growth in HEPES-buffered minimal salt medium with the indicated additions. Lane 1: Protein ladder, Lane 2: 0.1 % Glucose, Lane 3: 0.1% Glycerol, Lane 4: 0.1% Maltose, Lane 5: 0.1% Glucose + 0.2% CAA, Lane 6: 0.1% Glycerol + CAA, Lane 7: 0.1% Maltose + CAA, Lane 8: 0.1% Glucose + 0.2% CAA + 2.5 mM $KHPO_4$, Lane 9: 0.1% Glycerol + CAA+ 2.5 mM $KHPO_4$, Lane 10: 0.1% Maltose + CAA + 2.5 mM $KHPO_4$. Lane 11: Db11 grow in LB liquid media. Casamino acid (CAA) was added as a source of amino acids and $KHPO_4$ as a source of phosphate. Arrows show the band sizes associated with PhoE (39 kDa), LamB (48 kDa), OmpA (27 kDa) and OmpX (18 kDa). Digital images of stained gels were obtained used an Epson perfection V500 PHOTO scanner.

The production of OmpX and OmpA does not appear to be affected either in LB medium or in HEPES-buffered minimal salt medium for most of the growth conditions evaluated (Figure 3.10; lane 11). Only an increase in the intensity of the band associated with the molecular size for OmpX was observed in the lane 9 (0.1% Glycerol + CAA+ 2.5 mM $KHPO_4$).

The integral membrane proteins OmpX and OmpA form single-channel porin structures. In *E. coli*, OmpX is a cation-selective channel that is up-regulated by MarA and high osmolarity. Overexpression of OmpX correlates with low levels of OmpF, but its compensatory effect to modulate permeability in the absence of OmpF and OmpC is still unclear (Dupont *et al.*, 2007; Gao *et al.*, 2011). On the other hand, OmpA is involved in bacterial adhesion, invasion and virulence in addition to its structural function that is well conserved in *Enterobacteriaceae* (Confer & Ayalew, 2013; Smith *et al.*, 2007). Expression of OmpA increases serum resistance and pathogenicity of *E. coli* K-12 (Weiser & Gotschlich, 1991) and in *Y. pestis* it is required for intracellular survival in macrophages (Bartra *et al.*, 2012). OmpA is also regulated by growth rate, cAMP, nutrient availability, MicA and Hfq protein (Smith *et al.*, 2007). Taken together, it was initially thought that the loss of OmpF and OmpC porins would affect bacterial fitness or produced visible modifications in the protein profile that would indicate altered expression of other porins; however, none of these effects were observed under the environmental conditions performed in this study.

In summary, clean, unmarked deletions of *ompC*, *ompF* and *phoE* were generated in *S. marcescens* Db11 and were now available for the analysis of the roles in antibiotic resistance (Chapter 4). Similar strategy was used to generate *ampD* unmarked mutants.

CHAPTER 4. THE LACK OF OMPF, BUT NOT OMPC, CONTRIBUTES TO INCREASED ANTIBIOTIC RESISTANCE IN *Serratia marcescens*.

The results presented in this chapter were published in *Microbiology*, 2014: Moya-Torres, A., Mulvey, M.R., Kumar, A., Oresnik, I.J., Brassinga, A.K. (2014). The lack of OmpF, but not OmpC, contributes to increased antibiotic resistance in *Serratia marcescens*. Microbiology. 160:1882-92. doi: 10.1099/mic.0.081166-0.

Authors' contributions

Moya-Torres, A. (First author), designed and performed the work presented. Romeo Hinzon (National Microbiology Laboratory, Winnipeg, Manitoba, R3E 3R2.) performed the determination of the minimal inhibitory concentration assay of selected antibiotics and Dr. Mulvey (National Microbiology Laboratory, Winnipeg, Manitoba, R3E 3R2.) participated in the writing of the manuscript. Dr. Brassinga and Dr. Oresnik conceived the study and participated in the designing, discussion and writing of the manuscript. Dr. Kumar helped with the discussion of the results and writing of the manuscript.

4.1 ABSTRACT

The environmental organism *Serratia marcescens* is one of the primary causes of numerous nosocomial outbreaks and opportunistic infections. Multi-drug resistance is now a common feature among *S. marcescens* clinical isolates complicating the efficacy of treatment. Recent reports have attributed antibiotic resistance to altered porin expression as well as perturbation of the intrinsic AmpC β-lactamase production pathway. In this study, we aim to genetically correlate the absence of OmpF and OmpC classical porins with increased antibiotic resistance. In generating isogenic porin mutant strains, we avoided incorporating additional resistance through the use of antibiotic cassette in gene replacement and adopted an alternate strategy in creating clean unmarked mutant strains. We found that lack of OmpF,

but not OmpC, significantly increased antibiotic minimum inhibitory concentration (MIC) values to the β-lactam drugs such as ampicillin and cefoxitin as well as to nitrofurantoin. Furthermore, we found that cefoxitin did not induce intrinsic AmpC β-lactamase production indicating that the increased MIC values was a result of reduced permeability of cefoxitin due to the lack of OmpF. Genetic deletion of both *ompF* and *ompC* did not compromise the integrity of the bacterial cell envelope in optimal growth conditions suggesting that other outer membrane porins may function in a compensatory role to facilitate nutrient uptake and cell envelope integrity. Taken together, to our knowledge this is the first study that genetically correlates increased antibiotic resistance with altered porin expression in *S. marcescens.*

4.2. INTRODUCTION

The Gram-negative bacterium *Serratia marcescens*, normally an environmental organism, is one of the primary causative agents of numerous nosocomial outbreaks and opportunistic uropathogenic, septic, ocular and respiratory infections (Mahlen, 2011). It has been noted that in past few decades, there has been a steady rise in the number of cases of *S. marcescens* nosocomial infection as well as a surge in the number and type of multi-drug resistant (MDR) strains, increasing the burden on health care resources (Laupland *et al*., 2008; Mahlen, 2011). The increase and diversity of antibiotic resistance can be attributed in part to altered porin expression and intrinsic β-lactamase production.

In the outer membrane, classical porins enable the movement of hydrophilic molecules in a non-selective fashion facilitating the diffusion of nutrients and extrusion of waste products (Silhavy *et al*., 2010). OmpF and OmpC are the most studied classical porins in Gram negative bacteria and are highly conserved in the entire *Enterobacteriaceae* family (Tsai *et al*., 2011; Pagel *et al*., 2007; Masi & Pagès, 2013). The structure of these non-specific pore forming proteins comprise a trimeric assembly of monomeric 16-stranded β-

barrels, each containing its own hydrophilic channel that span the thickness of bacterial outer membrane (Fairman *et al*., 2011; Kumar *et al*., 2010). OmpF has a slightly larger pore size compared to OmpC; thus the proportions of these porins in the outer membrane can affect the type and amount of solutes that can enter the cell (Fernández & Hancock, 2012; Marisa & Page, 2012). Homologues of the OmpF and OmpC porins serve as ports of entry for β-lactam compounds in *E. coli* and other enterobacteria (Pagès *et al*., 2008; Yoshimura and Nikaido, 1985). Thus, members of the *Enterobacteriaceae* genus can modify their resistance to antibiotics by changing their outer membrane permeability as mediated by the outer membrane porins.

Environmental conditions can also dramatically alter OmpF and OmpC porin expression. In *E. coli* under conditions of low osmolarity, expression of OmpF is upregulated to facilitate the influx of nutrients. Conversely, in high osmolarity conditions (i.e. nutrient rich environment) or acidic pH, OmpF expression is downregulated and OmpC is upregulated, resulting in increased resistance to β-lactams. Other environmental signals (high temperature, oxidative stress, salicylate) also modulate porin expression, which is governed by the two-component regulator OmpR-EnvZ and antisense *micF*, a post-transcriptional regulator of OmpF translation (Barbosa & Levy, 2000; Fernández & Hancock, 2012; Hall & Silhavy, 1981). As in the *E. coli* system, expression levels of OmpF and OmpC in *S. marcescens* also appear to be influenced by physiochemical conditions that include pH, temperature and osmotic stress (Begic & Worobec, 2006).

Intrinsic β-lactamase production is a common feature among *Enterobacteriaceae* and a few other organisms. Disruption of peptidoglycan murein biosynthesis by a β-lactam agent leads to activation of the transcriptional regulator AmpR by the induction signal, an *N*-acetyl-anhydromuramylpeptide released from β-lactam-damaged peptidoglycan. AmpR initiates expression of chromosomally-encoded molecular class C β-lactamase AmpC. Induction of

AmpC is negatively controlled by AmpD, an *N*-acetyl-anhydromuramyl-L-alanine amidase, which degrades the induction signal (Jacoby 2009; Pfeifer *et al*., 2010). Carbapenem antibiotics are often used to combat AmpC-producing bacteria as the structure is highly resistant to most β-lactamases; however, resistance to carbapenems can arise in bacteria by mutations that reduce permeability (outer membrane porin loss) or increase efflux (efflux pump activation) (Jacoby, 2009; Pfeifer *et al*., 2010).

Several studies elsewhere have characterized multi-drug resistant *S. marcescens* clinical strains with altered or defective OmpF and/or OmpC porin expression as well as over-production of AmpC β-lactamase (Hechler *et al*., 1989; Hashizume *et al*., 1993; Weindorf *et al*., 1998; Suh *et al*., 2010). These reports prompted us to conduct direct genetic studies to further investigate the interplay between the presence of the porins and antibiotic susceptibility. Here we report an alternate process of the construction of single and combinatorial porin gene unmarked deletions in the *S. marcescens* Db11 strain background. We found that the deletion of OmpF, but not OmpC, altered the antibiotic susceptibility profile. To our knowledge, this study is the first study that describes, on the molecular level, the role of OmpF porin in antibiotic resistance of *S. marcescens*.

4.3. GENERATION OF PORIN MUTANT STRAINS.

S. marcescens Db11 is the first strain of *S. marcescens* to have its genome sequenced and annotated leading to a proposal of this strain as the reference standard strain by *S. marcescens* researchers (Petty *et al*., 2006). The identities were 96% and 99% for OmpF and OmpC, respectively, on the amino acid level when compared to orthologs in *S. marcescens* UOC-51. Figure 4.1 outlines the strategy for allelic gene exchange in generating Δ*ompF* (ATF101) and Δ*ompC* (ATC101) strains; and table 4.1 shows the primer used to generate the mutants. Creation of the double-porin Δ*ompF*Δ*ompC* (ATFC01) mutant strain was achieved

by general transduction. The genetic organization of the separate *ompF* and *ompC* loci in *S. marcescens* Db11 are schematically displayed in Figure 4.2.

4.4. OUTER MEMBRANE PROTEIN PROFILES.

The presence or absence of OmpF and/or OmpC porins in wild-type and isogenic mutants were evaluated by SDS-PAGE (Figure 4.3). The molecular size of the OmpF and OmpC porins is predicted to be at approximately 41 kDa and 40 kDa, respectively. In comparison to the wild-type protein profile, a high-intensity band corresponding to the size of 41 kDa was absent in the protein profile of the Δ*ompF* strain revealing the presence of the underlying low-intensity band corresponding to 40 kDa.

Due to the overlapping band sizes of 41 kDa and 40 kDa as well as the differential band intensities correlating to the amount of porin protein, the absence of the OmpC porin was not observed in the protein profile of the ATC101 strain. However, deletion of both OmpF and OmpC porins resulted in the complete absence of bands corresponding to the 41 kDa and 40 kDa sizes in the protein profile of the ATFC01 strain. No other alterations in the outer membrane protein profiles were observed. Complementation *in trans* of OmpF and OmpC appears to have returned the phenotype of the 41 kDa and 40 kDa size bands in the protein profiles of ATF242 and ATC50 strains, respectively. The band intensities appear to be higher, more so for ATF242 than for ATC50, in comparison to the wild-type protein profile which is presumably due to more than one plasmid-borne copy of the porin gene. To verify *in trans* complementation of OmpF and OmpC, the plasmid constructs pUFR::*ompF* and pUFR::*ompC* were introduced into the double porin Δ*ompF*Δ*ompC* deletion mutant strain, respectively, for SDS-PAGE analyses. Outer membrane protein profiles of ATFC242 and ATFC50 both indicated the return of the 41 kDa and 40 kDa size bands, respectively, in the double porin mutant strain background (Figure 4.3).

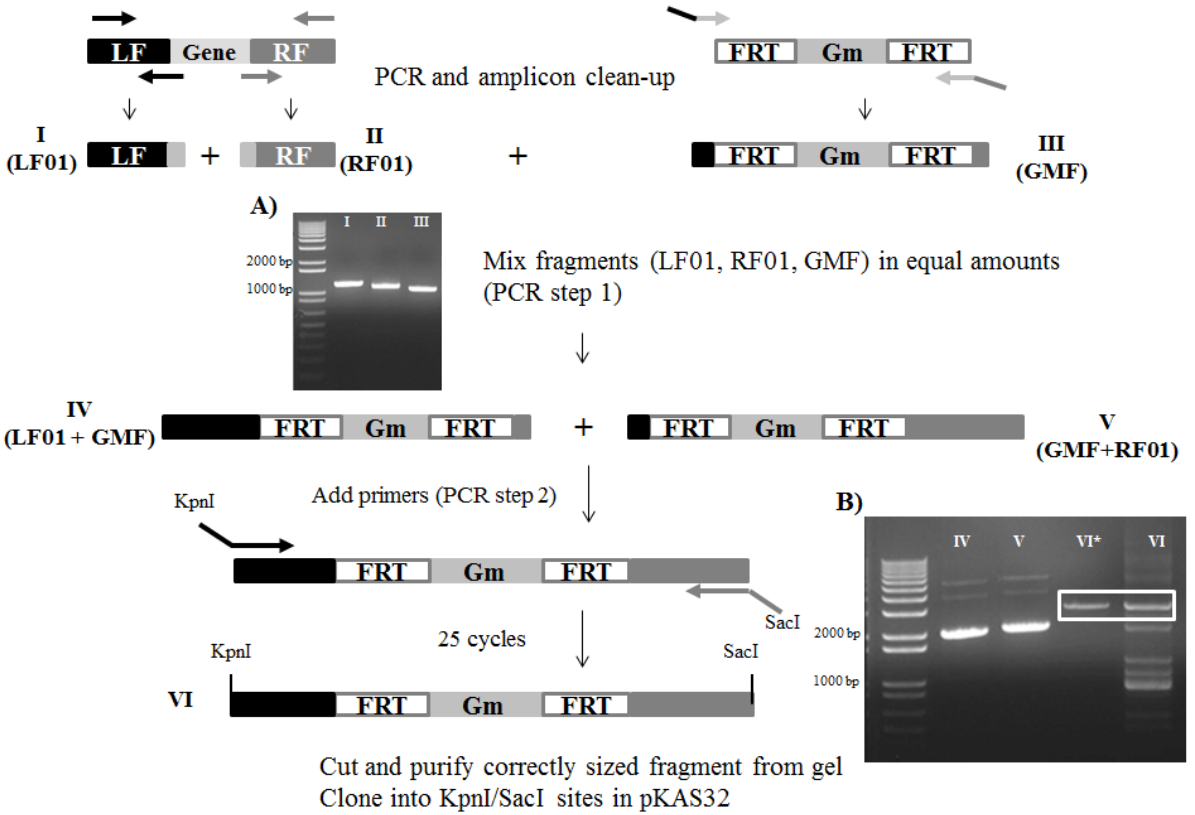

Figure 4.1. Schematic of the allelic gene exchange strategy employed to generate the Δ*ompF* (ATF101) isogenic mutant strains. Fragments I, II, and III refer to LF01, RF01, and GMF amplicons, respectively. Fragments IV and V refer to paired LF01+GMF and GMF+RF01 amplicons, respectively. Fragment VI refers to the 3,301 bp fragment comprising LF01+GMF+RF01 stitched together by PCR. Inset agarose gel photograph demonstrates the fragments IV, V, and VI. Fragment VI* refers to the correctly sized amplicon after purification. Please see text for further details.

TABLE 4.1. Primer sets and annealing temperatures of amplicons

Amplicon; (primer set); [annealing temperature °C]	**Product size (bp)**
LF01 (PF*ompF*/RB*ompF*01) [57.5]	892
RF01 (PR*ompF*/LE*ompF*01) [57.5]	1069
GMF(PF-FRTGm/PR-FRTGm) [54.3]	1107
LC01 (PF*ompC* left/PR(Left)*ompC*) [57.5]	1162
RC01 (PR*ompC* right/PF(Right)*ompC*) [57.5]	1032
GMC ((PF-FRTGmFRT (*ompC*) /PR-FRTGmFRT (*ompC*)) [60.3]	1107
CompF (PF*ompF*1 EcoRI/ PR*ompF SphI*) [57.5]	1458
CompC (PF*ompC*1 *EcoRI*/ PR*ompC SphI*) [57.5]	1362
FD (PF*ampD* Flk(*KpnI*)/ PR(N)*ampD* Left) [57.5]	1144
LD (PR *ampD* Flk *SacI*/PF *ampD* Right) [57.5]	1432
GMD (PF*ampD*(N)KO(Gm)/ PR*ampD* KO(Gm)) [57.5]	1112

4.5. GROWTH KINETICS AND CELL MORPHOLOGY OF PORIN DELETION STRAINS.

The absence of OmpF and/or OmpC porins may have affected the integrity of the cell envelope or perhaps alter the influx of nutrients into the cell. Interestingly, the growth curves of the wild-type, isogenic single and double porin mutant strains were similar in profile indicating that the growth rate of the mutant strains in LB media at 37^{o}C were unaffected in comparison to the wild-type (Figure 4.4). Likewise, growth of the single (ATF101, ATC101) and double porin (ATFC01) porin mutant strains on deoxycholate growth medium was unaffected in comparison to that of the wild-type Db11 strain (data not shown).

Differential Interference contrast (DIC) microscopic imaging of the single and double porin mutant strains did not reveal any drastic changes in cell morphology (Figure 4.5). Given that OmpF and OmpC belong to the classical porin family, it is quite possible that other members of the family are able to perform compensatory functions in the absence of OmpF and OmpC.

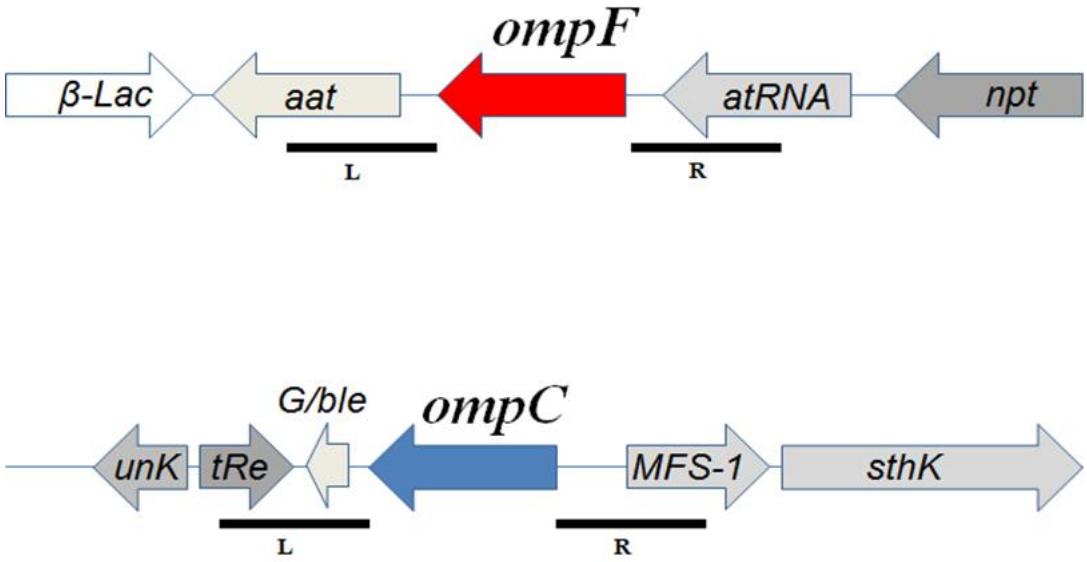

Figure 4.2. Physical map of the *ompF* and *ompC* genes in *S. marcescens* Db11. DNA fragments in red and blue are the region that were deleted from the *ompF* and *ompC* mutant strains, respectively. Flanking genes were annotated via alignment of *S. marcescens* Db11 with other *Serratia* strains with gene information available. Metallo β-lactamase domain (*β-lact*); aspartate aminotransferase (*aat*); Asparagine-tRNA ligase synthetase (*atRNA*); Nicotinate phosphoribosyl transferase (*npt)*; Unknown gene function (*unK)*; Transcriptional regulator TetR family (*tRe*); Glyoxalase/bleomycin resistan protein dioxygenase (*G/ble*); Major facilitator superfamily (MFS-1); Multi-sensor signal transduction histidine kinase (*sthK*).

4.6. ANTIBIOTIC SUSCEPTIBILITY OF PORIN DELETION STRAINS.

While the absence of OmpF and OmpC porins did not appear to have an effect on the influx of nutrients, it may be that the cell wall permeability of antibiotics was affected resulting in a change in antibiotic susceptibility profiles. To determine if deletion of *ompF* and/or *ompC* altered the antibiotic susceptibility profile, wild-type and porin mutant strains were subjected MIC assays with antibiotics currently used in clinical applications (Table 4.2). Increased MICs to the β-lactam agents (ampicillin, cefoxitin) and nitrofurantoin was observed in strains that lack *ompF*. Deletion of *ompC* did not appear to significantly alter the antibiotic susceptibility profile. However, an increase in the MIC value was noted for ertapenem in the double Δ*ompF*Δ*ompC* mutant strain. Interestingly, a decrease in MIC value was observed for tigecycline, a new member of the glycylcyclines (Namdari *et al.*, 2012), for strains lacking *ompF*, suggesting a possible implication of this porin with other antimicrobial mechanisms. *In trans* complementation of OmpF in the Δ*ompF*Δ*ompC* strain background (ATFC242) did not completely reverse the MIC changes to that of the single Δ*ompC* mutant strain. Increased resistance to ampicillin and cefoxitin as well as increased susceptibility to meropenem is still noted. However, *in trans* complementation of OmpC in the Δ*ompF*Δ*ompC* strain background (ATFC50) more closely matched the MICs to that of the single Δ*ompF* mutant strain; the only exception was for tigecycline which the MIC value matched that of wild-type.

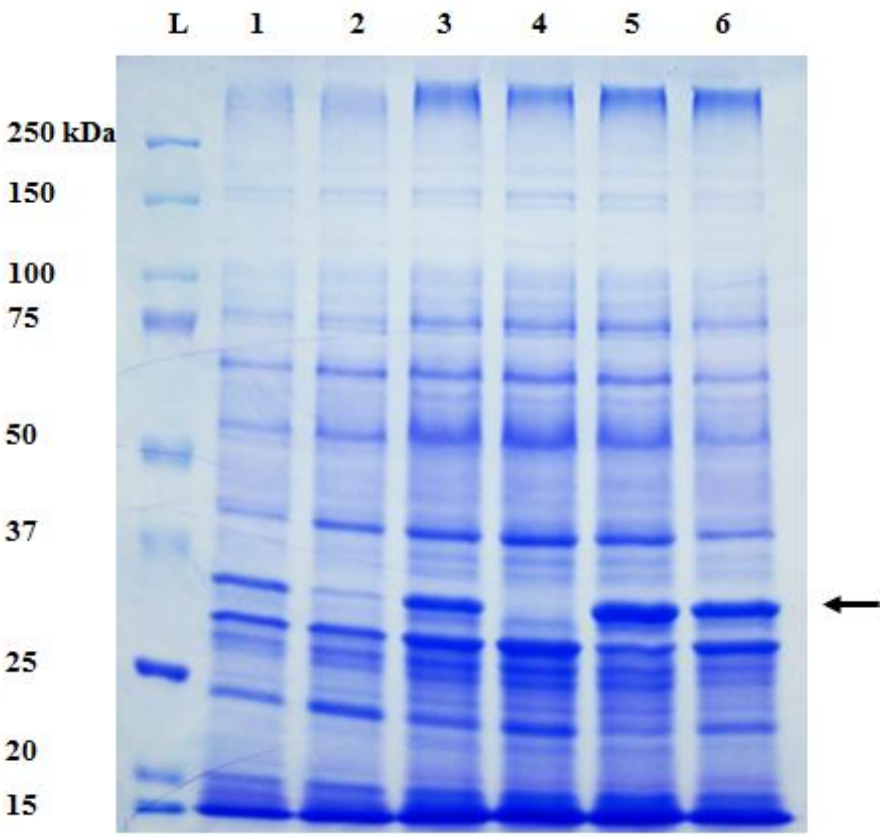

Figure 4.3. Outer membrane protein profiles from *S. marcescens* Db11 wild type, mutant and complemented strains grown in LB liquid medium at 37 °C. Lane L: Protein MW Ladder, lane 1: Db11 wild type, lane 2: ATF101 (Δ*ompF*), lane 3: ATC101 (Δ*ompC*), lane 4: ATFC01 (Δ*ompF*Δ*ompC*), lane 5: ATF242, lane 6: ATC50. An arrow indicates missing bands corresponding to overlapping predicted sizes of OmpF and OmpC porins.

Given the fact that the copies of *ompF* and *ompC* are plasmid-borne with low copy number and that the concentration of OmpF has been found elsewhere to be much higher than that of OmpC (Fernandez & Hancock, 2012; Masi & Page, 2013), it is appears that in our study that complementation is partially achieved with OmpF and overachieved with OmpC in the Δ*ompF*Δ*ompC* strain background. Sensitivity of wild-type, porin mutant and complemented strains to imipenem was unaltered.

4.7. OMPF MEDIATES UPTAKE OF CEFOXITIN.

Whereas the lack of OmpF can contribute to increased MIC values to some antibiotics, intrinsic AmpC β-lactamase production must also be considered as a possible contributing factor. To assess whether the deletion of OmpF and/or OmpC affected expression of AmpC β-lactamase when induced by cefoxitin, the β-lactamase activity assay was implemented (Table 4.3). As expected, cefoxitin induced β-lactamase activity by 150-fold for the wild-type strain Db11. In correlation with the MIC assays, deletion of *ompC* did not alter β-lactamase activity in comparison to wild-type. However, induction of β-lactamase was reduced more than 3 times in strains lacking *ompF* (ATF101 and ATFC01) in the presence of cefoxitin when compared with the wild-type strain. As induction of AmpC is negatively controlled by AmpD which degrades the induction signal (Pfeifer *et al*., 2010), it must be ascertained that the decrease in AmpC β-lactamase production is the result of reduced permeability of cefoxitin due to lack of OmpF porin and not due to increased intrinsic AmpD expression. To this end, single Δ*ampD* (ATD101) and combinatorial gene deletion Δ*ampD*Δ*ompF* (ATFD01) and Δ*ampD*Δ*ompF*Δ*ompC* (ATFCD1) strains were generated. Deletion of *ampD* did not alter the β-lactamase activity level in comparison to the wild-type. Thus, the decreases in the β-lactamase activity levels of the ATFD01 and ATFCD1 strains are attributed to the absence of *ompF* as levels are similar to those of

ATF101 and ATFC01, rather than altered *ampD* expression. Taken together, our data suggest that uptake of ceftoxin is mediated only by OmpF porin.

4.8. DISCUSSION

Mutations that lead to the loss, down-regulation or alteration of porins have a direct impact on the rate at which antibiotics can enter the cell, and enhance the influence of resistance mechanisms such as efflux pumps and β-lactamases (Fernandez & Hancock, 2012; Masi & Pages, 2013). The classical porins OmpF and OmpC serve as ports of entry for hydrophilic molecules such as β-lactams, and their absence or modification can limit the access of antimicrobials to most inner bacterial targets. In this study, we provide direct genetic evidence that increased antibiotic resistance of *S. marcescens* is correlated to the absence of the classical porin OmpF. Furthermore, to achieve our objectives in this study, we pioneered a protocol to generate clean unmarked deletion porin mutant strains via allelic exchange and FLP-*FRT* strategies thereby preventing incorporation of additional antibiotic resistance that would otherwise have been attributed to the use of an antibiotic marker cassette.

We found that in the absence of OmpF, increased MIC was noted for the β-lactam agents ampicillin and cefoxitin. It was reasonable to assume that the increased MIC was attributed to the reduced permeability of cefoxtin due to the lack of OmpF; however, it has been reported elsewhere that bacterial resistance to cefoxitin is also considered to be an indicator of AmpC production (Jacoby, 2009). β-lactam drugs, such as cefoxitin and meropenem, can induce AmpC β-lactamase production (Mahlen, 2011). Cefoxitin, a member of the β-lactam group called cephems, can penetrate Gram-negative bacteria outer membranes via entry through aqueous channels such as OmpF and OmpC porin structures, and is a strong inducer of AmpC β-lactamase (Vidal, 2005).

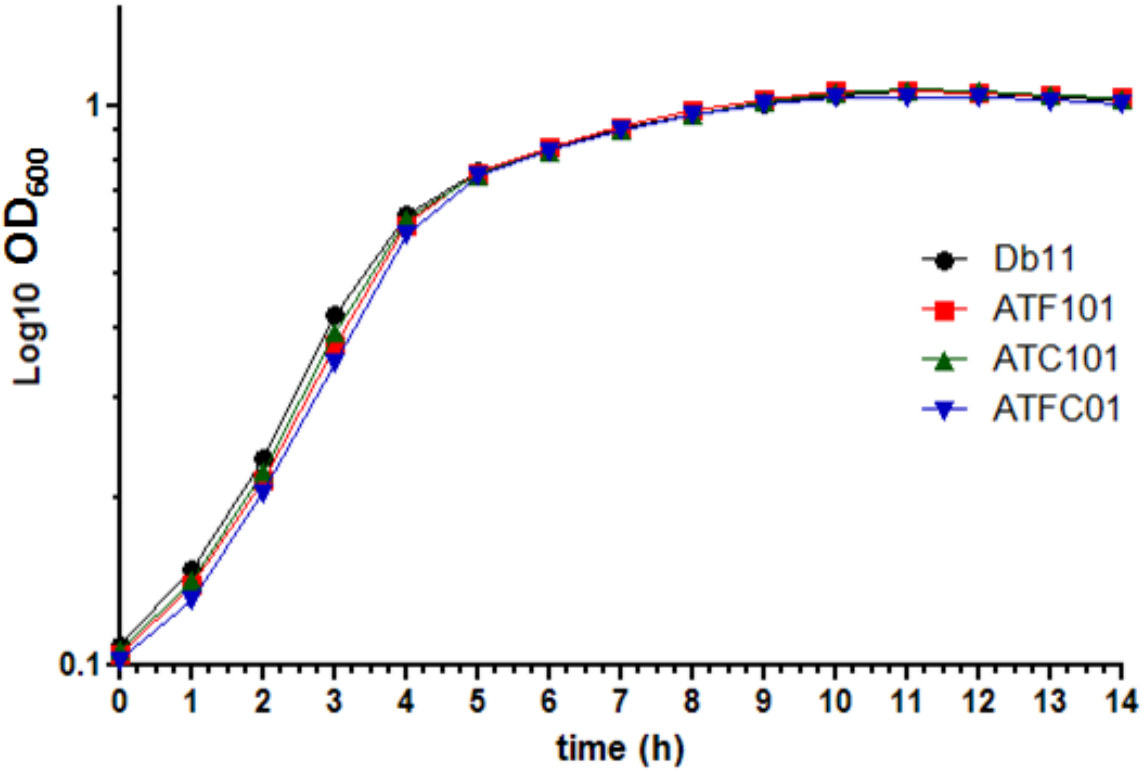

Figure 4.4. Growth curves of *S. marcescens* Db11 and isogenic porin mutants. Bacteria were grown in LB medium at 37 °C over a time period of 18 hours.

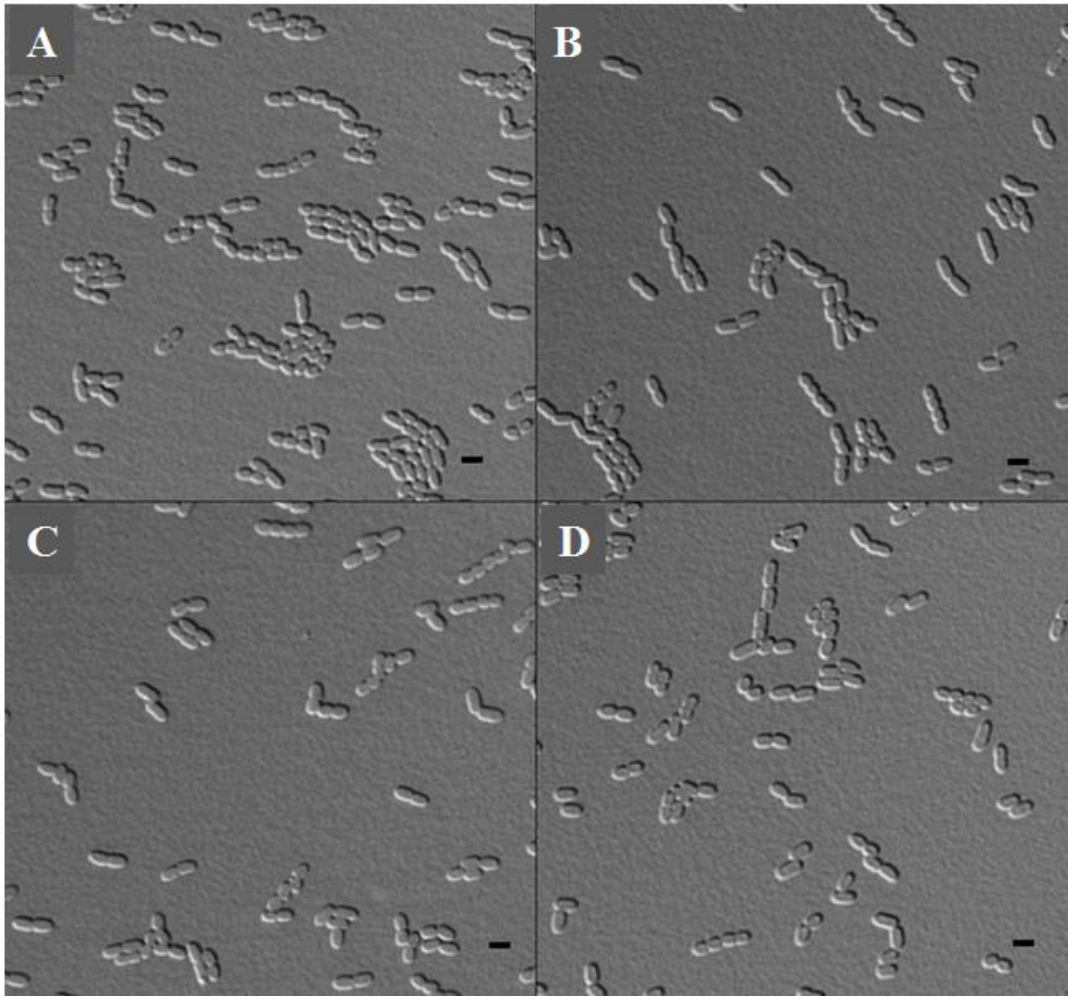

Figure 4.5. Bacterial morphology. Differential Interference Contrast (DIC) micrographs of *S. marcescens* strains grown to mid-log phase on LB agar. A: Db11 wild type; B: ATF101 (Δ*ompF*); C: ATC101 (Δ*ompC*); D: ATFC01 (Δ*ompF*Δ*ompC*). Scale bar is 2μm.

Specifically, perturbation (i.e. dysfunctional AmpD) in the induction pathway can lead to constitutive overexpression of *ampC* gene and increase the resistance to more β-lactam drugs (Mahlen *et al*., 2003). We investigated the involvement of the AmpC β-lactamase induction pathway in the absence of OmpF, and found that the genetic deletion of the inhibitor AmpD had no effect on the overall AmpC β-lactamase production in the absence of OmpF when induced by cefoxitin. In further support of the involvement of OmpF porin in modulating antibiotic resistance, we demonstrated that the antibiotic susceptibility phenotype could be partially complemented by plasmid-borne copy of *ompF*. Surprisingly, absence of the classical porin OmpC did not seem to significantly affect the antibiotic susceptibility profile suggesting that unlike *E. coli*, the *S. marcescens* OmpC porin does not play a role in the import of antibiotics tested in our study.

Our findings correlate with previous reports detailing altered porin expression and elevated intrinsic β-lactamase production in *S. marcescens* clinical isolates (Hechler *et al*., 1989; Hashiszume *et al*., 1993; Weindorf *et al*., 1998). One report examined two *S. marcescens* clinical isolates found to have altered porin (OmpF and OmpC) expression, which resulted in increased antibiotic resistance to penicillins and cephalosporins (Hashiszume *et al*., 1993). However, these strains were still susceptible to the carbapenem drug imipenem. Likewise, in this study the single and double isogenic porin mutants were also found to be sensitive to imipenem. Interestingly, another study reported the involvement of porins as well as the AmpC β-lactamase induction pathway. Several clinical isolates were characterized and found to possess differing MIC values isolates displaying resistance to the β-lactam antibiotics (cefotaxime, ceftizoxime, ceftazidime, aztreonam, latamoxef, cefoxitin, ampicillin, imipenem) featured intrinsic β-lactamase (AmpC) overproduction, whereas isolates with higher MICs harboured defects in one or both of the OmpF and OmpC porins in addition to AmpC overproduction (Hechler *et al*., 1989; Weindorf *et al*., 1998).

Table 4.2. Minimal inhibitory concentrations of selected antibiotics in *S. marcescens* Db11 wild type and isogenic mutant strains (≥2-fold differences in MIC values of the mutant strains when compared to the values of the wild-type Db11 strain are underlined).

Antimicrobial	**Db11**	**ATF101**	**ATC101**	**ATFC01**	**ATF242**	**ATC50**
	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)	MIC (μg/ml)
Ampicillin	16	≥32	16	≥32	≥32	≥32
Cefoxitin	8	≥64	8	≥ 64	32	≥ 64
Tigecycline	2	1	2	1	2	2
Ertapenem	≤0.5	≤ 0.5	≤0.5	1	≤0.5	≤0.5
Meropenem	≤0.25	≤ 0.25	≤0.25	≤0.25	≤0.25	≤0.25
Imipenem	0.75	0.75	0.75	1	0.75	1
Nitrofurantoin	128	256	128	256	128	256

Table 4.3. Levels of β-lactamase activity in cell lysate obtained from *S. marcescens* Db11 and isogenic mutant strains after induction with cefoxitin (10 μg/ml). Basal and induced activity was measure in units of nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

Strain	Average β-lactamase ± SD	
	Basal	Induced
Db11	5.7 ± 0.2	854.0 ± 71.1
ATF101	5.6 ± 0.3	259.0 ± 39.4
ATC101	6.5 ± 2.0	811.8 ± 161.9
ATFC01	7.8 ± 1.8	194.9 ± 13.6
ATD101	7.2 ± 1.6	920.7± 23.5
ATFD01	9.9 ± 0.6	156.3 ± 70.9
ATFCD1	9.9 ± 1.2	117.3 ± 40

Interestingly, overproduction of AmpC was further facilitated by mutations in *ampD* that encodes an inhibitor of AmpC production. Characterization of meropenem-resistant *S. marcescens* clinical isolates from a hospital outbreak in South Korea revealed AmpC overproduction and loss of OmpF porin due to disruption of the *ompF* gene by an *IS1* mobile genetic element (Suh *et al*., 2010). In light of these reports, our findings that the deletion of *ampD* did not increase AmpC β-lactamase production in the absence or presence of OmpF and/or OmpC were confounding. However, the possibility exists that there are paralogs of *ampD* in *S. marcescens* as such the case with *Pseudomonas aeruginosa*. Indeed, there are three *ampD* paralogs in *P. aeruginosa* of which all three must be inactivated to elevate β-lactamase levels (Schmidtke & Hanson, 2008). In this view, further BLAST analyses of *S. marcescens* Db11 genome revealed two *ampD* paralogs in addition to the *ampD* paralog that was initially deleted. Thus, efforts are underway to generate an isogenic mutant strain in which all three *ampD* paralogs are inactivated

Bacterial adaptations to the environment imply strict regulation of cell envelope permeability. OmpF has a slightly larger pore size compared to OmpC; thus the proportions of these porins in the outer membrane can affect the type and amount of solutes that can enter the cell. Surprisingly, deletion of one or both porins did not appear to affect bacterial growth kinetics nor did it significantly affect cell morphology of the single and combinatorial porin deletion strains. It is quite possible that other porins in the cell envelope may function in a compensatory role to facilitate intake of nutrients and maintain cell envelope integrity. In *E. coli,* the LamB porin and the PhoE porin are trimers responsible for the diffusion of maltodextrins and phosphate, respectively (Cowan *et al*., 1992; Schirmer *et al*., 1995). PhoE, a part of the Pho regulon, is expressed under phosphate starvations and regulated by the two component system

PhoB/PhoR (Lamarche *et al*., 2008). The *lamB* gene is part of the maltose operon and is regulated according the availability of sugars. Orthologous of LamB and PhoE are present in *S. marcescens*, but these porins have not yet been characterized. Thus, one may speculate that molecules may use these porins in a non-specific manner; however, this scenario is unlikely as LamB and PhoE are regulated by different systems. Nevertheless, studies are ongoing to ascertain the involvement, if any, of other porins in maintaining cell envelope integrity.

The presence of antibiotics coupled with physiochemical conditions in the human host may select for changes, on the molecular level, in porin expression and intrinsic β-lactamase production in *S. marcescens*. Taken together, these studies highlight the concern for the emergent rise of carbapenem resistance in *S. marcescens* due to antimicrobial pressure placed on the AmpC-producing nosocomial pathogens for positive selection of porin-deficient organisms.

ACKNOWLEDGEMENTS

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CHAPTER 5. BACTERIAL PATHOGENITICITY OF *S. marcescens* PORIN MUTANTS IN THE *C. elegans* HOST MODEL.

The genetically tractable microbivorous soil nematode *C. elegans* has been well established as a host model for immunopathogenesis studies on a variety of human bacterial pathogens including *S. aureus, S. epidermidis*, and *P. aeruginosa* (Kurz *et al.*, 2003; Powell & Ausubel, 2008; Sifri *et al.*, 2005). Once consumed as a food source, the pathogenic bacteria generally colonize the intestinal lumen overcoming the activated innate immune defense resulting in a shortened nematode lifespan. The transparency of the nematode body provides an advantageous feature in that the infectious process can be easily monitored over time using microscopy providing useful information with respect to microbe-host interactions.

C. elegans has been established as a suitable host model to demonstrate bacterial pathogenicity of *S. marcescens* strains (Kurz *et al.*, 2003; Kurz & Ewbank, 2000). This approach was employed in a previous study that involved the generation of a 2300-member transposon (mini-*Tn5*Cm) insertion library created in the *S. marcescens* Db11 strain background. Using the *C. elegans* host model, this library was screened for changes in bacterial pathogenicity of which 23 clones were found to be attenuated for virulence (Kurz *et al.*, 2003). In this work, the *C. elegans* host model was used to assess differences, if any, in virulence of the isogenic porin mutant strains when compared to the virulent phenotype established for the parental Db11 strain. As outlined in Chapter 3, environmental conditions (i.e. temperature and nutrient availability) influence expression of porins that aid in modulating bacterial cell membrane permeability to maintain homeostasis. Based on this premise, it is hypothesized that the porin mutant strains will be attenuated for virulence due to the environmental conditions present within the *C. elegans* intestinal lumen. Indeed, the pH of the intestinal lumen was recently determined to be acidic

ranging from 5.96 ± 0.31 in the anterior pharynx to 3.59 ± 0.09 in the posterior intestine (Chauchan *et al*., 2013). In addition to the grinder and intestinal enzymes, the pH of the intestinal lumen contributes to diminish the effect of pathogen in the gut; however pathogenic bacteria can survive and colonize the organ. The acidic pH in the *C. elegans* intestine helps to maintain active digestive enzymes for degradation of proteins and carbohydrates. Bacterial pathogens inside the intestine must to adapt to this environment during colonization. The cell envelope may play a crucial role in maintaining bacterial fitness during bacterial infection, by protecting the cell from the wide pH range. In lab conditions, the nematodes live in buffered media (generally NGM, pH 6), so to keep an acidic pH where digestive enzymes such as hydrolases (optimal activity pH 5) are active, the nematode has to maintain the optimal pH in the intestine. *S. marcescens* seems to be able to resist these conditions and to produce proteases that inactivate enzymes and protective mediators that normally control bacterial infection. Furthermore, the limited nutrient availability in the *C. elegans* intestinal lumen and in the nematode growth medium (NGM) coupled with the survival assay temperature (25 ^{o}C) may also further restrict bacterial growth kinetics and pathogenicity due to the lack of OmpF and/or OmpC. The hypothesis that the lack of OmpF and/or OmpC porins affects bacterial fitness thereby decreasing *S. marcescens* virulence is supported by experimental evidence that nematodes infected with a transposon insertion in *phoE* resulted in attenuated virulence (personal communication with Jonathan Ewbank). Given that PhoE is categorized as a classical porin, it is then surmised that lack of the classical porins OmpF and/or OmpC may also result in attenuated virulence in the *C. elegans* host model.

5.1. RESULTS.

5.1.1. Outer membrane profiles of *S. marcescens* strains grown in different environmental conditions.

Changes in environmental conditions may alter porin expression and thus affect bacterial fitness. However, the lack of one or more porins may dramatically affect the overall profile of porin expression, perhaps severely impacting bacterial fitness. In order to evaluate the effects of temperature and growth conditions on porin expression profiles of single and combinatorial porin mutant strains, outer membrane extracts were obtained from each mutant grown in LB and NGM, and compared using SDS-PAGE (Figure 5.1). NGM is the growth medium previously used for *C. elegans* survival assays to assess the pathogenesis of *S. marcescens* strains. NGM has lower levels of nutrients and NaCl than the LB medium commonly used to grow *S. marcescens* strains. The growth temperatures of 25 °C and 37 °C were also included in the environmental conditions test to assess changes, if any, in outer membrane profiles. It should be noted that all strains were grown in LB liquid medium at 37 °C prior to subculturing on NGM and LB liquid media at the two different growth temperatures. As observed in Figure 5.1, the pattern of band sizes corresponding to certain porin types differed in intensity when comparing the two growth medium types. In each LB sample at 25 °C and 37 °C, there is a band at approximately 20 kDa that is unknown and is not present in NGM-grown samples. For all samples evaluated, bands corresponding to the sizes of PhoE and LamB appear to not be highly induced although it is interesting to observe that the band associated with OmpA in the double mutant ATFC01 (Δ*ompF*Δ*ompC*) strain showed a high intensity. This protein has been well-characterized to contribue to maintainance of outer membrane integity (Confer & Ayalew, 2013; Smith *et al.*, 2007). In all samples at 25 °C and 37 °C, the band size associated with OmpX did not appear

altered. The optimal growth temperature of *S. marcescens* is 30 °C; however, it was interesting to see the influence of 25 °C and 37°C on the overall expression of porins; these are the same temperatures as nematode and human body respectively. The increase of temperature in the expression has been seen to affect the expression of *ompF* and *ompC* in *S. marcescens* (Begic & Worobec, 2006). This study indicated that the overall OMP profile appeared altered in NGM, in comparison to LB medium, in that the profile indicated reduced as well as increased levels of different OMP for all strains tested. This suggests the bacterial fitness of the strains may be dependent on the available nutrients and temperature in that the overall OMP profile changes to adapt to limiting nutrient conditions. Furthermore, adaptation to limiting nutrient conditions may also compromise bacterial pathogenicity of porin mutant strains.

5.1.2. *S. marcescens* porin mutant strains are hypervirulent in the *C. elegans* survival assay.

The *C. elegans* survival assay was used to detail the pathologic differences in nematodes infected with the porin mutant strains (ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*)) and the wild type Db11. Typically, L4 stage *C. elegans* nematodes are used in the survival assay. Younger larval stages are resistant to bacterial infection; thus, older nematodes are more suitable for the survival assay (Kurz & Tan, 2004). One possible reason for age-dependent resistance/susceptibility to infection is that the grinders are much more efficient in younger nematodes than in older nematodes (Kurz & Tan, 2004). Once in the intestinal lumen, the virulence of the bacterial pathogen can be mitigated by the triggered innate immune response which may or may not effectively eliminate the bacterial pathogen depending on the defense measures (i.e. biofilm, toxins, etc.) employed by the pathogen (Begun *et al.*, 2007; Irazoqui *et al.*, 2010b). Thus, the selection of the L4 stage is based on a compromise between susceptibility

and resistance that will allow the observation of hypo- and hypervirulent phenotypes of the bacterial species in question.

To confirm the survival rate of *C. elegans* N2 infected with *S. marcescens* Db11 previously reported by Kurz *et al.* (2003), a survival assay was conducted with Db11 as wild type strain, along with the previously characterized attenuated mutant strains 20C2 and 21C4 (Figure 5.2). The 20C2 and 21C4 strains were generated via transposon mutagenesis with mini-*Tn5*Cm. The 20C2 strain contains a defect in the expression of *wzm*, a gene involved in O-chain of LPS biosynthesis, and the 21C4 strain is a mutant for *shlB*, an outer membrane protein involved in the activation and secretion of ShlA hemolysin (Kurz *et al*. 2003). As shown in Figure 5.2, the survival rate of nematodes infected with Db11 was reduced in comparison to nematodes infected with the attenuated mutant 20C2 and 21C4 strains.

Approximately 50% of the nematode population survived at 5 days (120 hours) and 7 days (168 hours) post-infection with Db11 and 20C2, respectively. The 21C4 strain showed a slightly less attenuated phenotype than the 20C2 strain. Thus, the survival assay results trended with those reported in Kurz *et al*. (2003). Interestingly, for both 20C2 and 21C4 mutant strains, the nematode population steadily decreased over time on the assay plates. As the L4 stage nematodes developed into gravid adults giving rise to progeny on the assay plates, an effect on the development of nematode progeny was observed; maturation of the progeny was slowed.

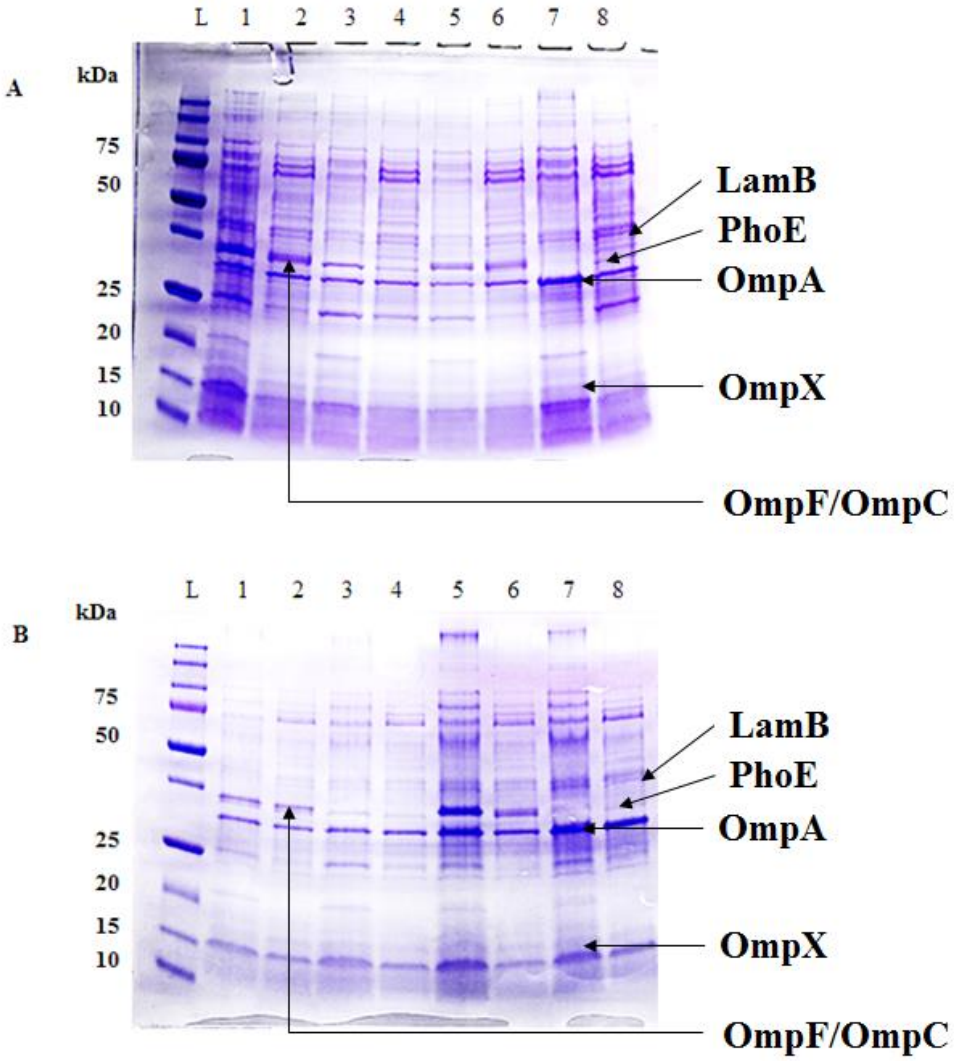


Figure 5.1. Environmental conditions affect outer membrane protein profiles. Outer membrane protein extracts were prepared from *S. marcescens* strains grown in two types of media (LB and NGM) at two temperatures A) 25 ^{o}C and B) 37 ^{o}C and run on SDS-PAGE gels. Lanes are molecular weight ladder (L), Db11 in LB (1) and NGM (2), ATF101 (Δ*ompF*)in LB (3) and NGM (4), ATC101 (Δ*ompC*) in LB (5) and NGM (6), and ATFC01 (Δ*ompFompC*) in LB (7) and NGM (8). Gels represent the results of one out of three identical PAGE.

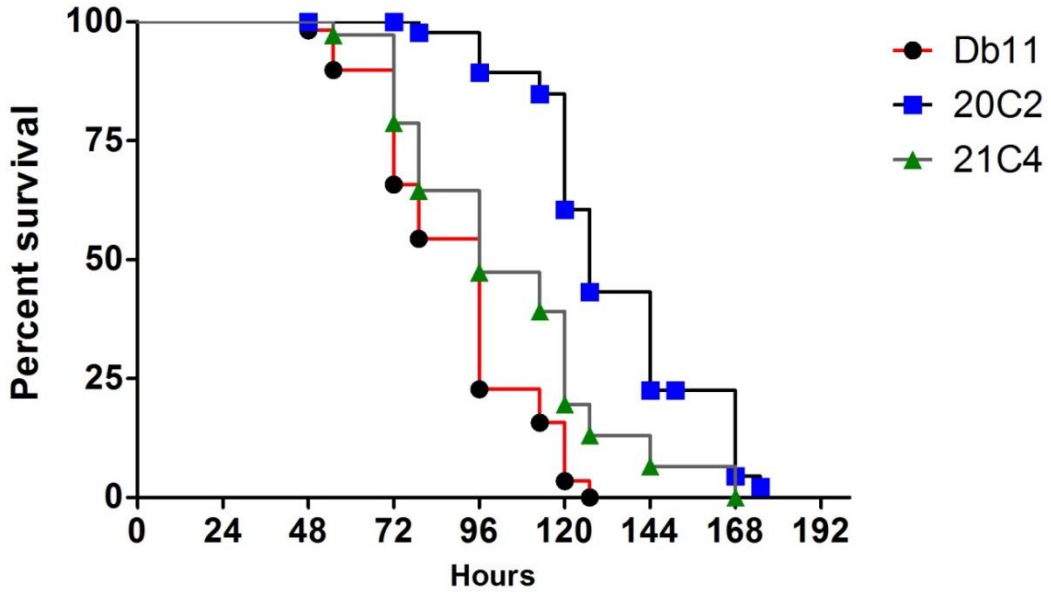


Figure 5.2. Survival of *C. elegans* nematodes infected with *S. marcescens* strains. Kaplan-Meier survival curve of *C. elegans* N2 nematodes infected with Db11 (circle, n= 114) and attenuated mutants 20C2 (squares, n= 101), 21C4 (triangle, n= 118). $P<0.0001$ by pairwise comparison (20C2 and 21C4) vs Db11 by the log-rank (Mantel-Cox) test. Survival curve is representative of one of three independent experiments. The strain 20C2 is a mutant for LPS O-antigen and strain 21C4 is a mutant for hemolysin production

Surprisingly, nematodes infected with single and combinatorial porin mutant strains appeared to have increased virulence in *C. elegans* as demonstrated by reduced survival rates in comparison to nematodes infected with Db11 (Figure 5.3). Nevertheless, the small but significant differences (log rank test), may indicate heightened virulence on a cellular level that is not immediately apparent on the organismal level. To further investigate the differences in the virulence mechanisms employed by the porin mutant strains, detailed microscopic examinations of the infected nematodes were required to shed light on the infectious process of the porin mutant strains.

5.1.3. Microscopic observation of the infectious process using GFP-tagged *S. marcescens*.

In order to collect more information of the infectious process inside the nematode and the organs affected, the infection was followed in detail over time using microscopy. It should be noted that nematodes are naturally autofluorescent, and that increased autofluorescence occurs when nematodes are infected by a bacterial pathogen (Baeriswyl *et al.*, 2010). The autofluorescence is produced by granules within intestinal cells commonly known as gut granules (Clokey & Jacobson, 1986). Gut granules have acidic pH and function as secondary lysosomes (Clokey & Jacobson, 1986; Schroeder *et al.*, 2007). However, the birefringence of the gut granules can be digitally muted using microscopy software to facilitate observation of GFP-tagged bacteria within infected nematodes.

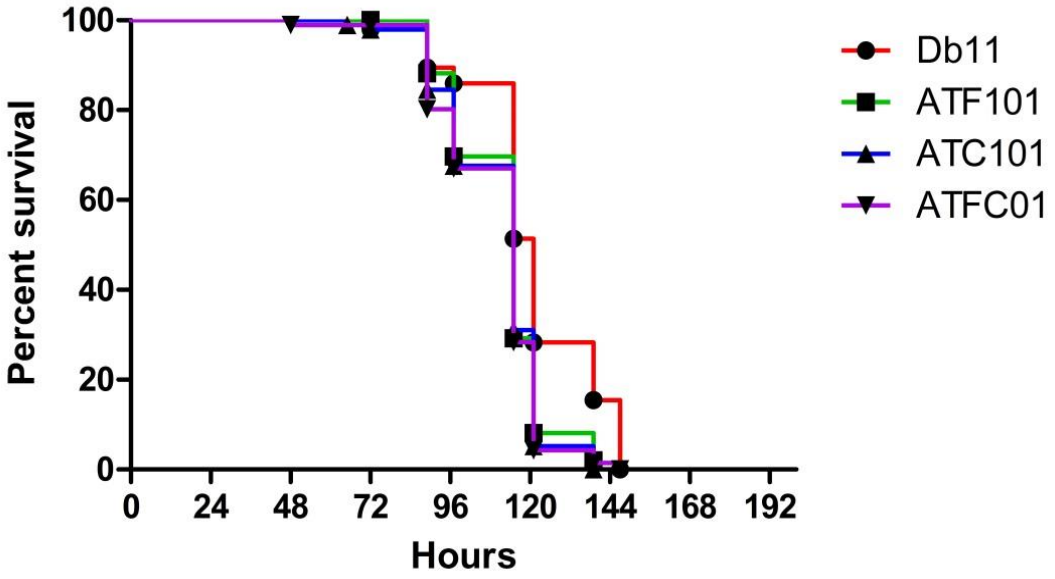


Figure 5.3. Survival of *C. elegans* infected with *S. marcescens* porin mutant strains. Kaplan–Meier survival of *C. elegans* N2 nematodes infected with Db11 (circle, n=90), ATF101 (square, n=90), ATC101 (triangle, n=90), and ATFC01 (inverted triangle, n=90). *P< 0.0123* by pairwise comparison (ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*)) versus Db11 by log-rank test (Mantel-Cox). Survival curve is representative of one of three independent experiments.

To enable clear observation of bacteria within the infected nematode using fluorescence, pUFR-GFP was transformed into *S. marcescens* Db11 and isogenic porin mutant strains. Nematodes fed/infected with GFP-tagged *S. marcescens* strains (Db11, ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*)) under standard survival assays conditions were monitored on a daily basis over 120 hours. It was not possible to monitor nematodes infected for more than 120 hours as the sample size proved to be insufficient in numbers due to the decreased survival rate of the population. Attenuated mutant 20C2 and 21C4 strains were used as virulence-attenuated controls (Kurz *et al*. 2003). Anatomical locations monitored were the head, the intestine, and the vulva.

Table 5.1 shows the timeline of the infectious process for each *S. marcescens* strain modeled in nematodes. For the Db11-GFP strain, bacteria were observed to enter the intestine in the first 24 hours and accumulate quickly by colonization inside the intestine. It should be noted that the grinder function of the nematode is further disrupted by the presence of Db11-GFP bacteria facilitating bacterial colonization as previously reported (Kurz *et al*., 2004). At 24 and 48 hours point-infection, colonization by Db11-GFP increased progressively with established colonization in the anterior portion of the intestine by 72 hours (Table 5.1; Figures 5.4 and 5.5A). At the time-point of 48 hours post-infection, a decrease in auto-fluorescence in the intestinal cells was also observed. Colonization of the uterus was also observed on day 3 and 4 post-infection (Table 5.1).

Beginning at 72 hours post-infection, Db11-GFP bacteria were observed to breach the confines of the pharyngeal grinder spreading into the surrounding tissue over time (Figures 5.5B and 5.5C). This event defined head invasion. It is supposed that the pressure of the distended intestine coupled with bacterial virulence factors compromised the integrity of the intestinal

lining allowing the bacteria to spread into the head region simultaneous with bacterial invasion of the pharyngeal grinder. Invasion of the vulva and uterus region was defined by bacteria entering the vulva breaching the vulvar lining spreading into the uterine tissue; this event was observed from 72 hours onwards to 120 hours post-infection. Thus, Db11-GFP was not only observed to colonize the intestinal lumen but also invade the grinder and vulva/uterus region; events that eventually lead to the demise of the infected nematode. It should be noted that in a previous study, grinder invasion was not reported and while invasion of the vulva/uterus region was reported, this event was a rarity (Kurz *et al*. 2003). However, it found that in this study that invasion of these two regions was on a more frequent but not common basis and was considered as a contributing factor to nematode death. Entry of the bacteria into the uterus from the vulva seems to be facilitated by the act of egg-laying by gravid adult nematodes 24 hours post-infection. This event was observed between 24 and 48 hours post-infection; however, viable nematodes on 96 and 120 hours post-infection rarely develop infection and invasion in the vulva and uterus region presumably due to the fact that the nematodes are no longer gravid. A characteristic feature of intestinal colonization is the progressive deterioration of the intestinal lining of the nematodes with a high volume of bacteria in the intestine (Irazoqui *et al.*, 2010b).

To monitor the infectious process by the Δ*ompF* porin mutant strain, nematodes colonized with GFP-expressing ATF101 (Δ*ompF*) were examined using microscopy (Figure 5.6). During the first few days of infection, ATF101-GFP bacteria accumulated in the intestinal tract but the colonization did not appear to be at the same rate as wild type Db11 (Table 5.1; Figure 5.4). In contrast to nematodes infected with Db11, invasion and accumulation of bacteria in the uterine region appears to occur at a higher frequency (Table 5.1; Figure 5.7). In some cases, the lining of the intestine and mid-point in close proximity to uterine region was observed

to be disrupted allowing the accumulated colonized ATF101-GFP bacteria to gain access to the uterus prior to the demise of the nematode. As bacterial colonization of the vulva and uterus has been reported as a rare event for the Db11 strain (Kurz *et al.*, 2003), it appears that this event occurred on a more frequent basis in nematodes infected with ATF101

Table 5.1. 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 belong to one independent representative experiment

		Hours fed on *S. marcescens* lawns				
Strains	Infection	24 h	48 h	72 h	96 h	120 h
Db11-GFP	Grinder			**	**	***
	Intestine	*	**	***	***	***
	Uterus			***	*	
Δ*ompF*-GFP	Grinder			***	***	***
	Intestine	*	*	***	***	***
	Uterus			*	***	**
Δ*ompC*-GFP	Grinder		**	***	***	***
	Intestine	*	*	***	***	***
	Uterus			*	***	***
Δ*ompF*Δ*ompC*-GFP	Grinder			*	**	**
	Intestine	*	**	***	**	**
	Uterus			**	**	**
20C2-GFP	Grinder				**	
	Intestine	*	*	*	*	*
	Uterus		*		*	
21C4-GFP	Grinder					
	Intestine	*	*	***	*	*
	Uterus				*	

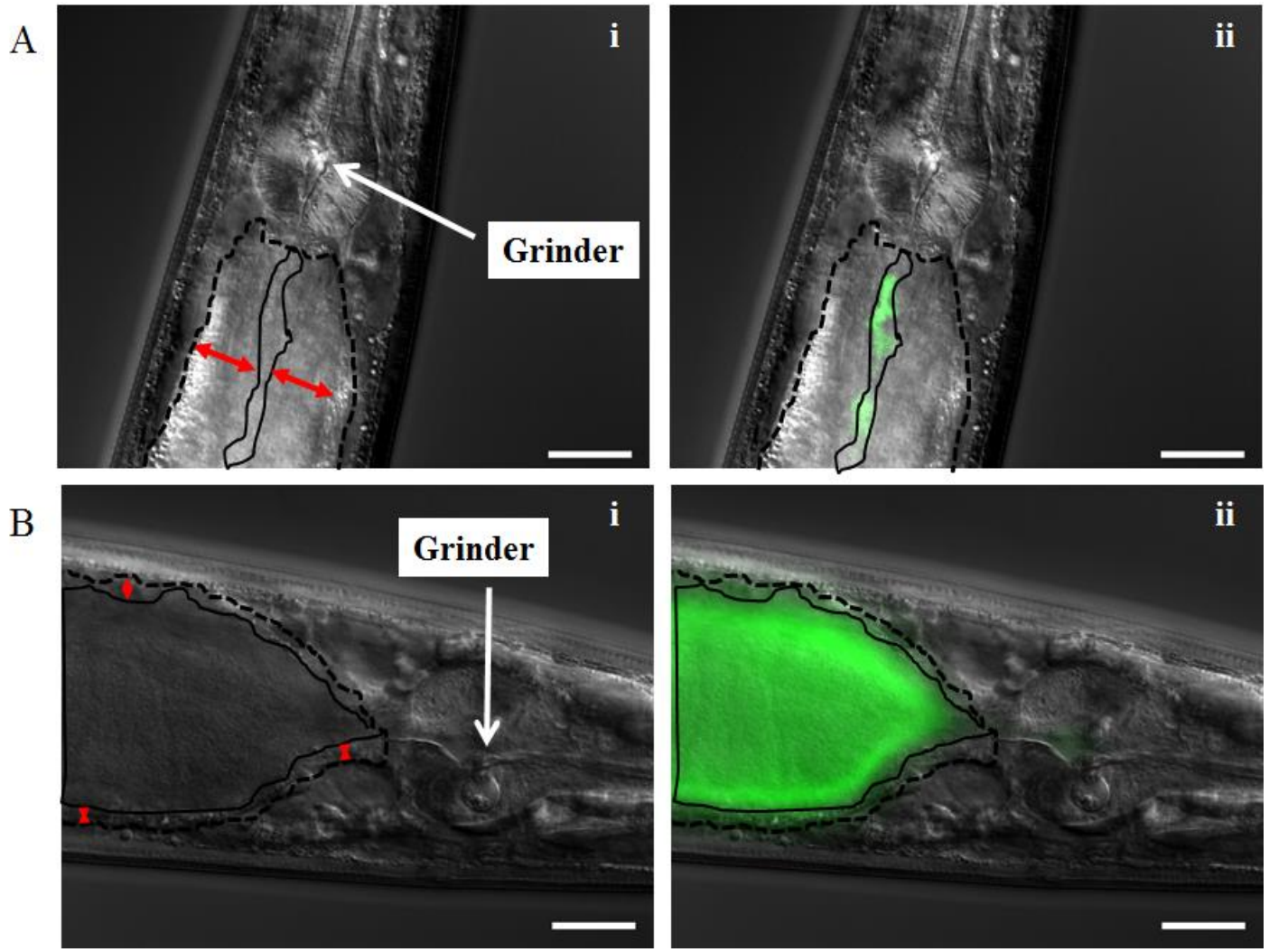


Figure 5.4. Intestinal colonization of *S. marcescens* Db11 in *C. elegans*. Accumulation of GFP-tagged Db11 in the intestinal tract at: (A) 24 hours and (B) 72 hours post infection. Accumulation of bacteria distends the intestinal lumen affecting reduction in intestinal cell volume. Red arrows in A and B show the changes in intestinal cell. Panels i and ii correspond to DIC and overall images with DIC and green channels respectively. Scale bar is 20 µm.

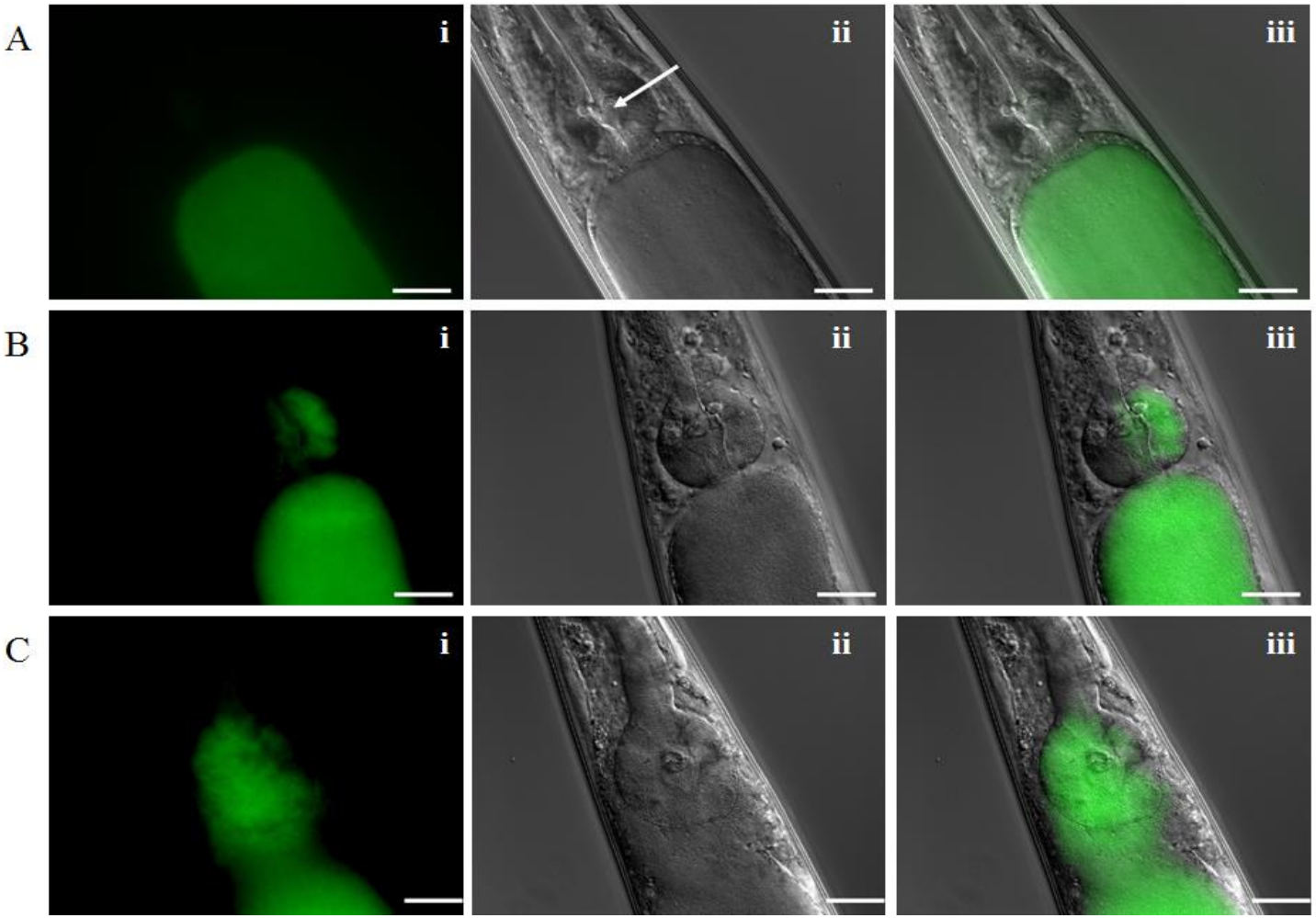


Figure 5.5. *S. marcescens* Db11-GFP colonization of *C. elegans.* DIC and confocal microscopy of *C. elegans* nematode with Db11-GFP. A) 72 hours post-infection with colonization of the anterior region of the lumen; B) 96 hours post-infection with invasion of the pharyngeal grinder and C) progression of bacterial spread into head region from the pharyngeal grinder and disrupted lining of the colonized intestinal lumen. Note the location of the pharyngeal grinder. Panel i,ii, and iii correspond to green channel, DIC and overlaid images, respectively. Scale bar is 20 µm.

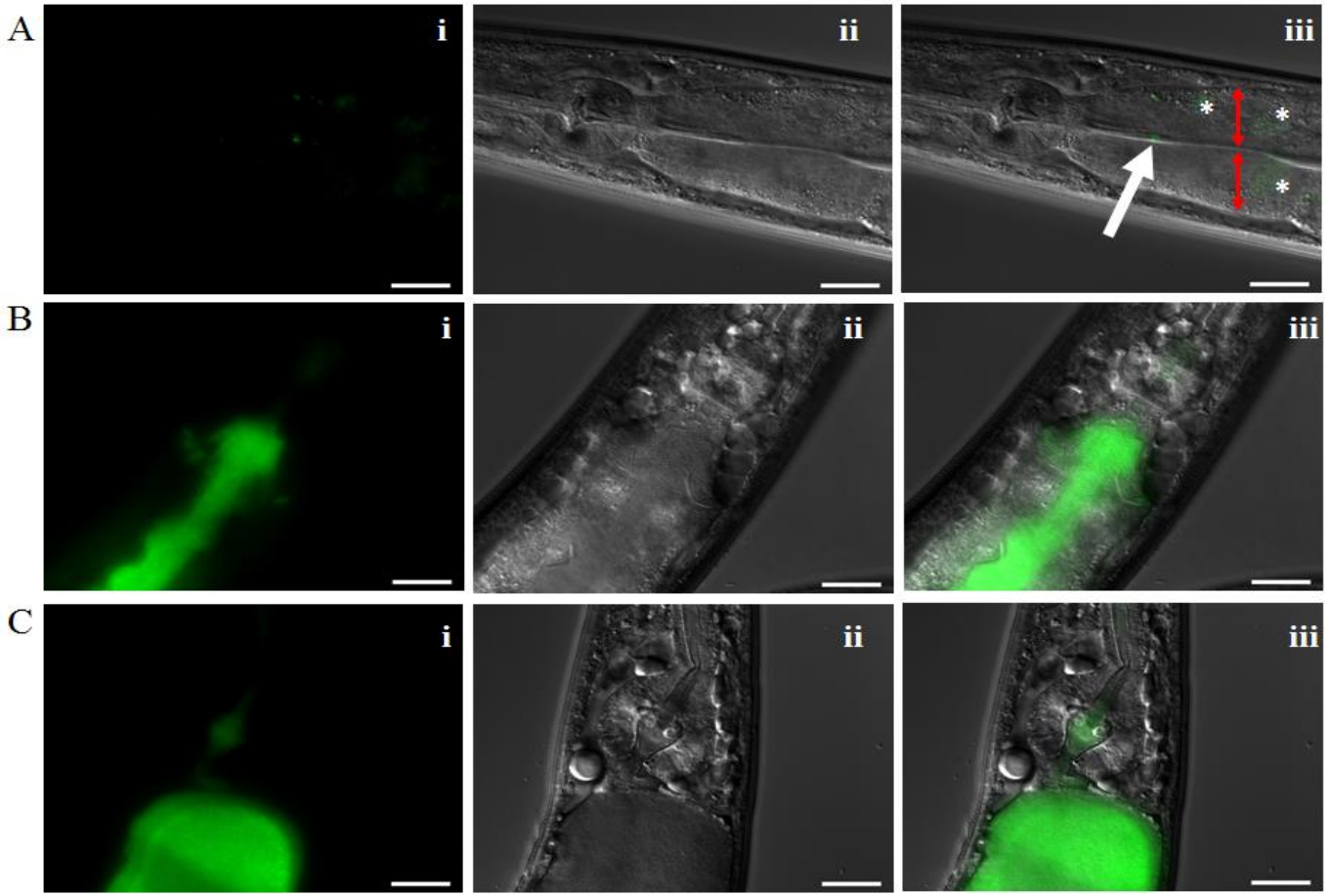


Figure 5.6. *C. elegans* colonized with *S. marcescens* ATF101 (Δ*ompF*). Microscopy images of nematodes (A) 24 hours, (B) 48 hours and (C) 72 hours post-infection. Panel A shows the presence of bacteria (white arrow) in the intestinal tract after passage through the grinder. Note the presence of autofluorescent granules (asterisk) located within intestinal cell and progressive bacterial colonization leading to intestinal distension. Panels i, ii and iii correspond to green channel, DIC and overlaid images, respectively. Scale bar is 20 µm.

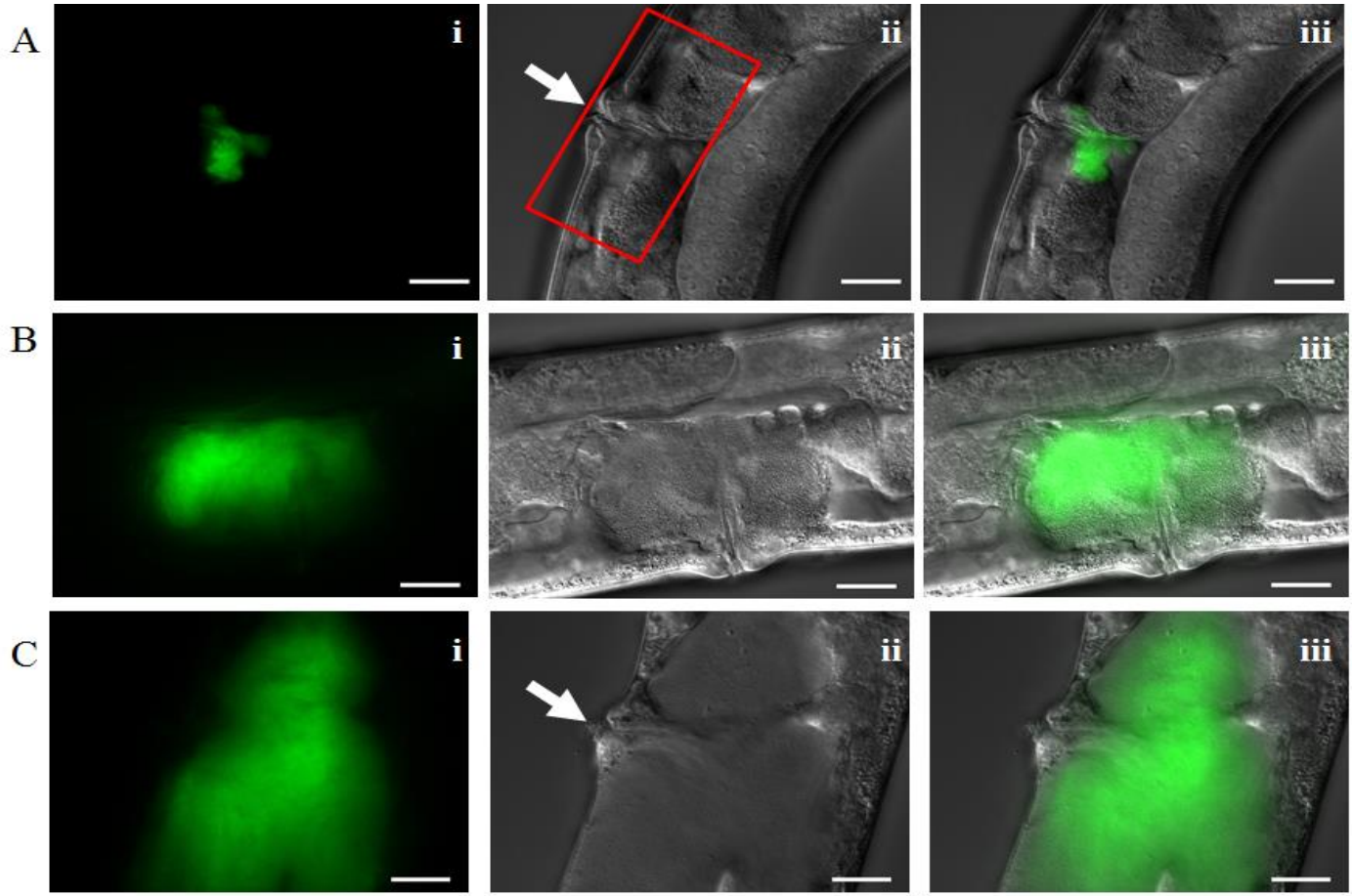


Figure 5.7. ATF101-GFP colonization of the *C. elegans* uterus. Colonization of the uterine region with GFP-expressing ATF101 (Δ*ompF*) in *C. elegans* (A) 72 hours and (B) and (C) 96 hours post-infection. The uterus (red box) and the vulva (white arrow) are indicated. Note the progressive accumulation of bacteria in the uterine over time. Panels i, ii, and iii correspond to green channel, DIC and overlaid images, respectively. Scale bar is 20 μm. This image is representative of what was observed in the other isogenic mutants.

In nematodes fed with Δ*ompF*-GFP, bacterial colonization of the intestine appeared to occur at the same rate as observed for nematodes fed with Db11-GFP. However, colonization of the uterine and grinder regions occurred at a much higher frequency rate in comparison to nematodes fed Db11-GFP (Figure 5.3; Table 5.1). Furthermore, colonization of the grinder occurred earlier and colonization of the uterus occurred later, in nematodes infected with Δ*ompF*-GFP in comparison to nematodes fed Db11-GFP, respectively The increased frequency of colonization of uterine and grinder regions was also observed for nematodes fed Δ*ompC*-GFP (Figure 5.8; Table 5.1). The increased pathology in nematodes infected with Δ*ompF*-GFP and Δ*ompC*-GFP corroborated with the hypervirulent phenotype observed in the survival assay (Figure 5.3). For both mutant strains, bacteria accumulated in the intestine distending the organ and compromising the structural integrity. Interestingly, the double porin mutant strain Δ*ompF*Δ*ompC*, demonstrated reduced colonization of the intestine, uterine and grinder regions in comparison with ATF101 and ATC101 (Figure 5.9; Table 5.1). This observation was in contrast to the hypervirulent phenotype demonstrated in the survival assay (Figure 5.3). ATFC01 (Δ*ompF*Δ*ompC*) is able to colonize the worm and kill it faster than the wild type, but the way it accumulates in the intestine differs from the single porin mutants. ATFC01 (Δ*ompF*Δ*ompC*) growth rate seems to be not affected when ATFC01 grows in LB media (Figure 4.4); however the deletion of *ompF* and *ompC* alters the colonization. The factors that are present are unknown but it seems that in addition to the modification of outer membrane permeability, other factors are sufficient to disrupt the nematode defense mechanisms, finally killing the nematode.

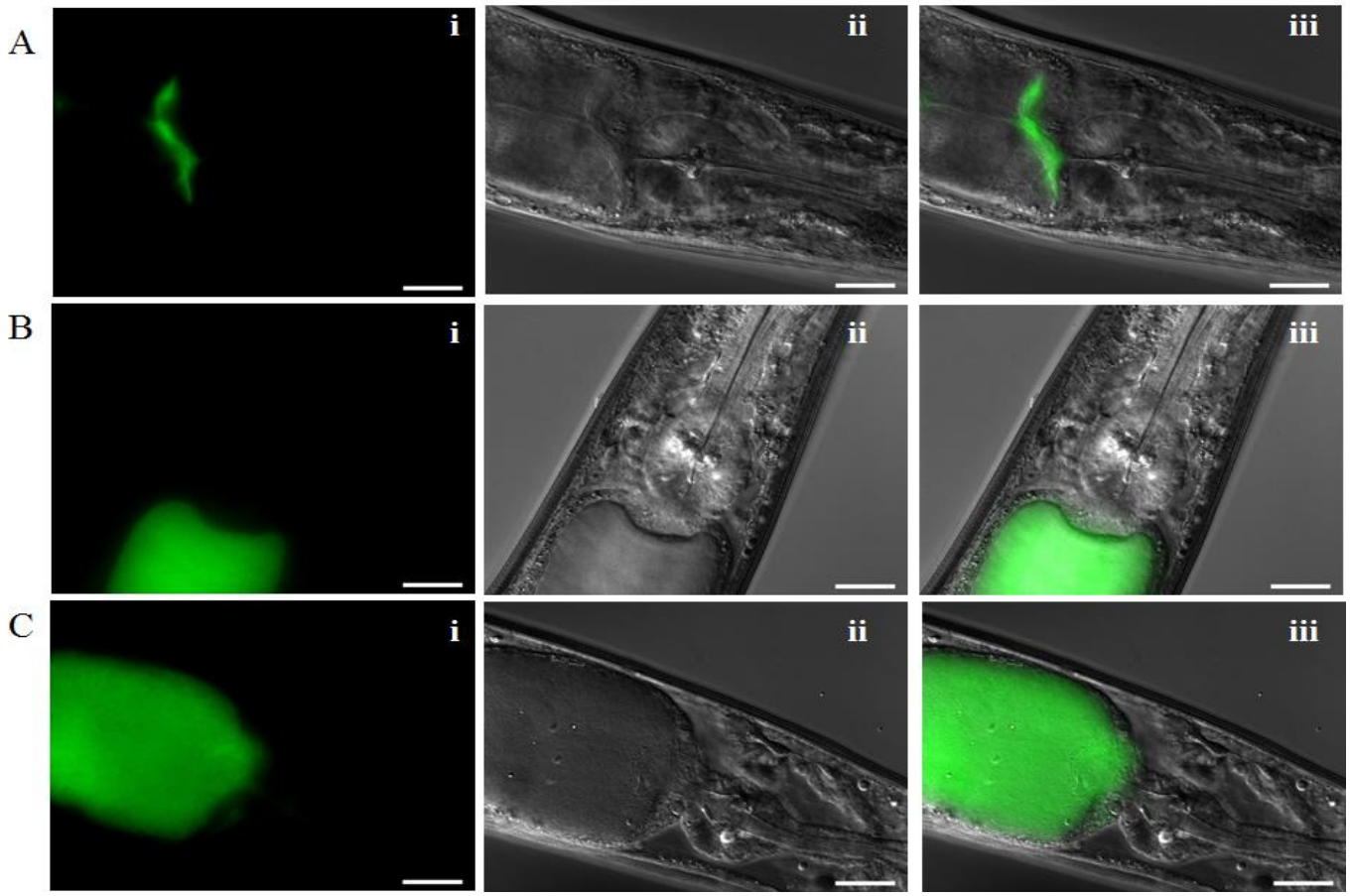


Figure 5.8. *C. elegans* colonized with *S. marcescens* ATC101 (Δ*ompC*). Microscopic images of nematodes (A) 24 hours; (B) 48 hours and (C) 72 hours post-infection. Panel A shows the presence of bacteria (white arrow) in the intestinal tract after passage through the grinder. Note the progressive bacterial colonization leading to intestinal distension and eventual deterioration of the anterior intestinal epithelium juxtaposition to the grinder. Panels I, ii and iii correspond to green channel DIC and overlaid images respectively. Scale bar in 20 µm.

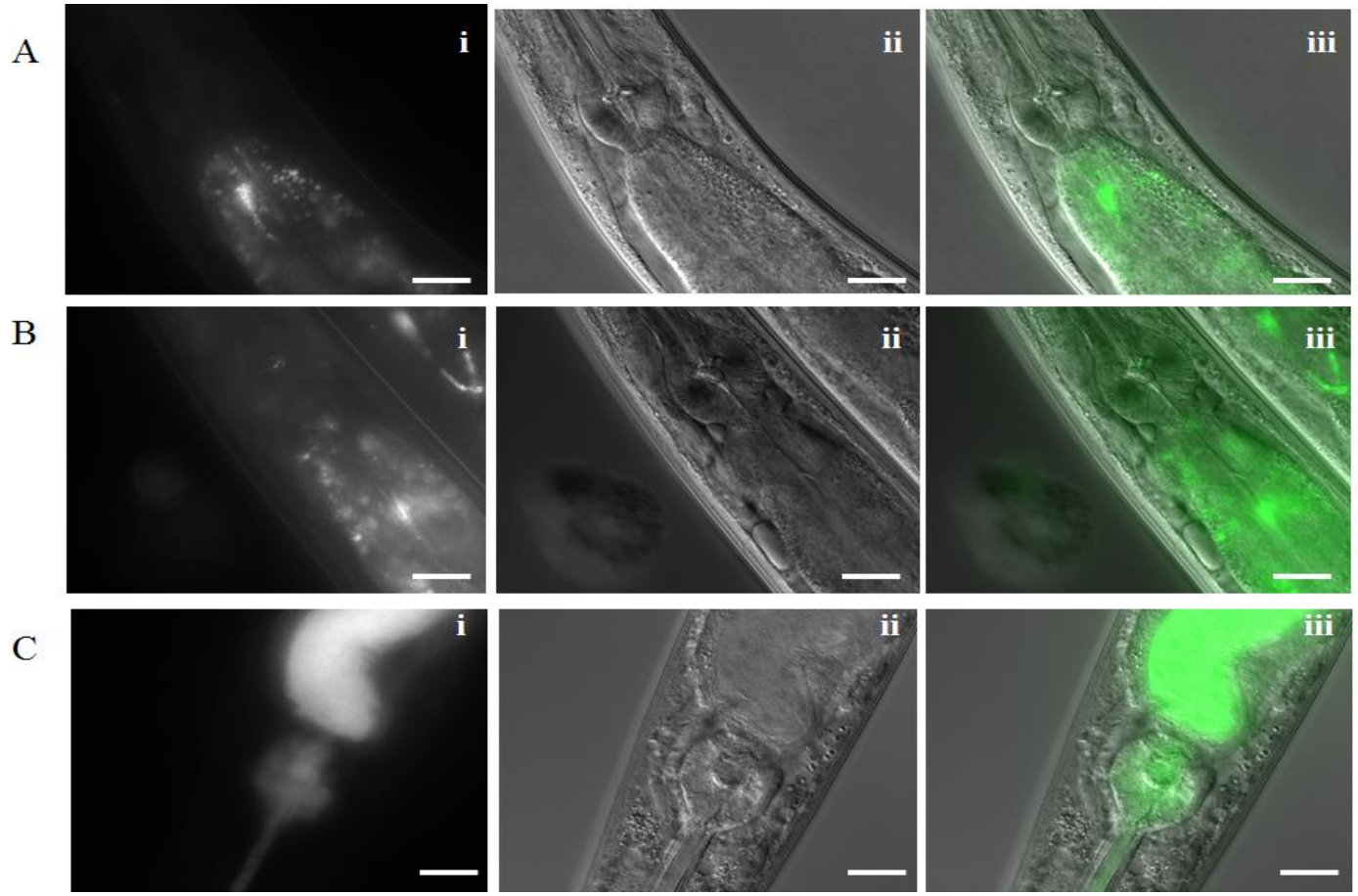


Figure 5.9. *C. elegans* colonized with *S. marcescens* ATFC01 (Δ*ompF*Δ*ompC*). Microscopic images of nematodes (A) 24 hours, (B) 48 hours and (C) 72 hours post infection. Note the presence of autofluorescent granules located within intestinal cell and starting the progressive bacterial colonization. Panels i, ii and iii correspond to green channel, DIC and overlaid images respectively. Scale bar is 20 µm. Note that the image in panel iii in (C) is overexposed to highlight the GFP-tagged bacteria infecting the grinder.

Attenuated mutants strains 20C2 and 21C4 were used as comparative controls in this study. In the survival assay, nematodes fed either 20C2 or 21C4 showed a significantly higher survival rate in comparison to nematodes fed on Db11. Microscopic examination of nematodes fed on the 20C2-GFP, indicated the presence of intact bacteria in the intestinal tract; however, intestinal distension was not observed indicating the lack of accumulated bacterial mass (Table 5.1; Figure 5.10). Nematodes fed 21C4-GFP showed a higher rate of colonization in comparison to nematodes fed on 20C2 on 72 hours, but decreased to low levels of colonization on subsequent days (Table 5.1; Figure 5.11). For both 20C2-GFP and 21C4-GFP strains, bacterial invasion and colonization of the uterine and grinder was observed on an infrequent basis (Table 5.1).

It should be noted that for all bacterial strains, bacteria adhered to the vulva entrance for most of the nematodes observed from the first 24 hours of exposure except for the 20C2-GFP strain. For 20C2-GFP, bacteria were observed to adhere to the vulva entrance 96 hours post-infection. It is possible that lack of O-polysaccharide chain of LPS has affected the ability of bacteria to efficiently adhere to surfaces. Nevertheless, the adherence of the bacteria to the vulva entry may facilitate the entrance and subsequent invasion of the uterine region.

5.1.4. Observation of refractive vesicles

The presence of vesicles with bright crystalline appearance was also observed lining the intestinal lumen. These vesicles migrated from the intestine to the posterior region into the rectum where they accumulated interrupting normal defecation via rhythmic peristalsis (Figure 5.12). Observation of these vesicles accumulating in the posterior region of the intestine has been reported by Kurz *et al*. (2003); however, the nature of the vesicles is unknown.

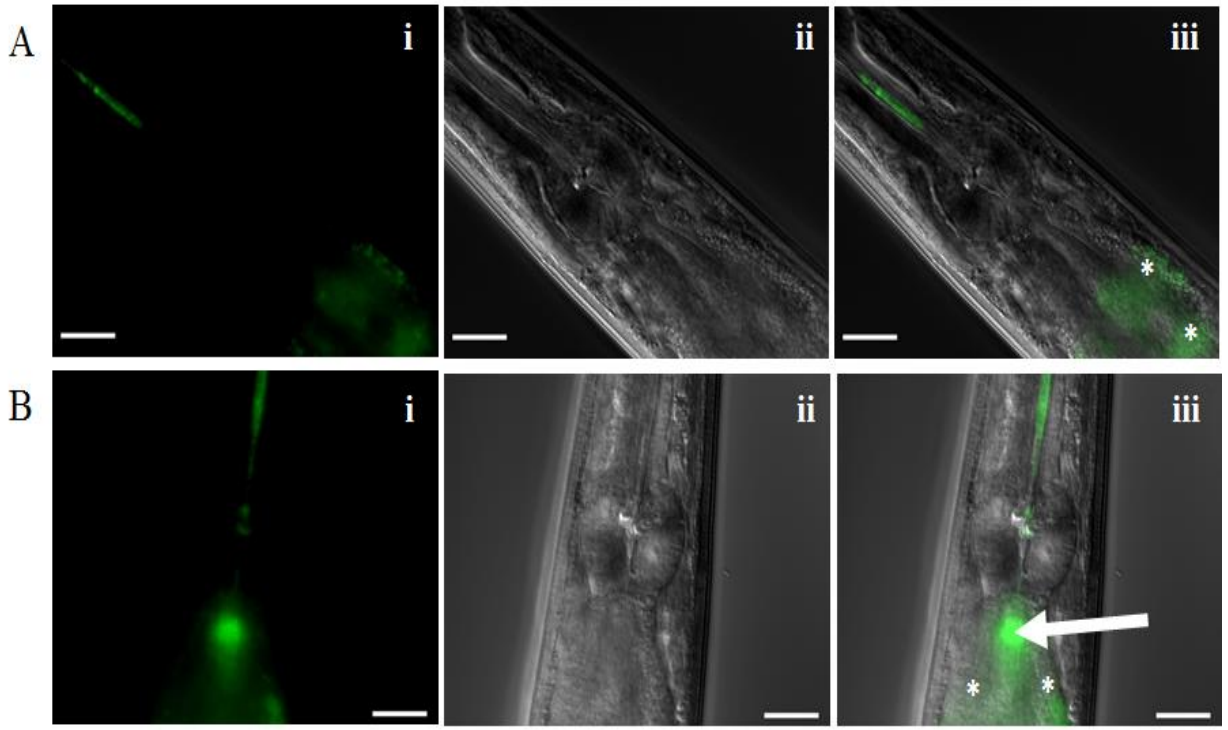


Figure 5.10. *C. elegans* colonized with *S. marcescens* 20C2. Microscopic images of nematodes (A) 72 hours and (B) 120 hours post infection. Note the presence of autofluorescent granules (denoted by asterisk). The presence of GFP-expressing 20C2 is indicated (white arrow) within the intestinal tract. It should be noted that the amount of bacteria inside the intestinal tract remains low over infection time. Panels i, ii, and iii correspond to green channel, DIC and overlaid images, respectively. Scale bar is 20 µm.

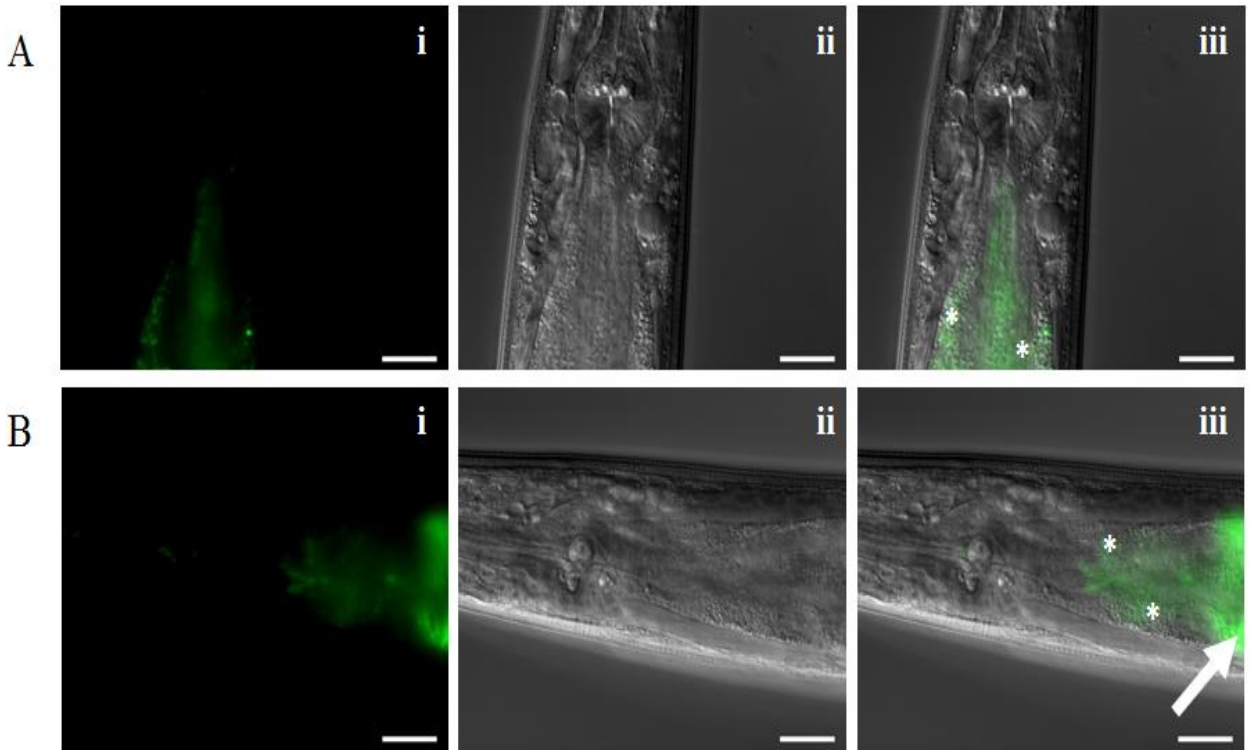


Figure 5.11. *C. elegans* colonized with *S. marcescens* 21C4. Microscopic images of nematodes (A) 72 hours and (B) 120 hours post infection. Note the presence of autofluorescent granules (denoted by asterisk). The presence of GFP-expressing 21C4 is indicated (white arrow) within the intestinal tract. It should be noted that the amount of bacteria inside the intestinal tract remains low over infection time. Panels i, ii, and iii correspond to green channel, DIC and overlaid images, respectively. Scale bar is 20 µm.

It is speculated that because the vesicles budded from the interior membrane of the intestine, they could contain antimicrobial products to counteract the bacterial infection (De *et al.*, 1995; Irazoqui *et al.*, 2010b). Accumulation of the vesicles do not appear to directly cause the demise of the nematodes but may contribute as an indirect factor by impeding the ability of the nematode to defecate that would otherwise reduce the amount of colonized bacteria. During *S. marcescens* infection of nematode gut, refractive vesicles were observed to move within the intestinal tract toward the rectum (Figure 4.12). The number of vesicles increased over time in nematodes colonized with Db11, *ΔompF*, Δ*ompC* and Δ*ompF*Δ*ompC*. The refractive vesicles were not observed for nematodes colonized with 20C2 and 21C4 mutants.

5.1.5. Quantification of bacterial mass in nematodes infected with *S. marcescens*

Bacterial accumulation within the infected nematode is an indicator of the colonization process and thus can be used to quantitatively compare nematodes colonized with wild-type and porin mutant strains. Briefly, quantification of bacterial colonization entailed lysing 10 infected nematodes and then performing serial dilution plating to obtain bacterial counts. For the Db11 strain, 8.2×10^4 CFU/nematode was quantified at 24 hours post-infection and this increased by two logs to 3.8×10^6 CFU/nematode at 48 hours post-infection (Figure 5.13). This observation correlated with pronounced distension of the intestine due to accumulated bacterial mass when the nematodes were examined microscopically (Table 5.1; Figures 5.4 and 5.5).

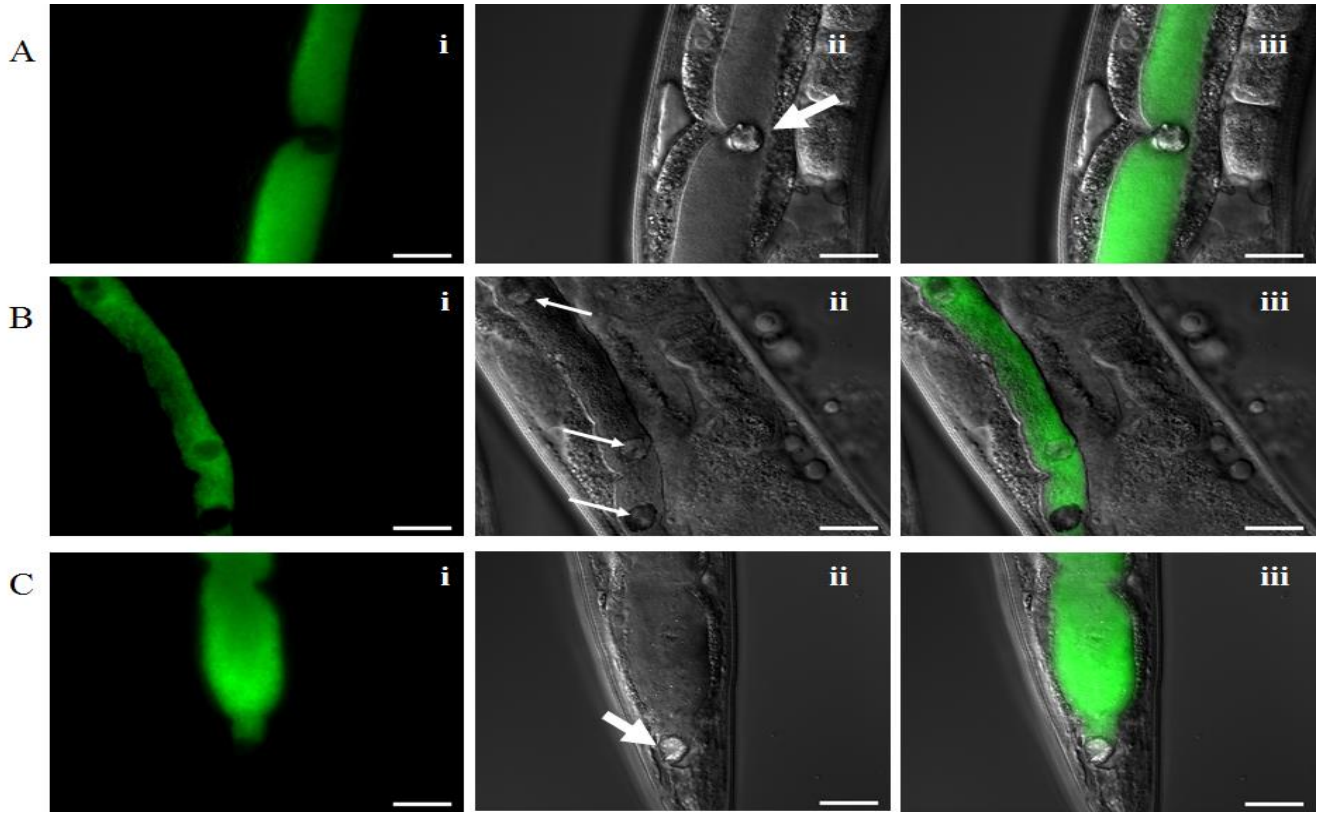


Figure 5.12. Vesicles in the intestinal tract of *C. elegans* colonized with *S. marcescens*. Nematode colonized with GFP-expressing *S. marcescens* Db11. Formation of small round vesicles (indicated by white arrows) (A) budding from the lining of the intestinal tract; (B) migrating through the intestinal tract; and (C) accumulating in the posterior region of the intestinal tract juxtaposition to the anal region. Panels i ii, and iii correspond to green channel, DIC and overlaid images, respectively. Scale bar is 20 μm.

Elevated counts of colonized bacteria were noted for the ATF101 (Δ*ompF*)strain progressing from 3.5 x 10^6 CFU/nematode at 24 hours to 5.3 x 10^8 CFU/nematode at 48 hours. Although this trend did not at first corroborate with decreased colonization rate of the intestinal lumen observed for Δ*ompF*-infected nematodes in comparison to Db11-infected nematodes (Table 5.1), bacterial counts from uterine and head invasion sites in Δ*ompF*-infected nematodes were also included in the quantification accounting for the elevated level. Δ*ompC* and Δ*ompF*Δ*ompC* mutant strains were able to colonize the *C. elegans* intestine, but not in the same manner as Δ*ompF* that at the 48 hour time-point had the higher CFU/nematode. Among the porin mutant strains, the double mutant Δ*ompF*Δ*ompC* strain showed the lowest amount of bacteria within the colonized nematode; however, this variation was not significant (Figure 5.13).

The 20C2 strain was included in this assay as the representative attenuated virulence strain. At 24 hours post-infection, the quantified level of bacterial colonization (1.1 x 10^3 CFU/nematode) was similar to levels determined for Db11 and Δ*ompF*Δ*ompC* at that time-point. However, bacterial colonization (4.1 x 10^3 CFU/nematode) did not increase by the 48 hour post-infection time-point as observed for the other bacterial strains. Taken together, it appears that the lack of OmpF contributed to significantly increased levels of bacterial colonization in the infected nematodes.

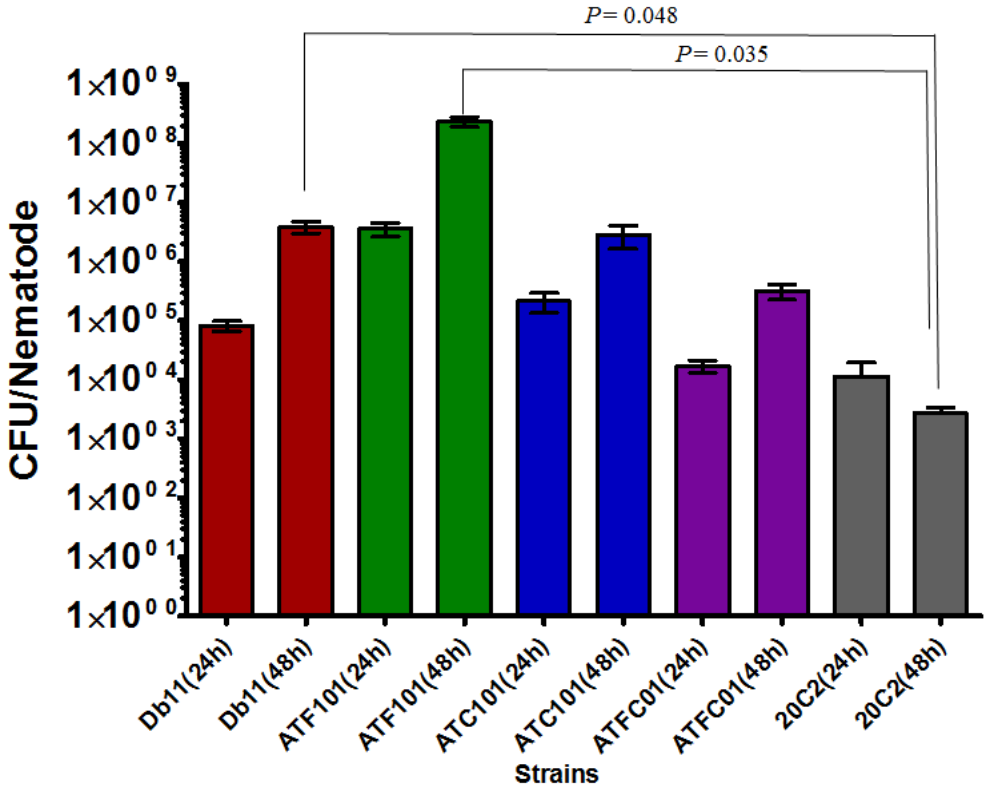


Figure 5.13. Quantification of nematode intestinal colonization. *C. elegans* N2 nematodes were infected with *S. marcescens* strains and the bacterial colonization quantified over time. Data show the mean numbers of CFU/nematode from three independent experiments, with error bars signifying standard errors of measurement. Red bars: Db11, green bars: ATF101 (Δ*ompF*), blue bars: ATC101 (Δ*ompC*), violet bars ATFC01 (Δ*ompF*Δ*ompC*), gray bars: 20C2. Significant values were found for Db11(48h) vs ATF101 (48h) (*P*=0.0349); Db11 (48h) vs 20C2 (48h) (*P* = 0.0482) and ATF101 vs 20C2 (48h) (*P=0.035)* by Paired t test).

5.2. DISCUSSION

In this study, *S. marcescens* strains lacking OmpF and/or OmpC porins demonstrated increased virulence as demonstrated in the *C. elegans* host model. The increased virulence was not immediately apparent in the *C. elegans* survival assay as the biological significance appears to be small, regardless of the inferred statistical significance of the differences between the survival curves. However, the microscopic examination of infected nematodes provided more informative details of the infectious process indicating that the elusive virulence differences were more substantial on a cellular level. Surprisingly, the absence of both OmpF and OmpC in Δ*ompF*Δ*ompC* did not generate attenuation in virulence, but rather appeared to enhance the virulence phenotype in the survival assay even though the colonization level was substantially less in comparison to the single porin mutant and wild-type strains. The absence of these porins may have indeed compromised bacterial fitness as indicated by the low colonization level; however, this did not appear to affect the enhanced virulence phenotype demonstrated by the double porin mutant strain.

It was previously reported that *S. marcescens* colonization was restricted to the intestinal lumen and that uterine colonization, if any, was a rare event (Kurz *et al*., 2003). It was also noted that chitinases secreted from the colonized bacteria in the intestinal lumen were able to dissolve the eggshells of the oocytes in gravid adult nematodes (Kurz *et al*., 2003). Our findings showed that in nematodes infected with the *S. marcescens* Db11, uterine colonization was observed on a more frequent basis than previously reported and occurred specifically when gravid nematodes were actively egg-laying. Uterine colonization presented as an opaque "bubble" centred over the vulva opening and below the intestine when viewed using a stereomicroscope. This characteristic feature has been seen elsewhere in nematodes colonized with the soil microorganism *Leucobacter chromiireducens* (Muir & Tan, 2008). *L. chromiireducens* was postulated to

establish uterine colonization by entry through the vulva as uterine colonization was absent in vulva-less nematodes (Muir & Tan, 2008). Thus, on this basis of comparison, it is surmised that Db11 bacteria also establish uterine colonization by the same route.

Pharyngeal grinder colonization was also observed in nematodes infected with *S. marcescens* Db11, a feature not previously reported by Kurz and colleagues (2003). Pharyngeal grinder colonization has been observed elsewhere in hypo-immune (loss-of-function *tol-1*) nematodes infected with *Salmonella enterica* Typhimurium (Tenor & Aballay, 2008). The *tol-1* pathway is linked with innate immunity by controlling pathogen avoidance behaviour, a trait connected to olfactory sensing of pathogenic bacteria that are potentially harmful and is partially controlled by *tol-1,* a Toll-Like Receptor (TLR) homologue (Tenor & Aballay, 2008). Thus, lack of functional Tol-1 leads to colonization of the pharyngeal grinder and subsequent invasion of the head tissue. The fact that *S. marcescens* Db11 is able to colonize the grinders of wild-type nematodes with a functional innate immune system on a frequent basis is considered to be a novel finding.

Another interesting finding was vulva colonization. Most of studies on *C. elegans*-pathogen infection are focused on the gut. Structures that have not been referred to in most of the studies of pathogenicity of *S. marcescens* are the vulva and uterus. The *C. elegans* vulva is used to study mechanisms of cell specification and tissue morphogenesis (Gupta *et al.*, 2012). The vulva connects the uterus to the external milieu. Nematodes infected with single porin mutant strains showed elevated levels of uterine colonization and pharyngeal grinder invasion. Furthermore, uterine colonization peaked in nematodes that were no longer engaged in egg-laying activity indicating that perhaps uterine colonization was facilitated by invasion from the intestinal lumen. Interestingly, nematodes infected with the double-porin mutant strain showed

decreased levels of colonization of the intestine, grinder and uterine region. Given that OmpF and OmpC are classical porins responsible for maintaining homeostasis by controlling the influx of substrates under certain environmental conditions, it is possible that the lack of OmpF or OmpC porins induced increased nutrient scavenging by increasing expression of virulence factors such as type VI secretion system, hemolysin, proteases, and siderophores (Coulthurst, 2013; Faraldo-Gómez & Sansom, 2003; Iguchi *et al.*, 2014; Kurz *et al.*, 2003). Additionally, other outer membrane porins may contribute to bacterial virulence as reported for bacterial species. Members of the OmpA family promote adhesion of the bacterial cell to mucosal surfaces, invasion of the host tissue cells and host cell activation (Smith *et al*., 2007). An increase in OmpA expression was observed in Δ*ompF*Δ*ompC* (Figure 5.1,A), that could contribute to the infection. OmpX belongs to a family of proteins that promote bacterial adhesion, invasion and interfere with the human complement defense system (Vogt & Schulz, 1999). Thus, it is quite possible that increased expression of virulence factors and/or porins may contribute to increased bacterial virulence. The decreased levels of virulence associated with the lack of both OmpF and OmpC suggests that bacterial fitness and cell envelope integrity may have been compromised within the *C. elegans* host environment that includes activated innate immune defense measures. Further studies are required to ascertain changes in the *in vivo* expression profile of genes encoding virulence factors and porins in the presence and absence of OmpF and OmpC.

CHAPTER 6. THESIS CONCLUSIONS

In Gram-negative bacteria, classical porins play an important role in outer membrane permeability by regulating the influx and efflux of hydrophilic solutes to maintain homeostasis in response to changing environmental conditions (Liu & Ferenci, 2001b; Nikaido, 2003; Pratt *et al.*, 1996). Due to their non-specific properties, classical porins also take up antibiotic compounds when encountered in the environment placing selective pressure for changes on the molecular level to reduce or suspend expression of classical porins to enhance bacterial survival (Fernández & Hancock, 2012). In addition to its primary role in maintaining homeostasis, studies have implicated that classical porins play a secondary role in effecting bacterial pathogenicity such as adhesion and invasion of mammalian cells (Confer & Ayalew, 2013; Vogt & Schulz, 1999). Gram-negative clinical isolates sourced from bacterial infections in humans have been increasingly characterized to feature deficient porin expression, in particular OmpF and OmpC, leading to increased antibiotic resistance (Suh *et al.*, 2010; Thiolas *et al.*, 2004). One commonly isolated bacterium, *S. marcescens*, has become a prevalent opportunistic bacterial pathogen with increasing propensity to feature multi-drug resistance, partially due to defective porin expression, complicating the efficacy of treatment (Suh *et al.*, 2010). Thus, this thesis work was undertaken to genetically correlate the absence of OmpF and OmpC porins with increased antibiotic resistance in *S. marcescens*, and to establish if this increased advantage comes as a cost to bacterial fitness and pathogenicity in fluctuating environmental conditions.

Given the close relatedness between members of the *Enterobacteriaceae* genus, it is presumed that *S. marcescens* has similar levels of orthologous OmpF and OmpC porins in the outer membrane as does *E. coli*. Thus, the deletion of OmpF and/or OmpC should, in theory,

compromise the ability of *S. marcescens* (i.e. bacterial fitness) in response to changing environmental conditions.

The work presented in this thesis centers on the development of an efficient strategy to generate mutant strains in *S. marcescens*. In previous studies, mutant strains were generated from *S. marcescens* UOC67 (ATCC13880) (Begic, 2009), using the TargeTron gene knockout system involving the insertion of an antibiotic resistance cassette into the target gene (Nasiri, 2011). However, as this work involves profiling changes in the antibiotic susceptibility of porin mutant strains in comparison to that of the wild-type parental strain, the use of an antibiotic resistance cassette marker was not considered as a viable approach as this would introduce additional antibiotic resistance thereby potentially biasing the results. Thus, a strategy was developed to create in-frame unmarked gene deletion mutants to circumvent the potential problem of biased results. In addition, the *S. marcescens* Db11 strain was used in place of *S. marcescens* UOC67 for three reasons: firstly, this strain has been fully sequenced and annotated allowing for the use of bioinformatics approaches to identify orthologous of porin genes; secondly, the availability of a generalized transducing phage IF3 to move marked gene targets between strain backgrounds prior to removal of the antibiotic resistance cassette by FRT-Flp strategy; and thirdly, the pathogenicity of *S. marcescens* Db11 is well-characterized in the infection model *C. elegans* providing the opportunity to assess changes in bacterial virulence of isogenic porin mutant strains (Iguchi *et al.*, 2014; Kurz *et al.*, 2003; Petty *et al.*, 2006).

In this thesis work, it was determined that lack of OmpF, but not OmpC, contributed to increased antibiotic resistance to beta-lactam drugs such as ampicillin and cefoxitin as well as to nitrofurantoin. It was also determined that intrinsic beta-lactamase activity encoded by the chromosomal gene *ampC* was not induced by the presence of cefoxitin indicating that the

increased antibiotic resistance is attributed solely to the absence of OmpF. The findings of this work align with the findings of previous studies (Suh *et al.*, 2010), that indicated increased antibiotic resistance to beta-lactam drugs resulted from defective or lack of OmpF production due to mutations or insertion elements within the coding sequence for OmpF. Additionally, these clinical isolates featured increased intrinsic AmpC beta-lactamase levels due to defective or absence of the inhibitor AmpD. Thus, to genetically correlate this finding, attempts were made to induce AmpC beta-lactamase activity in wild-type and porin mutant strains by deleting the gene encoding the inhibitor AmpD. However, similar to the related bacterial species *P. aeruginosa*, it was determined that *S. marcescens* Db11 contained three paralogues of *ampD* in the chromosome, and thus it is most likely similar to the situation with *P. aeruginosa* that all three paralogous genes must be deleted to achieve unrestricted induction of AmpC beta-lactamase activity. Taken together, the findings of this work specifically correlates, on a molecular level, increased antibiotic resistance with the deletion of the OmpF porin highlighting the concern of unintentional positive selection of porin-deficient antibiotic resistant *S. marcescens* strains in clinical settings.

The absence of OmpF porin may benefit *S. marcescens* with increased resistance to beta-lactam drugs; however, would this advantage come at a cost to bacterial fitness, and in turn, compromise bacterial pathogenicity? To answer this question, the *C. elegans* infection model was implemented in which the pathogenesis of *S. marcescens* Db11 has been well characterized (Kurz *et al.*, 2003; Mallo *et al.*, 2002). The hypothesis that porin mutant strains would feature virulence attenuation was supported by the observation that a *phoE* insertion mutant was attenuated for virulence (personal communication with J. Ewbank). Like OmpF and OmpC, PhoE is considered to be a classical porin but substrate-specific; in low-nutrient conditions,

influx of phosphate is increased to supplement the metabolic needs of the bacterial cell. As further support for the hypothesis, it has been reported elsewhere that *Klebsiella pneumonia ompF* and *ompC* mutant strains were attenuated for virulence in the *C. elegans* host model (Bialek *et al*., 2010). Surprisingly, it was determined in this thesis work that single *ompF* and *ompC* porin mutants displayed hypervirulent phenotypes when assessed in *C. elegans* survival assays. This hypervirulent phenotype was further supported by the observation of increased invasion by the single porin mutant strains facilitating bacterial spread in the uterine and head tissues. Conversely, the pathogenicity of the double porin mutant was not as severe on the cellular level although a hypervirulent phenotype was demonstrated in the *C. elegans* survival assay. This result could indicate that the loss of both porins could have compromised the virulence mechanisms on a cellular level, but not to the extent that this is observed in the *C. elegans* survival assay.

It should be noted that porins are generally not considered virulence factors but are implicated in the way bacteria sense the environment and adapt (Achouak *et al.*, 2011; Nikaido, 2003). The outer membrane functions as a protective barrier to the hostile environment in the *C. elegans* intestine and protects bacteria during colonization; thus the location and function of porins may have a role during infection. Deletion of porin expression seems to be involved, albeit indirectly, in the resistance of bacteria to the conditions present within the *C. elegans* intestine and to the host-defense mechanisms. It is not clear how the deletion of *ompF* or *ompC* affects the overall gene expression profile. It is possible that expression of other porin types may have increased in a compensatory role. For example, OmpA is upregulated in porin-deficient mutants and has been implicated elsewhere in adhesion and invasion of eukaryotic cells (Hashizume *et al*., 1993; Smith *et al*., 2007). In addition, *P. aeruginosa* OprF, which shares high

homology with the OmpA in *Enterobacteriaceae*, is involved in virulence. Deletion of *oprF* in *P. aeruginosa* produces virulence attenuation in the *C. elegans* host-model (Fito-Boncompte *et al.*, 2011). It was determined that deletion of *oprF* affected the activity of other virulence factors such as quorum sense and type III secretion system such that the overall virulence of the strain was decreased. Alternatively, components comprising the type VI secretion system could be overexpressed in response to the deletion of either porin in *S. marcescens*. The Type VI secretion system has been shown in *Serratia marcescens* to pierce eukaryotic cells for translocation of effector molecules to facilitating cytotoxicity and cell death (Coulthurst, 2013; Iguchi *et al.*, 2014; Murdoch *et al.*, 2011).

In summary, this thesis work has demonstrated that the genetic deletion of *ompF* results in increased antibiotic resistance. Deletion of *ompC* did not appear to affect the overall antibiotic susceptibility profile. Interestingly, despite the prevalence of OmpF and OmpC in the outer membrane, the single and combinatorial deletion of *ompF* and *ompC* genes did not appear to affect bacterial growth kinetics in optimal growth conditions. However, changes in the outer membrane profile were noted when the strains were grown in limited nutrient conditions (i.e.NGM) in comparison to strains grown in rich nutrient conditions (i.e. LB media). The virulence of the single porin strains increased, less so for the double porin mutant strain, in comparison to the wild-type parental strain in the *C. elegans* infection model. Additional studies are needed to determine the cause of the altered virulence in the porin mutant strains. While *S. marcescens* Db11 has been designated as an insect pathogen, recent genome sequence comparison between *S. marcescens* Db11 and the multi-drug resistant clinical isolate *S. marcescens* SM39 revealed a conserved set of virulence-related genes within the core genome

shared between the two strains (Iguchi *et al*., 2014). The results and conclusions presented in this thesis are relevant to human clinical infections.

6.2. FUTURE STUDIES.

The characterization of the OmpF and OmpC porins in *S. marcescens* in relation to antibiotic resistance and virulence is the first step in understanding the role of porins in the bacterial pathogenesis of *S. marcescens*. Characterization of the other porins (LamB, PhoE, OmpA and OmpX) is required to understand their roles, if any, in antibiotic resistance and virulence of *S. marcescens*, and to determine if their functions are different from orthologous functions characterized elsewhere.

6.2.1. Characterization of porin gene expression profiles.

The gene expression and expression protein profiles need to be ascertained when one or more porin genes are deleted to determine compensatory effects, if any, via alteration in expression levels of the remaining porin genes. For example, OmpA and OmpX have been shown elsewhere to adhere and invade mammalian cells (Bartra *et al.*, 2012; Vogt & Schulz, 1999). Thus, increased levels of OmpA and/or OmpX may explain the increased virulence of single *ompF* and *ompC* porin deletion strains. Porin genes to be evaluated in response to changing environmental conditions are *ompA* and *ompX* as well as *lamb* and *phoE*. Quantitative PCR (qPCR) and custom antibodies generated against recombinant porin proteins will aid in the determination of transcript and protein levels, respectively, *in vitro*. For comparison, the gene expression profile of the four porin genes (*ompA, ompX, lamB, phoE*) should also be assessed in the *C. elegans* infection model. Briefly, total RNA from bacteria isolated from infected nematodes will be extracted on a daily basis and analyzed using qPCR to determine relative transcript levels or RNAseq to look at the transcriptome profile.

Recombinant porin proteins used for the generation of the custom antibodies could also be subjected to X-ray crystallography to determine the structure of porin for comparison with established structural orthologous, and provide important information for the design of effector molecules to inhibit the function of porin protein in question. The implication of specific modification at the amino acid level, generate information to understand how porins interact with the solute and with other porins (Baslé *et al.*, 2004; Naveed *et al.*, 2012; Varma *et al.*, 2006).

The regulatory mechanisms involved in controlling porin expression in response to changing environmental conditions also need to be investigated in *S. marcescens*. Regulatory factors characterized in *E. coli* to be involved in porin gene regulation (EnvZ/OmpR, *micF*, *rpoS*, *lrp*) as well as metabolic regulators such as cAMP could serve as first targets for evaluating the role of its orthologs in *S. marcescens*. Experimental approaches could include DNA/protein binding studies, promoter reporter assays, and genetic deletions of the regulatory gene or cognate binding site(s). To simulate changing environmental conditions, levels and types of nutrients should be varied as well as a range of pH and temperatures should be implemented on an individual and combinatorial basis, in order to ascertain the effect on the porin gene expression profile.

6.2.2. Generation of *ampC* and *ampD* mutant strains.

The intrinsic AmpC beta-lactamase pathway should also be investigated further. It should be determined if deletion of all three paralogous *ampD* genes will result in unrestricted AmpC induction as the case is with *P. aeruginosa* (Moya *et al.*, 2008). The deletion of *ampC* will validate the presence of only one β-lactamase as determined by genome sequence analysis (Mahlen *et al.*, 2003). Beta-lactamase activity assays should be complemented by qPCR assays to correlate transcript levels with β-lactamase expression levels. The investigation could be

expanded to include the positive regulator AmpR to confirm its involvement in modulating *ampC* transcription in the presence/absence of AmpD.

6.2.3. Clinically relate genetic studies to *S. marcescens* isolates.

The results of the genetic studies in this thesis and future investigations should be related to human *S. marcescens* clinical isolates. One approach is to characterize the clinical isolates for altered classical porin expression by qPCR and immunoblot with custom antibodies in correlation with antibiotic resistance profiling. The results of this investigation would provide an indicator of the prevalence of altered porin expression leading to enhanced antibiotic resistance of clinical isolates.

6.2.4. Relating the *C. elegans* infection model to mammalian systems.

The *C. elegans* host model serves as an excellent infection model to demonstrate the bacterial pathogenesis of *S. marcescens* (Kurz *et al.*, 2003; Mallo *et al.*, 2002). Although components of the *C. elegans* innate immune system are functionally conserved with orthologous in mammalian systems (Schulenburg *et al.*, 2004; Sifri *et al.*, 2005), results obtained with the *C. elegans* infection model should be related to mammalian cells. The CHO cell-line has been recently established as a versatile model to study *S. marcescens* adherence and invasion (Fedrigo *et al.*, 2011). Surprisingly, *S. marcescens* is able to survive and proliferate in autophagic-like vacuoles with the cell, and is able to delay or prevent fusion with lysosomal compartments. This autophagic feature is dependent on live bacteria and requires vacuole acidification (Fedrigo *et al.*, 2011). An interesting question to pose is to investigate if the autophagic process is porin-mediated. For relevancy to *C. elegans* model, the pH of the intestinal lumen of *C. elegans* ranges from 5.9 in the anterior pharynx to 3.5 in the posterior intestine (Chauhan *et al.*, 2013); thus, it is quite possible that acidic pH may enhance the invasiveness

properties of porin mutant strains (Wu *et al.*, 2009). Therefore, in addition to bacterial adherence and intracellular growth kinetic studies of porin mutant strains with CHO cells, immunofluorescence utilizing lysosomal markers should also be employed to track the autophagy process of internalized porin mutant strains.

CHAPTER 7. REFERENCES

Achouak, W., Heulin, T., Pagès, J. M., Mark, B. L., Vocadlo, D. J., Oliver, A. 2011. Multiple facets of bacterial porins. *Future Microbiology*, 6:1–7. doi:10.2217/fmb.11.128

Albrecht, R., Zeth, K. 2011. Structural basis of outer membrane protein biogenesis in bacteria. *The Journal of Biological Chemistry*, 286:27792–803. doi:10.1074/jbc.M111.238931

Alegado, R. A, Campbell, M. C., Chen, W. C., Slutz, S. S., Tan, M. -W. 2003. Characterization of mediators of microbial virulence and innate immunity using the *Caenorhabditis elegans* host-pathogen model. *Cellular Microbiology*, 5:435–44. doi:10.1046/j.1462-5822.2003.00287.x

Alper, S., McBride, S. J., Lackford, B., Freedman, J. H. & Schwartz, D. 2007. Specificity and complexity of the *Caenorhabditis elegans* innate immune response. *Molecular and Celular Biology*, 27:5544–53. doi:10.1128/MCB.02070-06

Altun, Z. F., Hall, D. H. 2009. Alimentary system, rectum and anus. In *WormAtlas*. doi:10.3908/wormatlas.1.5

Arnold, T., Poynor, M., Nussberger, S., Lupas, A. N., Linke, D. 2007. Gene duplication of the eight-stranded beta-barrel OmpX produces a functional pore: a scenario for the evolution of transmembrane beta-barrels. *Journal of Molecular Biology*, 366:1174–84. doi:10.1016/j.jmb.2006.12.029

Arora, A., Rinehart, D., Szabo, G., Tamm, L. K. 2000. Refolded outer membrane protein A of *Escherichia coli* forms ion channels with two conductance states in planar lipid bilayers. *The Journal of Biological Chemistry*, *275*:1594–600. doi:10.1074/jbc.275.3.1594

Baeriswyl, S., Diard, M., Mosser, T., Leroy, M., Manière, X., Taddei, F., Matic, I. 2010. Modulation of aging profiles in isogenic populations of *Caenorhabditis elegans* by bacteria causing different extrinsic mortality rates. *Biogerontology*, 11:53–65. doi:10.1007/s10522-009-9228-0

Balasubramaniam, D., Arockiasamy, A., Kumar, P. D., Sharma, A., Krishnaswamy, S. 2012. Asymmetric pore occupancy in crystal structure of OmpF porin from *Salmonella typhi*. *Journal of Structural Biology*, 178:233–244. doi:10.1016/j.jsb.2012.04.005

Barchiesi, J., Castelli, M. E., Di Venanzio, G., Colombo, M. I., García Véscovi, E. 2012. The PhoP/PhoQ system and its role in *Serratia marcescens* pathogenesis. *Journal of Bacteriology*, 194:2949–61. doi:10.1128/JB.06820-11

Bartra, S. S., Gong, X., Lorica, C. D., Jain, C., Nair, M. K., Schifferli, D., Qian, L., Li, Z., Plano, G.V., Schesser, K. 2012. The outer membrane protein A (OmpA) of *Yersinia pestis*

promotes intracellular survival and virulence in mice. *Microbial Pathogenesis*, 52:41–6. doi:10.1016/j.micpath.2011.09.009

Baslé, A., Rummel, G., Storici, P., Rosenbusch, J. P., Schirmer, T. 2006. Crystal structure of osmoporin OmpC from *E. coli* at 2.0 A. *Journal of Molecular Biology*, 362:933–42. doi:10.1016/j.jmb.2006.08.002

Bawono, P., Heringa, J., Editor, D. J. R. 2014. PRALINE: a versatile multiple sequence alignment toolkit. *Methods in Molecular Biology*, 1079:245–62. doi:10.1007/978-1-62703-646-7_16

Begic, S. 2009. The role of OmpF and OmpC outer membrane porins and RND efflux pumps in antibiotic resistance of *Serratia marcescens*. PhD thesis, University of Manitoba. Department of Microbiology, Winnipeg, Manitoba. http://hdl.handle.net/1993/21396

Begic, S., Worobec, E. 2006. Regulation of *Serratia marcescens ompF* and *ompC* porin genes in response to osmotic stress, salicylate, temperature and pH. *Microbiology*, 152:485–91. doi:10.1099/mic.0.28428-0

Begic, S., Worobec, E. 2007. Site-directed mutagenesis studies to probe the role of specific residues in the external loop (L3) of OmpF and OmpC porins in susceptibility of *Serratia marcescens* to antibiotics. *Canadian Journal of Microbiology*, 53:710–9. doi:10.1139/W07-018

Begun, J., Gaiani, J. M., Rohde, H., Mack, D., Calderwood, S. B., Ausubel, F. M., Sifri, C. D. 2007. Staphylococcal biofilm exopolysaccharide protects against *Caenorhabditis elegans* immune defenses. *PLoS Pathogens*, 3:e57. doi:10.1371/journal.ppat.0030057

Bennion, D., Charlson, E. S., Coon, E., Misra, R. 2010. Dissection of β-barrel outer membrane protein assembly pathways through characterizing BamA POTRA 1 mutants of *Escherichia coli*. *Molecular Microbiology*, 77:1153–71. doi:10.1111/j.1365-2958.2010.07280.x

Berlanga, M., Ruiz, N., Hernandez-Borrell, J., Montero, T., Viñas, M. 2000. Role of the outer membrane in the accumulation of quinolones by *Serratia marcescens*. *Canadian Journal of Microbiology*, 46:716–22. doi:10.1139/w00-052

Bewick, V.,Cheek, L.,Ball, J. 2004. Statistics review 12: survival analysis. *Critical Care*. 8:389-94.

Bölter, B., Soll, J. 2001. Ion channels in the outer membranes of chloroplasts and mitochondria : open doors or regulated gates? *EMBO Journal*. 20:935–940. doi:10.1093/emboj/20.5.935

Boos, W., Shuman, H. 1998. Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiology and Molecular Biology Reviews*, 62: 204-29.

Borgonie, G., Van Driessche, R., Leyns, F., Arnaut, G., De Waele, D., Coomans, A. 1995. Germination of *Bacillus thuringiensis* spores in bacteriophagous nematodes (Nematoda: Rhabditida) *Journal of Invertebrate Pathology*, 65:61–7. doi:10.1006/jipa.1995.1008

Bos, M. P., Robert, V., Tommassen, J. 2007. Biogenesis of the Gram-negative bacterial outer membrane. *Annual Review of Microbiology*, 61:191–214. doi:10.1146/annurev.micro.61.080706.093245

Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics*, 77:71–94.

Brogden, K. A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews. Microbiology*, 3:238–50. doi:10.1038/nrmicro1098

Carbonell, G. V, Della Colleta, H. H., Yano, T., Darini, A. L., Levy, C. E., Fonseca, B. A. 2000. Clinical relevance and virulence factors of pigmented *Serratia marcescens*. *FEMS Immunology and Medical Microbiology*, 28:143–9. doi:10.1111/j.1574-695X.2000.tb01469.x

Chancey, S. T., Zähner, D., Stephens, D. S. 2012. Acquired inducible antimicrobial resistance in Gram-positive bacteria. *Future Microbiology*, 7:959–78. doi:10.2217/fmb.12.63

Chauhan, V. M., Orsi, G., Brown, A., Pritchard, D. I., Aylott, J. W. 2013. Mapping the pharyngeal and intestinal pH of *Caenorhabditis elegans* and real-time luminal pH oscillations using extended dynamic range pH-sensitive nanosensors. *ACS Nano*, 7:5577–87. doi:10.1021/nn401856u

Chiang, S. M., Schellhorn, H. E. 2012. Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. *Archives of Biochemistry and Biophysics*, 525:161–9. doi:10.1016/j.abb.2012.02.007

Choi, K.-H., Mima, T., Casart, Y., Rholl, D., Kumar, A., Beacham, I. R., Schweizer, H. P. 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. *Applied and Environmental Microbiology*, 74:1064–75. doi:10.1128/AEM.02430-07

Chow, C., Xu, H., Blanchard, J. S. 2013. Kinetic characterization of hydrolysis of nitrocefin, cefoxitin, and meropenem by β-lactamase from *Mycobacterium tuberculosis*. *Biochemistry*, 52:4097–104. doi:10.1021/bi400177y

Chubiz, L. M., Rao, C. V. 2011. Role of the *mar-sox-rob* regulon in regulating outer membrane porin expression. *Journal of Bacteriology*, 193:2252–60. doi:10.1128/JB.01382-10

Clokey, G. V, Jacobson, L. A. 1986. The autofluorescent “lipofuscin granules” in the intestinal cells of *Caenorhabditis elegans* are secondary lysosomes. *Mechanisms of Ageing and Development*, 35:79–94. doi:10.1016/0047-6374(86)90068-0

Coderch, N., Piqué, N., Lindner, B., Abitiu, N., Merino, S., Izquierdo, L., Jimenez, N., Tomás, J.M., Holst, O., Regue, M. 2004. Genetic and structural characterization of the core region of the lipopolysaccharide from *Serratia marcescens* N28b (serovar O4) *Journal of Bacteriology*, 186:978–88. doi:10.1128/JB.186.4.978

Confer, A. W., Ayalew, S. 2013. The OmpA family of proteins: roles in bacterial pathogenesis and immunity. *Veterinary Microbiology*, 163:207–22. doi:10.1016/j.vetmic.2012.08.019

Consortium, T. *C. elegans* S. 1998. Genome sequence of the nematode *C. elegans*: A platform for investigating biology. *Science*, 282:2012–2018. doi:10.1126/science.282.5396.2012

Coulthurst, S. J. 2013. The Type VI secretion system - a widespread and versatile cell targeting system. *Research in Microbiology*, 164:640–54. doi:10.1016/j.resmic.2013.03.017

Coulthurst, S. J., Barnard, A. M. L., Salmond, G. P. C. 2005. Regulation and biosynthesis of carbapenem antibiotics in bacteria. *Nature Reviews. Microbiology*, 3:295–306. doi:10.1038/nrmicro1128

Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J.N., Rosenbusch, J. P. 1992. Crystal structures explain functional properties of two *E. coli* porins. *Nature*, 358:727–33. doi:10.1038/358727a0

Crépin, S., Chekabab, S.-M., Le Bihan, G., Bertrand, N., Dozois, C. M., Harel, J. 2011. The Pho regulon and the pathogenesis of *Escherichia coli*. *Veterinary Microbiology*, 153:82–8. doi:10.1016/j.vetmic.2011.05.043

Davin-Regli, A., Bolla, J.-M., James, C. E., Lavigne, J.-P., Chevalier, J., Garnotel, E., Molitor, A., Pagès, J.-M. 2008. Membrane permeability and regulation of drug "influx and efflux" in Enterobacterial pathogens. *Current Drug Targets*, 9:750–9. doi:10.2174/138945008785747824

De Cock, H., Struyvé, M., Kleerebezem, M., van der Krift, T., Tommassen, J. 1997. Role of the carboxy-terminal phenylalanine in the biogenesis of outer membrane protein PhoE of *Escherichia coli* K-12. *Journal of Molecular Biology*, 269:473–8. doi:10.1006/jmbi.1997.1069

De, D., Borgonle, G., Claeys, M., Waele, D. De, Coomans, A. 1995. Ultrastructure of the intestine of the bacteriophagous nematodes *Caenorhabditis elegans*, *Panagrolaimus superbus* and *Acrobeloides maximus* (Nematoda : Rhabditida) *Fundamental and Applied Nematology*, 18:123–133.

De Feyter, R., Yang, Y., Gabriel, D. W. 1993. Gene-for-genes interactions between cotton R genes and *Xanthomonas campestris* pv. *malvacearum* avr genes. *Molecular Plant-Microbe Interactions*, 6:225–37.

De la Cruz, M. A., Calva, E. 2010. The complexities of porin genetic regulation. *Journal of Molecular Microbiology and Biotechnology*, 18:24–36. doi:10.1159/000274309

Delcour, A. H. 2003. Solute uptake through general porins. *Frontiers in Bioscience*, 8:1055–71.

Delihas, N. 2012. Regulating the regulator: MicF RNA controls expression of the global regulator Lrp. *Molecular Microbiology*, 84:401–4. doi:10.1111/j.1365-2958.2012.08030.x

Delihas, N., Forst, S. 2001. MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. *Journal of Molecular Biology*, 313:1–12. doi:10.1006/jmbi.2001.5029

Driessen, A. J. M., Nouwen, N. 2008. Protein translocation across the bacterial cytoplasmic membrane. *Annual Review of Biochemistry*, 77:643–67. doi:10.1146/annurev.biochem.77.061606.160747

Dupont, M., Dé, E., Chollet, R., Chevalier, J., Pagès, J.-M. 2004. *Enterobacter aerogenes* OmpX, a cation-selective channel mar- and osmo-regulated. *FEBS Letters*, 569:27–30. doi:10.1016/j.febslet.2004.05.047

Dupont, M., James, C. E., Chevalier, J., Pagès, J.-M. 2007. An early response to environmental stress involves regulation of OmpX and OmpF, two *Enterobacter*ial outer membrane pore-forming proteins. *Antimicrobial Agents and Chemotherapy*, 51:3190–8. doi:10.1128/AAC.01481-06

Eberl, L., Winson, M. K., Sternberg, C., Stewart, G. S., Christiansen, G., Chhabra, S. R., Williams P., Molin S., Givskov, M. 1996. Involvement of N-acyl-L-hormoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Molecular Microbiology*, 20:127–36. doi:10.1111/j.1365-2958.1996.tb02495.x

Ellis, R. E., Stanfield, G. M. 2014. The regulation of spermatogenesis and sperm function in nematodes. *Seminars in Cell & Developmental Biology*, 29:17–30. doi:10.1016/j.semcdb.2014.04.005

Engelmann, I., Griffon, A., Tichit, L., Montañana-Sanchis, F., Wang, G., Reinke, V., Waterston R.,H., Hillier L,W., Ewbank, J. J. 2011. A comprehensive analysis of gene expression changes provoked by bacterial and fungal infection in *C. elegans*. *PloS One*, 6:e19055. doi:10.1371/journal.pone.0019055

Engelmann, I., Pujol, N. 2010. Invertebrate Immunity. In Advances in Experimental Medicine and Biology, pp. 105–121. Edited by Kenneth Söderhäll . Boston, MA: Springer US. ISBN 978-1-4419-8059-5. doi:10.1007/978-1-4419-8059-5_6

English, G., Trunk, K., Rao, V. A., Srikannathasan, V., Hunter, W. N., Coulthurst, S. J. 2012. New secreted toxins and immunity proteins encoded within the Type VI secretion system

gene cluster of *Serratia marcescens*. *Molecular Microbiology*, 86:921–36. doi:10.1111/mmi.12028

Ewbank, J. J. 2002. Tackling both sides of the host-pathogen equation with *Caenorhabditis elegans*. *Microbes and Infection*, 4:247–56.

Ewbank, J. J. 2006. Signaling in the immune response. *WormBook : The Online Review of C. elegans Biology*, 1–12. doi:10.1895/wormbook.1.83.1

Ewbank, J. J., Zugasti, O. 2011. *C. elegans*: model host and tool for antimicrobial drug discovery. *Disease Models & Mechanisms*, 4:300–4. doi:10.1242/dmm.006684

Fairman, J. W., Noinaj, N., Buchanan, S. K. 2011. The structural biology of β-barrel membrane proteins: a summary of recent reports. *Current Opinion in Structural Biology*, 21:523–31. doi:10.1016/j.sbi.2011.05.005

Faraldo-Gómez, J. D., Sansom, M. S. P. 2003. Acquisition of siderophores in Gram-negative bacteria. *Nature Reviews. Molecular Cell Biology*, 4:105–16. doi:10.1038/nrm1015

Fedrigo, G.V., Campoy, E.M., Di Venanzio, G., Colombo, M.I., García-Véscovi E. 2011. *Serratia marcescens* is able to survive and proliferate in autophagic-like vacuoles inside non-phagocytic cells. *PLoS One*, 6:e24054. doi:10.1371/journal.pone.0024054

Ferenci, T. 1996. Adaptation to life at micromolar nutrient levels: the regulation of *Escherichia coli* glucose transport by endoinduction and cAMP. *FEMS Microbiology Reviews*, 18:301–17.

Fernández, L., Hancock, R. E. W. 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clinical Microbiology Reviews*, 25:661–81. doi:10.1128/CMR.00043-12

Filloux, A. 2013. The rise of the Type VI secretion system. *F1000prime Reports*, 5:52. doi:10.12703/P5-52

Fito-Boncompte, L., Chapalain, A., Bouffartigues, E., Chaker, H., Lesouhaitier, O., Gicquel, G., Bazire, A., Madi, A., Connil, N., Véron, W., Taupin, L., Toussaint, B., Cornelis, P., Wei, Q., Shioya, K., Déziel, E., Feuilloley, M.G., Orange, N., Dufour, A., Chevalier, S. 2011. Full virulence of *Pseudomonas aeruginosa* requires OprF. *Infection and Immunity*, 79:1176–86. doi:10.1128/IAI.00850-10

Fjell, C. D., Hiss, J. a, Hancock, R. E. W., Schneider, G. 2012. Designing antimicrobial peptides: form follows function. *Nature Reviews. Drug Discovery*, 11:37–51. doi:10.1038/nrd3591

Flyg, C., Kenne, K., Boman, H. G. 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to Cecropia immunity and a

decreased virulence to Drosophila. *Journal of General Microbiology*, 120:173–81. doi:10.1099/00221287-120-1-173

Forst, S. A., Delgado, J., Inouye, M. 1989. DNA-binding properties of the transcription activator (OmpR) for the upstream sequences of *ompF* in *Escherichia coli* are altered by *envZ* mutations and medium osmolarity. *Journal of Bacteriology*, 171:2949–55.

Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K., Ramos, J. L. 1997. Arac/XylS family of transcriptional regulators. *Microbiology and Molecular Biology Reviews*, 61:393–410.

Gao, H., Zhang, Y., Yang, L., Liu, X., Guo, Z., Tan, Y., Han, Y., Huang, X., Zhou, D., Yang, R. 2011. Regulatory effects of cAMP receptor protein (CRP) on porin genes and its own gene in *Yersinia pestis*. *BMC Microbiology*, 11:40. doi:10.1186/1471-2180-11-40

García-Sureda, L., Doménech-Sánchez, A., Barbier, M., Juan, C., Gascó, J., Albertí, S. 2011. OmpK26, a novel porin associated with carbapenem resistance in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*, 55:4742–7. doi:10.1128/AAC.00309-11

García-Sureda, L., Juan, C., Doménech-Sánchez, A., Albertí, S. 2011. Role of *Klebsiella pneumoniae* LamB porin in antimicrobial resistance. *Antimicrobial Agents and Chemotherapy*, 55:1803–5. doi:10.1128/AAC.01441-10

Gelder, P. Van, Dumas, F., Bartoldus, I., Saint, N., Prilipov, A., Winterhalter, M., Wang, Y.,Philippsen, A.,Rosenbusch, J.P., Schirmer, T. 2002. Sugar transport through maltoporin of *Escherichia coli*: role of the greasy slide. *Journal of Bacteriology*, 184:2994–9. doi:10.1128/JB.184.11.2994

Götzke, H., Palombo, I., Muheim, C., Perrody, E., Genevaux, P., Kudva, R., Müller, M., Daley, D.O. 2014. YfgM is an ancillary subunit of the SecYEG translocon in *Escherichia coli*. Journal of Biological Chemistry. 289:19089–97. doi: 10.1074/jbc.M113.541672

Greco-Stewart, V. S., Brown, E. E., Parr, C., Kalab, M., Jacobs, M. R., Yomtovian, R. a, Ramírez-Arcos, S. M. 2012. *Serratia marcescens* strains implicated in adverse transfusion reactions form biofilms in platelet concentrates and demonstrate reduced detection by automated culture. *Vox Sanguinis*, 102:212–20. doi:10.1111/j.1423-0410.2011.01550.x

Green, D. W. 2002. The bacterial cell wall as a source of antibacterial targets. *Expert Opinion on Therapeutic Targets*, 6:1–19. doi:10.1517/14728222.6.1.1

Greer, E. L., Maures, T. J., Ucar, D., Hauswirth, A. G., Mancini, E., Lim, J. P., Benayoun, B.A., Shi, Y., Brunet, A. 2011. Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans*. *Nature*, 479:365–71. doi:10.1038/nature10572

Grimont, P. A, Grimont, F. 1978. The genus *Serratia*. *Annual Review of Microbiology*, 32:221–48. doi:10.1146/annurev.mi.32.100178.001253

Gristwood, T., Fineran, P. C., Everson, L., Williamson, N. R., Salmond, G. P. 2009. The PhoBR two-component system regulates antibiotic biosynthesis in *Serratia* in response to phosphate, 14:1–14. doi:10.1186/1471-2180-9-112

Guasch, J. F., Ferrer, S., Enfedaque, J., Viejo, M. B., Regué, M. 1995. A 17 kDa outer-membrane protein (Omp4) from *Serratia marcescens* confers partial resistance to bacteriocin 28b when expressed in *Escherichia coli*. *Microbiology*, 141:2535–42. doi:10.1099/13500872-141-10-2535

Gumienny, T. L., Savage-Dunn, C. 2013. TGF-β signaling in *C. elegans. WormBook* 1–34. doi:10.1895/wormbook.1.22.2.

Gupta, B. P., Hanna-Rose, W., Sternberg, P. W. 2012. Morphogenesis of the vulva and the vulval-uterine connection. *WormBook : The Online Review of C. elegans Biology*, 1–20. doi:10.1895/wormbook.1.152.1

Gutmann, L., Williamson, R., Moreau, N., Kitzis, M.-D. D., Collatz, E., Acar, J. F., Goldstein, F. W. 1985. Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella, Enterobacter*, and *Serratia*. *Journal of Infectious Diseases*, 151:501–507. doi:10.1093/infdis/151.3.501

Haag, E. S. 2005. The evolution of nematode sex determination: *C. elegans* as a reference point for comparative biology. *WormBook : The Online Review of C. elegans Biology*, 1–14. doi:10.1895/wormbook.1.120.1

Hall, M. N., Silhavy, T. J. 1981. Genetic analysis of the *ompB* locus in *Escherichia coli* K-12. *Journal of Molecular Biology*, 151:1–15. doi:10.1016/0022-2836(81)90218-7

Haltia, T., Freire, E. 1995. Forces and factors that contribute to the structural stability of membrane proteins. *Biochimica et Biophysica Acta*, 1241:295–322. doi:10.1016/0304-4157(94)00161-6

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology*, 166:557–80. doi:10.1016/S0022-2836(83)80284-8

Hancock, R. E., Chapple, D. S. 1999. Peptide antibiotics. *Antimicrobial Agents and Chemotherapy*, 43:1317–23.

Haney, E. F., Hancock, R. E. W. 2013. Peptide design for antimicrobial and immunomodulatory applications. *Biopolymers*, 100:572–83. doi:10.1002/bip.22250

Hanson, N. D. 2003. AmpC beta-lactamases: what do we need to know for the future? *The Journal of Antimicrobial Chemotherapy*, 52:2–4. doi:10.1093/jac/dkg284

Harris, A. K. P., Williamson, N. R., Slater, H., Cox, A., Abbasi, S., Foulds, I., Simonsen, H.T., Leeper, F.J. Salmond, G. P. C. 2004. The *Serratia* gene cluster encoding biosynthesis

of the red antibiotic, prodigiosin, shows species- and strain-dependent genome context variation. *Microbiology*, 150:3547–60. doi:10.1099/mic.0.27222-0

Hejazi, A., Falkiner, F. R. 1997. *Serratia marcescens*. *Journal of Medical Microbiology*, 46:903–12. doi:10.1099/00222615-46-11-903

Henning, U., Sonntag, I., Hindennach, I. 1978. Mutants (*ompA*) affecting a major outer membrane protein of *Escherichia coli* K12. *European Journal of Biochemistry / FEBS*, 92:491–8. doi:10.1111/j.1432-1033.1978.tb12771.x

Hermann, G. J., Schroeder, L. K., Hieb, C. A., Kershner, A. M., Rabbitts, B. M., Fonarev, P., Grant, B.D., Priess, J. R. 2005. Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*. *Molecular Biology of the Cell*, 16:3273–88. doi:10.1091/mbc.E05-01-0060

Hinchliffe, P., Symmons, M. F., Hughes, C., Koronakis, V. 2013. Structure and operation of bacterial tripartite pumps. *Annual Review of Microbiology*, 67:221–42. doi:10.1146/annurev-micro-092412-155718

Hoang, T. T., Karkho, R. R., Kutchma, A. J., Schweizer, H. P. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences : application for isolation of unmarked *Pseudomonas aeruginosa* mutants, 212:77–86. doi:10.1016/S0378-1119(98)00130-9

Holmqvist, E., Unoson, C., Reimegård, J., Wagner, E. G. 2012. A mixed double negative feedback loop between the sRNA MicF and the global regulator Lrp. *Molecular Microbiology*, 84:414–27. doi:10.1111/j.1365-2958.2012.07994.x

Höltje, J. V, Kopp, U., Ursinus, A., Wiedemann, B. 1994. The negative regulator of beta-lactamase induction AmpD is a N-acetyl-anhydromuramyl-L-alanine amidase. *FEMS Microbiology Letters*, 122:159–64. doi:10.1111/j.1574-6968.1994.tb07159.x

Hope, I.A. 1999. C. elegans: a practical approach. In: Hope, I.A. (ed) The practical approach series. Oxford University Press, Oxford, pp 181-199.

Hsieh, Y.-J., Wanner, B. L. 2010. Global regulation by the seven-component Pi signaling system. *Current Opinion in Microbiology*, 13:198–203. doi:10.1016/j.mib.2010.01.014

Huang, K. J., Schieberl, J. L. Igo, M. M. 1994. A distant upstream site involved in the negative regulation of the *Escherichia coli ompF* gene. *Journal of Bacteriology*, 176:1309–15.

Hume, E. B., Stapleton, F., Willcox, M. D. P. 2003. Evasion of cellular ocular defenses by contact lens isolates of *Serratia marcescens*. *Eye and Contact Lens*, 29:108–12. doi:10.1097/01.ICL.0000062461.24391.7F

Huovinen, P. 2001. Resistance to trimethoprim-sulfamethoxazole. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, 32:1608–14. doi:10.1086/320532

Hutsul, J.A., Worobec, E.A. 1994. Molecular characterization of a 40 kDa OmpC-like porin from *Serratia marcescens*. *Microbiology*, 140:379–87. doi:10.1099/13500872-140-2-379

Hutsul, J.A., Worobec, E.A. 1997. Molecular characterization of the *Serratia marcescens* OmpF porin, and analysis of S. marcescens OmpF and OmpC osmoregulation. *Microbiology*, 143:2797–806. doi:10.1099/00221287-143-8-2797

Iguchi, A., Nagaya, Y., Pradel, E., Ooka, T., Ogura, Y., Katsura, K., Kurokawa, K., Oshima, K., Hattori, M., Parkhill, J., Sebaihia, M., Coulthurst, S.J., Gotoh, N., Thomson, N.R., Ewbank, J.J., Hayashi, T. 2014. Genome evolution and plasticity of *Serratia marcescens*, an important multidrug-resistant nosocomial pathogen. *Genome Biology and Evolution*, 6:2096–110. doi:10.1093/gbe/evu160

Irazoqui, J. E., Ausubel, F. M. 2010. 99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: *Caenorhabditis elegans* as a model to study tissues involved in host immunity and microbial pathogenesis. *Clinical and Experimental Immunology*, 160:48–57. doi:10.1111/j.1365-2249.2010.04122.x

Irazoqui, J. E., Troemel, E. R., Feinbaum, R. L., Luhachack, L. G., Cezairliyan, B. O., Ausubel, F. M. 2010. Distinct pathogenesis and host responses during infection of *C. elegans* by P. aeruginosa and S. aureus. *PLoS Pathogens*, 6:e1000982. doi:10.1371/journal.ppat.1000982

Irazoqui, J. E., Urbach, J. M., Ausubel, F. M. 2010. Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. *Nature Reviews. Immunology*, 10:47–58. doi:10.1038/nri2689

Jacoby, G. A. 2009. AmpC beta-lactamases. *Clinical Microbiology Reviews*, 22:161–82. doi:10.1128/CMR.00036-08

Jenssen, H., Hamill, P., Hancock, R. E. W. 2006. Peptide antimicrobial agents. *Clinical Microbiology Reviews*, 19:491–511. doi:10.1128/CMR.00056-05

Johnson, J. W., Fisher, J. F., Mobashery, S. 2013. Bacterial cell-wall recycling. *Annals of the New York Academy of Sciences*, 1277: 54–75. doi:10.1111/j.1749-6632.2012.06813.x

Joris, B., De Meester, F., Galleni, M., Masson, S., Dusart, J., Frère, J. M., Van Beeumen, J., Bush, K., Sykes, R. 1986. Properties of a class C beta-lactamase from *Serratia marcescens*. *The Biochemical Journal*, 239:581–6.

Juan, C., Moyá, B., Pérez, J. L., Oliver, A. 2006. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level beta-lactam resistance

involves three AmpD homologues. *Antimicrobial Agents and Chemotherapy*, 50:1780–7. doi:10.1128/AAC.50.5.1780-1787.2006

Kaczmarek, F. M., Dib-Hajj, F., Shang, W., Gootz, T. D. 2006. High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of bla(ACT-1) beta-lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin phoe. *Antimicrobial Agents and Chemotherapy*, 50:3396–406. doi:10.1128/AAC.00285-06

Kalivoda, E. J., Stella, N. a, O'Dee, D. M., Nau, G. J., Shanks, R. M. Q. 2008. The cyclic AMP-dependent catabolite repression system of *Serratia marcescens* mediates biofilm formation through regulation of type 1 fimbriae. *Applied and Environmental Microbiology*, 74:3461–70. doi:10.1128/AEM.02733-07

Kaniga, K., Delor, I., Cornelis, G. R. 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene*, 109:137–41. doi:10.1016/0378-1119(91)90599-7

Kenney, L. J., Bauer, M. D., Silhavy, T. J. 1995. Phosphorylation-dependent conformational changes in OmpR, an osmoregulatory DNA-binding protein of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, 92:8866–70.

Kerff, F., Petrella, S., Mercier, F., Sauvage, E., Herman, R., Pennartz, A., Zervosen, A., Luxen, A., Frère, J.M., Joris, B.,Charlier, P. 2010. Specific structural features of the N-acetylmuramoyl-L-alanine amidase AmiD from *Escherichia coli* and mechanistic implications for enzymes of this family. *Journal of Molecular Biology*, 397:249–59. doi:10.1016/j.jmb.2009.12.038

Kesika, P., Karutha Pandian, S., Balamurugan, K. 2011. Analysis of *Shigella flexneri*-mediated infections in model organism *Caenorhabditis elegans*. *Scandinavian Journal of Infectious Diseases*, 43:286–95. doi:10.3109/00365548.2010.548400

Kim, K. H., Aulakh, S., Paetzel, M. 2012. The bacterial outer membrane β-barrel assembly machinery. *Protein Science : A Publication of the Protein Society*, 21:751–68. doi:10.1002/pro.2069

Knowles, T. J., Jeeves, M., Bobat, S., Dancea, F., McClelland, D., Palmer, T., Overduin M, Henderson, I. R. 2008. Fold and function of polypeptide transport-associated domains responsible for delivering unfolded proteins to membranes. *Molecular Microbiology*, 68:1216–27. doi:10.1111/j.1365-2958.2008.06225.x

Koebnik, R., Locher, K. P., Van Gelder, P. 2000. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Molecular Microbiology*, 37:239–53. doi:10.1046/j.1365-2958.2000.01983.x

Konaklieva, M. 2014. Molecular targets of β-lactam-based antimicrobials: Beyond the usual suspects. *Antibiotics*, 3:128–142. doi:10.3390/antibiotics3020128

Kong, K.-F., Schneper, L., Mathee, K. 2010. Beta-lactam antibiotics: from antibiosis to resistance and bacteriology. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, 118:1–36. doi:10.1111/j.1600-0463.2009.02563.x

Korteland, J., Tommassen, J., Lugtenberg, B. 1982. PhoE protein pore of the outer membrane of *Escherichia coli* K12 is a particularly efficient channel for organic and inorganic phosphate. *Biochimica et Biophysica Acta*, 690:282–9.

Kumar, A., Schweizer, H. P. 2005. Bacterial resistance to antibiotics: active efflux and reduced uptake. *Advanced Drug Delivery Reviews*, 57:1486–513. doi:10.1016/j.addr.2005.04.004

Kumar, A., Worobec, E. A. 2005. Cloning , sequencing , and characterization of the SdeAB multidrug efflux pump of *Serratia marcescens*, 49:1495–1501. doi:10.1128/AAC.49.4.1495

Kurz, C.L., Chauvet, S., Andrès, E., Aurouze, M., Vallet, I., Michel, G.P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J.A., Finlay, B.B., Gorvel, J.P., Ferrandon, D., Ewbank, J.J. 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. *The EMBO Journal*, 22:1451–60. doi:10.1093/emboj/cdg159

Kurz, C. L., Ewbank, J. J. 2000. *Caenorhabditis elegans* for the study of host-pathogen interactions. *Trends in Microbiology*, 8:142–4. doi: 10.1016/S0966-842X(99)01691-1

Kurz, C. L., Ewbank, J. J. 2003. *Caenorhabditis elegans*: an emerging genetic model for the study of innate immunity. *Nature Reviews. Genetics*, 4:380–90. doi:10.1038/nrg1067

Kurz, C. L., Tan, M.-W. 2004. Regulation of aging and innate immunity in *C. elegans*. *Aging Cell*, 3:185–93. doi:10.1111/j.1474-9728.2004.00108.x

Kyriakis, J. M., Avruch, J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiological Reviews.* 81:807–69.

Labbate, M., Queck, S. Y., Koh, K. S., Rice, S. A., Givskov, M., Kjelleberg, S. 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. *Journal of Bacteriology*, 186:692–8. doi:10.1128/JB.186.3.692

Labbate, M., Zhu, H., Thung, L., Bandara, R., Larsen, M. R., Willcox, M. D., Givskov, M., Rice, S.A., Kjelleberg, S. 2007. Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. *Journal of Bacteriology*, 189:2702–11. doi:10.1128/JB.01582-06

Lam, J. S., Taylor, V. L., Islam, S. T., Hao, Y., Kocíncová, D. 2011. Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. *Frontiers in Microbiology*, 2:118. doi:10.3389/fmicb.2011.00118

Lamarche, M. G., Wanner, B. L., Crépin, S., Harel, J. 2008. The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *FEMS Microbiology Reviews*, 32:461–73. doi:10.1111/j.1574-6976.2008.00101.x

Lauman, B., Pagel, M., Delcour, A. H. 2008. Altered pore properties and kinetic changes in mutants of the *Vibrio cholerae* porin OmpU. *Molecular Membrane Biology*, 25:498–505. doi:10.1080/09687680802454637

Laupland, K. B., Parkins, M. D., Gregson, D. B., Church, D. L., Ross, T., Pitout, J. D. D. 2008. Population-based laboratory surveillance for *Serratia* species isolates in a large Canadian health region. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*, 27:89–95. doi:10.1007/s10096-007-0400-7

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K., Wertheim, H.F., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D., Cars, O. 2013. Antibiotic resistance-the need for global solutions. *The Lancet Infectious Diseases*, 13:1057–98. doi:10.1016/S1473-3099(13)70318-9

Leiman, P. G., Basler, M., Ramagopal, U. a, Bonanno, J. B., Sauder, J. M., Pukatzki, S., Burley, S.K., Almo, S.C. Mekalanos, J. J. 2009. Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proceedings of the National Academy of Sciences of the United States of America*, 106:4154–9. doi:10.1073/pnas.0813360106

Li, X.-Z., Nikaido, H. 2004. Efflux-mediated drug resistance in bacteria. *Drugs*, 64:159–204. doi:10.2165/00003495-200464020-00004

Li, X.-Z., Nikaido, H. 2009. Efflux-mediated drug resistance in bacteria: an update. *Drugs*, 69:1555–623. doi:10.2165/11317030-000000000-00000

Lin, J., Huang, S., Zhang, Q. 2002. Outer membrane proteins: key players for bacterial adaptation in host niches. *Microbes and Infection / Institut Pasteur*, 4:325–31. doi: 10.1016/S1286-4579(02)01545-9

Lister, P. D., Wolter, D. J., Hanson, N. D. 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clinical Microbiology Reviews*, 22:582–610. doi:10.1128/CMR.00040-09

Liu, X., Ferenci, T. 1998. Regulation of porin-mediated outer membrane permeability by nutrient limitation in *Escherichia coli*. *Journal of Bacteriology*, 180:3917–22.

Liu, X., Ferenci, T. 2001. An analysis of multifactorial influences on the transcriptional control of *ompF* and *ompC* porin expression under nutrient limitation. *Microbiology*, 147:2981–9.

Livermore, D. M. 1995. Beta-Lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews*, 8:557–84.

Lolicato, M., Reina, S., Messina, A., Guarino, F., Winterhalter, M., Benz, R., De Pinto, V. 2011. Generation of artificial channels by multimerization of β-strands from natural porin. *Biological Chemistry*, 392:617–24. doi:10.1515/BC.2011.063

Mahlen, S. D. 2011. *Serratia* infections: from military experiments to current practice. *Clinical Microbiology Reviews*, 24:755–91. doi:10.1128/CMR.00017-11

Mahlen, S. D., Morrow, S. S., Abdalhamid, B., Hanson, N. D. 2003. Analyses of *ampC* gene expression in *Serratia marcescens* reveal new regulatory properties. *The Journal of Antimicrobial Chemotherapy*, 51:791–802. doi:10.1093/jac/dkg133

Mallo, G. V, Kurz, C. L., Couillault, C., Pujol, N., Granjeaud, S., Kohara, Y., Ewbank, J. J. 2002. Inducible antibacterial defense system in *C. elegans*. *Current Biology*, 12:1209–14. doi:10.1016/S0960-9822(02)00928-4

Malouin, F., Campbell, G. D., Halpenny, M., Becker, G. W., Parr, T. R. 1990. Outer membrane and porin characteristics of *Serratia marcescens* grown in vitro and in rat intraperitoneal diffusion chambers. *Infection and Immunity*, 58:1247–1253.

Mark, B. L., Vocadlo, D. J., Oliver, A. 2011. Providing β-lactams a helping hand: targeting the AmpC β-lactamase induction pathway. *Future Microbiology*, 6:1415–27. doi:10.2217/fmb.11.128

Martin, R. J., Verma, S., Levandoski, M., Clark, C. L., Qian, H., Stewart, M., Robertson,A.P. 2005. Drug resistance and neurotransmitter receptors of nematodes: recent studies on the mode of action of levamisole. *Parasitology*, 131:S71–84. doi:10.1017/S0031182005008668

Masi, M., Pagès, J. 2013. Structure, function and regulation of outer membrane proteins involved in drug transport in *Enterobactericeae*: the OmpF/C - TolC case. *The Open Microbiology Journal*.7:22-33. doi:10.2174/1874285801307010022

Matsuo, T., Chen, J., Minato, Y., Ogawa, W., Mizushima, T., Kuroda, T., Tsuchiya, T. 2008. SmdAB, a heterodimeric ABC-type multidrug efflux pump, in *Serratia marcescens*. *Journal of Bacteriology*, 190:648–54. doi:10.1128/JB.01513-07

Matsuura, M. 2013. Structural modifications of bacterial lipopolysaccharide that facilitate Gram-negative bacteria evasion of host innate immunity. *Frontiers in Immunology*.4:109. doi: 10.3389/fimmu.2013.00109.

Matsuyama, T., Kaneda, K., Nakagawa, Y., Isa, K., Hara-Hotta, H., Yano, I. 1992. A novel extracellular cyclic lipopeptide which promotes flagellum-dependent and -independent spreading growth of *Serratia marcescens*. *Journal of Bacteriology*, 174:1769–76.

Matsuyama, T.,Tanikawa, T., Nakagawa, Y. 2011. Serrawettins and other surfactants produced by *Serratia*. In Biosurfactants, pp 93-120. Edited by Gloria Soberón-Chávez. Berlin, Heidelberg: Springer Berlin Heidelberg. ISBN 978-3-642-14490-5 doi:10.1007/978-3-642-14490-5

McGhee, J. D. 2007 The *C. elegans* intestine. *WormBook : The Online Review of C. elegans Biology*, 1–36. doi:10.1895/wormbook.1.133.1

Meyer, J. R., Dobias, D. T., Weitz, J. S., Barrick, J. E., Quick, R. T., Lenski, R. E. 2012. Repeatability and contingency in the evolution of a key innovation in phage lambda. *Science*, 335:428–32. doi:10.1126/science.1214449

Miller, V. L. & Mekalanos, J. J. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. *Journal of Bacteriology*, 170:2575–83.

Minato, Y., Shahcheraghi, F., Ogawa, W., Kuroda, T., Tsuchiya, T. 2008. Functional gene cloning and characterization of the SsmE multidrug efflux pump from *Serratia marcescens*. *Biological & Pharmaceutical Bulletin*, 31:516–9.

Mochii, M., Yoshida, S., Morita, K., Kohara, Y., Ueno, N. 1999. Identification of transforming growth factor-beta - regulated genes in *Caenorhabditis elegans* by differential hybridization of arrayed cDNAs. *Proceedings of the National Academy of Sciences*, 96:15020–15025. doi:10.1073/pnas.96.26.15020

Moy, T. I., Ball, A. R., Anklesaria, Z., Casadei, G., Lewis, K., Ausubel, F. M. 2006. Identification of novel antimicrobials using a live-animal infection model. *Proceedings of the National Academy of Sciences of the United States of America*, 103:10414–9. doi:10.1073/pnas.0604055103

Moya, B., Juan, C., Albertí, S., Pérez, J. L., Oliver, A. 2008. Benefit of having multiple *ampD* genes for acquiring beta-lactam resistance without losing fitness and virulence in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 52:3694–700. doi:10.1128/AAC.00172-08

Moya-Torres, A., Mulvey, M. R., Kumar, A., Oresnik, I. J., Brassinga, A. K. C. 2014. The lack of OmpF, but not OmpC, contributes to increased antibiotic resistance in *Serratia marcescens*. *Microbiology*, 160:1882–92. doi:10.1099/mic.0.081166-0

Muir, R. E., Tan, M.-W. 2008. Virulence of Leucobacter chromiireducens subsp. solipictus to *Caenorhabditis elegans*: characterization of a novel host-pathogen interaction. *Applied and Environmental Microbiology*, 74:4185–98. doi:10.1128/AEM.00381-08

Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E., Coulthurst, S. J. 2011. The opportunistic pathogen *Serratia marcescens* utilizes type VI secretion to target bacterial competitors. *Journal of Bacteriology*, 193:6057–69. doi:10.1128/JB.05671-11

Nakae, T. 1975. Outer membrane of *Salmonella typhi*murium: Reconstitution of sucrose-permeable membrane vesicles. *Biochemical and Biophysical Research Communications*, 64:1224-30. doi:10.1016/0006-291X(75)90823-2

Nakae, T. .1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. *Biochemical and Biophysical Research Communications*, 71:877–884. doi:10.1016/0006-291X(76)90913-X

Nakashima, R., Sakurai, K.,Yamasaki, S.,Nishino, K.,Yamaguchi, A. 2011. Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. Nature. 480:565-9. doi:10.1038/nature10641.

Namdari, H., Tan, T. Y., Dowzicky, M. J. 2012. Activity of tigecycline and comparators against skin and skin structure pathogens: global results of the Tigecycline evaluation and surveillance trial, 2004-2009. *International Journal of Infectious Diseases.* 16:e60–6. doi:10.1016/j.ijid.2011.09.021

Nasiri, B. J. 2011. Identification and analysis of Rob, a transcriptional regulator from *Serratia marcescens*. MSc thesis, University of Manitoba. Department of Microbiology, Winnipeg, Manitoba. http://hdl.handle.net/1993/4403

Naveed, H., Jimenez-Morales, D., Tian, J., Pasupuleti, V., Kenney, L. J., Liang, J. 2012. Engineered oligomerization state of OmpF protein through computational design decouples oligomer dissociation from unfolding. *Journal of Molecular Biology*, 419:89–101. doi:10.1016/j.jmb.2012.02.043

Nestorovich, E. M., Danelon, C., Winterhalter, M., Bezrukov, S. M. 2002. Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. *Proceedings of the National Academy of Sciences of the United States of America*, 99:9789–94. doi:10.1073/pnas.152206799

Nguyen, T. X., Alegre, E. R., Kelley, S. T. 2006. Phylogenetic analysis of general bacterial porins: a phylogenomic case study. *Journal of Molecular Microbiology and Biotechnology*, 11:291–301. doi:10.1159/000095631

Nikaido, H. 1992. Porins and specific channels of bacterial outer membranes. *Molecular Microbiology*, 6:435–42. doi:10.1111/j.1365-2958.1992.tb01487.x

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*, 67:593–656. doi:10.1128/MMBR.67.4.593-656.2003

Nikaido, H., Rosenberg, E. Y. 1983. Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. *Journal of Bacteriology*, 153:241–52.

Nikaido, H., Vaara, M. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews*, 49:1–32.

Noinaj, N., Fairman, J. W., Buchanan, S. K. 2011. The crystal structure of BamB suggests interactions with BamA and its role within the BAM complex. *Journal of Molecular Biology*, 407:248–60. doi:10.1016/j.jmb.2011.01.042

Noinaj, N., Kuszak, A. J., Balusek, C., Gumbart, J. C., Buchanan, S. K. 2014. Lateral opening and exit pore formation are required for BamA function. *Structure*, 22:1055–62. doi:10.1016/j.str.2014.05.008

Noinaj, N., Kuszak, A. J., Gumbart, J. C., Lukacik, P., Chang, H., Easley, N. C., Lithgow, T., Buchanan, S. K. 2013. Structural insight into the biogenesis of β-barrel membrane proteins. *Nature*, 501:385-90. doi:10.1038/nature12521

O'Callaghan, C. H., Morris, A., Kirby, S. M., Shingler, A. H. 1972. Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate. *Antimicrobial Agents and Chemotherapy*, 1:283–8. doi:10.1128/AAC.1.4.283

Pagès, J. 2009. Bacterial porin and antibiotic susceptibility. *Médecine sciences.* 20:346–351.

Pagès, J.-M., James, C. E., Winterhalter, M. 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nature Reviews. Microbiology*, 6:893–903. doi:10.1038/nrmicro1994

Park, B. S., Lee, J.-O. 2013. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental & Molecular Medicine*, 45:e66. doi:10.1038/emm.2013.97

Park, J. T., Uehara, T. 2008. How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan) *Microbiology and Molecular Biology Reviews*, 72:211–27, table of contents. doi:10.1128/MMBR.00027-07

Pell, L. G., Kanelis, V., Donaldson, L. W., Howell, P. L., Davidson, A. R. 2009. The phage lambda major tail protein structure reveals a common evolution for long-tailed phages and the type VI bacterial secretion system. *Proceedings of the National Academy of Sciences of the United States of America*, 106:4160–5. doi:10.1073/pnas.0900044106

Petty, N. K., Foulds, I. J., Pradel, E., Ewbank, J. J., Salmond, G. P. C. 2006. A generalized transducing phage (phiIF3) for the genomically sequenced *Serratia marcescens* strain Db11:

a tool for functional genomics of an opportunistic human pathogen. *Microbiology*, 152:1701–8. doi:10.1099/mic.0.28712-0

Pfaller, M., Segreti, J. 2006. Overview of the epidemiological profile and laboratory detection of extended-spectrum beta-lactamases. *Clinical Infectious Diseases*, 42:S153–63. doi:10.1086/500662

Pfeifer, Y., Cullik, A., Witte, W. 2010. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *International Journal of Medical Microbiology*, 300:371–9. doi:10.1016/j.ijmm.2010.04.005

Phale, P. S., Philippsen, A., Kiefhaber, T., Koebnik, R., Phale, V. P., Schirmer, T., Rosenbusch, J. P. 1998. Stability of trimeric OmpF porin: the contributions of the latching loop L2. *Biochemistry*, 37:15663–70. doi:10.1021/bi981215c

Pirovano, W., Feenstra, K. A., Heringa, J. 2008. PRALINE^{TM}: a strategy for improved multiple alignment of transmembrane proteins. *Bioinformatics*, 24:492–7. doi:10.1093/bioinformatics/btm636

Poole, K., Braun, V. 1988. Influence of growth temperature and lipopolysaccharide on hemolytic activity of *Serratia marcescens*. *Journal of Bacteriology*, 170:5146–52.

Poole, K., Schiebel, E., Braun, V. 1988. Molecular characterization of the hemolysin determinant of *Serratia marcescens*. *Journal of Bacteriology*, 170:3177-88.

Portnoy, D., A. 2005. Manipulation of innate immunity by bacterial pathogens. *Current Opinion in Immunology*. 17:25–28.

Powell, J. R., Ausubel, F. M. 2008. Models of *Caenorhabditis elegans* infection by bacterial and fungal pathogens. *Methods in Molecular Biology*, 415:403–27. doi:10.1007/978-1-59745-570-1_24

Powell, J. R., Kim, D. H., Ausubel, F. M. 2009. The G protein-coupled receptor FSHR-1 is required for the *Caenorhabditis elegans* innate immune response. *Proceedings of the National Academy of Sciences of the United States of America*, 106:2782–7. doi:10.1073/pnas.0813048106

Pozo, J. L., Patel, R. 2007. The challenge of treating biofilm-associated bacterial Infections, *Clinical Pharmacology & Therapeutics*, 82:204-9. doi:10.1038/sj.clpt.6100247

Pradel, E., Zhang, Y., Pujol, N., Matsuyama, T., Bargmann, C. I., Ewbank, J. J. 2007. Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marcescens* by *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, 104:2295–300. doi:10.1073/pnas.0610281104

Pratt, L. A., Hsing, W., Gibson, K. E., Silhavy, T. J. 1996. From acids to osmZ: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. *Molecular Microbiology*, 20:911-7.

Puig, M., Fusté, C., Viñas, M. 1993. Outer membrane proteins from *Serratia marcescens*. *Canadian Journal of Microbiology*, 39:108–111.

Quinn, H. J., Cameron, A. D. S., Dorman, C. J. 2014. Bacterial regulon evolution: distinct responses and roles for the identical OmpR proteins of *Salmonella typhimurium* and *Escherichia coli* in the acid stress response. *PLoS Genetics*, 10:e1004215. doi:10.1371/journal.pgen.1004215

Ramachandran, G. 2013. Gram-positive and Gram-negative bacterial toxins in sepsis: A brief review. *Virulence*, 5:196–201. doi:10.4161/viru.27024

Ramani, N., Huang, L. Freundlich, M. 1992. In vitro interactions of integration host factor with the ompF promoter-regulatory region of *Escherichia coli*. *Molecular and General Genetics*, 231:248–55.

Rao, N. N., Torhani, A., Torriani, A. 1990. Molecular aspects of phosphate transport in *Escherichia coli*. *Molecular Microbiology*, 4:1083–1090. doi:10.1111/j.1365-2958.1990.tb00682.x

Records, A. R. 2011. The type VI secretion system: a multipurpose delivery system with a phage-like machinery. *Molecular Plant-Microbe Interactions*, 24:751–7. doi:10.1094/MPMI-11-10-0262

Rice, S. A., Koh, K. S., Queck, S. Y., Labbate, M., Lam, K. W., Kjelleberg, S. 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *Journal of Bacteriology*, 187:3477–85. doi:10.1128/JB.187.10.3477-3485.2005

Rieber, H., Frontzek, A., Pfeifer, Y. 2012. Emergence of metallo-β-lactamase GIM-1 in a clinical isolate of *Serratia marcescens*. *Antimicrobial Agents and Chemotherapy*, 56:4945–7. doi:10.1128/AAC.00405-12

Ringler, P., Schulz, G. E. 2002. OmpA membrane domain as a tight-binding anchor for lipid bilayers. *Chembiochem : A European Journal of Chemical Biology*, 3:463–6. doi:10.1002/1439-7633(20020503)3:5<463::AID-CBIC463>3.0.CO;2-P

Ronquist, F., Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574. doi:10.1093/bioinformatics/btg180

Salomao, R., Brunialti, M. K. C., Rapozo, M. M., Baggio-Zappia, G. L., Galanos, C., Freudenberg, M. 2012. Bacterial sensing, cell signaling, and modulation of the immune response during sepsis. *Shock*, 38:227–42. doi:10.1097/SHK.0b013e318262c4b0

Samuelsson, A., Isaksson, B., Hanberger, H., Olhager, E. 2014. Late-onset neonatal sepsis, risk factors and interventions: an analysis of recurrent outbreaks of *Serratia marcescens*, 2006-2011. *The Journal of Hospital Infection*, 86:57–63. doi:10.1016/j.jhin.2013.09.017

Schembri, M. A, Klemm, P. 2001. Coordinate gene regulation by fimbriae-induced signal transduction. *The EMBO Journal*, 20:3074–81. doi:10.1093/emboj/20.12.3074

Schirmer, T., Keller, T. A., Wang, Y. F., Rosenbusch, J. P. 1995. Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution. *Science*, 267:512–4. doi:10.1126/science.7824948

Schleifer, K. H., Kandler, O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews*, 36:407–77.

Schmidtke, A. J., Hanson, N. D. 2008. Role of *ampD* homologs in overproduction of AmpC in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 52:3922–7. doi:10.1128/AAC.00341-08

Schroeder, L. K., Kremer, S., Kramer, M. J., Currie, E., Kwan, E., Watts, J. L., Lawrenson, A.L., Hermann, G. J. 2007. Function of the *Caenorhabditis elegans* ABC transporter PGP-2 in the biogenesis of a lysosome-related fat storage organelle. *Molecular Biology of the Cell*, 18:995–1008. doi:10.1091/mbc.E06-08-0685

Schulenburg, H., Ewbank, J. J. 2004. Diversity and specificity in the interaction between *Caenorhabditis elegans* and the pathogen *Serratia marcescens*. *BMC Evolutionary Biology*, 4:49. doi:10.1186/1471-2148-4-49

Schulenburg, H., Kurz, C. L., Ewbank, J. J. 2004. Evolution of the innate immune system: the worm perspective. *Immunological Reviews*, 198:36–58. doi:10.1111/j.0105-2896.2004.0125.x

Schulz, G. E. 2002. The structure of bacterial outer membrane proteins. *Biochimica et Biophysica Acta*, 1565:308–17. doi:10.1016/S0005-2736(02)00577-1

Shahcheraghi, F., Minato, Y., Chen, J., Mizushima, T., Ogawa, W., Kuroda, T., Tsuchiya, T. 2007. Molecular cloning and characterization of a multidrug efflux pump, SmfY, from *Serratia marcescens*. *Biological and Pharmaceutical Bulletin*, 30:798–800. doi:10.1248/bpb.30.798

Shanks, R. M. Q., Stella, N. A., Kalivoda, E. J., Doe, M. R., O'Dee, D. M., Lathrop, K. L., Guo, F.L., Nau, G. J. 2007. A *Serratia marcescens* OxyR homolog mediates surface attachment and biofilm formation. *Journal of Bacteriology*, 189:7262–72. doi:10.1128/JB.00859-07

Sheldon, A. T. 2005. Antibiotic resistance: a survival strategy. *Clinical Laboratory Science : Journal of the American Society for Medical Technology*, 18:170–80.

Sifri, C. D., Begun, J., Ausubel, F. M. 2005. The worm has turned-microbial virulence modeled in *Caenorhabditis elegans*. *Trends in Microbiology*, 13:119–27. doi:10.1016/j.tim.2005.01.003

Silhavy, T. J., Kahne, D., Walker, S. 2010. The bacterial cell envelope. *Cold Spring Harbor Perspectives in Biology*, 2:a000414. doi:10.1101/cshperspect.a000414

Simossis, V. A., Heringa, J. 2005. PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. *Nucleic Acids Research*, 33:W289–94. doi:10.1093/nar/gki390

Sklar, J. G., Wu, T., Kahne, D., & Silhavy, T. J. 2007. Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes and Development*, 21:2473–84. doi:10.1101/gad.1581007

Skorupski, K., Taylor, R. K. 1996. Positive selection vectors for allelic exchange. *Gene*, 169:47–52. doi:10.1016/0378-1119(95)00793-8

Slater, H., Crow, M., Everson, L., Salmond, G. P. C. 2003. Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. *Molecular Microbiology*, 47:303–20. doi:10.1046/j.1365-2958.2003.03295.x

Smith, S. G. J., Mahon, V., Lambert, M. A., Fagan, R. P. 2007. A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiology Letters*, 273:1–11. doi:10.1111/j.1574-6968.2007.00778.x

Soo, P.-C., Horng, Y.-T., Chang, Y.-L., Tsai, W.-W., Jeng, W.-Y., Lu, C.-C., Lai, H.-C. 2014. ManA is regulated by RssAB signaling and promotes motility in *Serratia marcescens*. *Research in Microbiology*, 165:21–9. doi:10.1016/j.resmic.2013.10.005

Staudinger, B. J., Oberdoerster, M. a, Lewis, P. J., Rosen, H. 2002. mRNA expression profiles for *Escherichia coli* ingested by normal and phagocyte oxidase-deficient human neutrophils. *The Journal of Clinical Investigation*, 110:1151–63. doi:10.1172/JCI15268

Stenkova, A. M., Isaeva, M. P., Shubin, F. N., Rasskazov, V. A., Rakin, A. V. 2011. Trends of the major porin gene (ompF) evolution: insight from the genus *Yersinia*. *PloS One*, 6:e20546. doi:10.1371/journal.pone.0020546

Stock, I., Burak, S., Sherwood, K. J., Gruger, T., Wiedemann, B. 2003. Natural antimicrobial susceptibilities of strains of "unusual" *Serratia* species: *S. ficaria, S. fonticola, S. odorifera, S. plymuthica* and *S. rubidaea. The Journal of Antimicrobial Chemotherapy*, 51:865–85. doi:10.1093/jac/dkg156

Stock, I., Grueger, T., Wiedemann, B. 2003. Natural antibiotic susceptibility of strains of *Serratia marcescens* and the *S. liquefaciens* complex: *S. liquefaciens sensu stricto, S.*

proteamaculans and *S. grimesii*. *International Journal of Antimicrobial Agents*, 22:35–47. doi:10.1016/S0924-8579(02)00163-2

Straud, S., Lee, I., Song, B., Avery, L., You, Y.-J. 2013. The jaw of the worm: GTPase-activating protein EAT-17 regulates grinder formation in *Caenorhabditis elegans*. *Genetics*, 195:115–25. doi:10.1534/genetics.113.152538

Struyvé, M., Moons, M., Tommassen, J., Struyvk, M. 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. *Journal of Molecular Biology*, 218:141–8. doi:10.1016/0022-2836(91)90880-F

Sugawara, E., Nikaido, H. 2012. OmpA is the principal nonspecific slow porin of *Acinetobacter baumannii*. *Journal of Bacteriology*, 194:4089–96. doi:10.1128/JB.00435-12

Suh, B., Bae, I. K., Kim, J., Jeong, S. H., Yong, D., Lee, K. 2010. Outbreak of meropenem-resistant *Serratia marcescens* comediated by chromosomal AmpC beta-lactamase overproduction and outer membrane protein loss. *Antimicrobial Agents and Chemotherapy*, 54:5057–61. doi:10.1128/AAC.00768-10

Tan, Y., Kagan, J. C. 2014. A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. *Molecular Cell*, 54:212–223. doi:10.1016/j.molcel.2014.03.012

Tarr, D. E. 2012. Distribution and characteristics of ABFs, cecropins, nemapores, and lysozymes in nematodes. *Developmental and Comparative Immunology*, 36:502–20. doi:10.1016/j.dci.2011.09.007

Tchetina, E., Newman, E. B. 1995. Identification of Lrp-regulated genes by inverse PCR and sequencing: regulation of two mal operons of *Escherichia coli* by leucine-responsive regulatory protein. *Journal of Bacteriology*, 177:2679–83.

Tenor, J. L., Aballay, A. 2008. A conserved Toll-like receptor is required for *Caenorhabditis elegans* innate immunity. *EMBO Reports*, 9:103–9. doi:10.1038/sj.embor.7401104

Tenover, F. C. 2006. Mechanisms of antimicrobial resistance in bacteria. *American Journal of Infection Control*, 34:S3–10. doi:10.1016/j.ajic.2006.05.219

Thompson, J. N. 2012. Evolution. The role of coevolution. *Science*, 335:410–1. doi:10.1126/science.1217807

Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A., Salmond, G. P. 2000. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. *Molecular Microbiology*, 36:539–56. doi:10.1046/j.1365-2958.2000.01872.x

Tokuda, H. 2009. Biogenesis of outer membranes in Gram-negative bacteria. *Bioscience, Biotechnology, and Biochemistry*, 73:465–473. doi:10.1271/bbb.80778

Tracz, D. M., Boyd, D. a, Bryden, L., Hizon, R., Giercke, S., Van Caeseele, P., Mulvey, M. R. 2005. Increase in *ampC* promoter strength due to mutations and deletion of the attenuator in a clinical isolate of cefoxitin-resistant *Escherichia coli* as determined by RT-PCR. *The Journal of Antimicrobial Chemotherapy*, 55:768–72. doi:10.1093/jac/dki074

Tracz, D.M., Boyd, D.A., Hizon, R., Bryce, E., McGeer, A., Ofner-Agostini, M., Simor, A.E., Paton, S., Mulvey, M.R.; Canadian nosocomial infection surveillance program. 2007. *ampC* gene expression in promoter mutants of cefoxitin-resistant *Escherichia coli* clinical isolates. *FEMS Microbiology Letters*, 270:265–71. doi:10.1111/j.1574-6968.2007.00672.x

Van der Ley, P., Bekkers, A., Van Meersbergen, J., Tommassen, J. 1987. A comparative study on the *phoE* genes of three Enterobacterial species. Implications for structure-function relationships in a pore-forming protein of the outer membrane. *European Journal of Biochemistry / FEBS*, 164, 469–75.

Vershinina, O. A., namenskaia, L. V. 2002. The Pho regulons of bacteria. *Mikrobiologiia*, 71:581–95.

Vidal, S., Bredin, J., Pagès, J.-M., Barbe, J. 2005. Beta-lactam screening by specific residues of the OmpF eyelet. *Journal of Medicinal Chemistry*, 48:1395–400. doi:10.1021/jm049652e

Voelz, A., Müller, A., Gillen, J., Le, C., Dresbach, T., Engelhart, S., Exner, M., Bates, C.J. Simon, A. 2010. Outbreaks of *Serratia marcescens* in neonatal and pediatric intensive care units: clinical aspects, risk factors and management. *International Journal of Hygiene and Environmental Health*, 213:79–87. doi:10.1016/j.ijheh.2009.09.003

Vogt, J., Schulz, G. E. 1999. The structure of the outer membrane protein OmpX from *Escherichia coli* reveals possible mechanisms of virulence. *Structure*, 7:1301–9. doi:10.1016/S0969-2126(00)80063-5

von Krüger, W.M., Lery, L.M., Soares, M.R., de Neves-Manta, F.S., Batista e Silva, C.M., Neves-Ferreira, A.G., Perales, J., Bisch, P.M. 2006. The phosphate-starvation response in *Vibrio cholerae* O1 and phoB mutant under proteomic analysis: disclosing functions involved in adaptation, survival and virulence. *Proteomics*, 6:1495–511. doi:10.1002/pmic.200500238

Wager, B., Baslé, A., Delcour, A. H. 2010. Disulfide bond tethering of extracellular loops does not affect the closure of OmpF porin at acidic pH. *Proteins*, 78:2886–94. doi:10.1002/prot.22807

Walsh, T.R., Toleman, M.A., Poirel, L., Nordmann, P. 2005. Metallo-beta-lactamases: the quiet before the storm? *Clinical Microbiology Reviews*. 18:306-25.

Walther, D. M., Rapaport, D., Tommassen, J. 2009. Biogenesis of beta-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. *Cellular and Molecular Life Sciences*, 66:2789–804. doi:10.1007/s00018-009-0029-z

Walthers, D., Go, A., Kenney, L.J. 2004. Regulation of porin gene expression by the two-component regulatory system EnvZ/OmpR. In Bacterial and Eukaryotic Porins: Structure, Function, Mechanism, pp. 1–24. Edited by R. Benz. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. ISBN 3-527-30775-3. doi:10.1002/3527603875.ch1

Weindorf, H., Schmidt, H., Martin, H. H. 1998. Contribution of overproduced chromosomal beta-lactamase and defective outer membrane porins to resistance to extended-spectrum beta-lactam antibiotics in *Serratia marcescens*. *The Journal of Antimicrobial Chemotherapy*, 41:189–95. doi:10.1093/jac/41.2.189

Weiser, J. N., Gotschlich, E. C. 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. *Infection and Immunity*, 59:2252–8.

Wheat R.P., Zuckerman A, R. L. A. 1951. Infection due to chromobacteria; report of 11 cases. *A.M.A. Archives of Internal Medicine.*, 88:461–6. doi:10.1001/archinte.1951.03810100045004

Williams, K. M., Bigley, E. C., Raybourne, R. B. 2000. Identification of murine B-cell and T-cell epitopes of *Escherichia coli* outer membrane protein F with synthetic polypeptides. *Infection and Immunity*, 68:2535–45. doi:10.1128/IAI.68.5.2535-2545.2000.Updated

Williamson, N. R., Fineran, P. C., Leeper, F. J., Salmond, G. P. C. 2006. The biosynthesis and regulation of bacterial prodiginines. *Nature Reviews. Microbiology*, 4:887–99. doi:10.1038/nrmicro1531

Wilson, D. N. 2014. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature Reviews. Microbiology*, 12:35–48. doi:10.1038/nrmicro3155

Wilson, R. K. 1999. How the worm was won. The *C. elegans* genome sequencing project. *Trends in Genetics*, 15:51–8. doi:10.1016/S0168-9525(98)01666-7

Woodward, H. M. M., Clarke, K. B. 1913. A case of infection in man by the bacterium prodigiosum. *The Lancet*, 181:314–315. doi:10.1016/S0140-6736(00)76133-2

Wu, L., Lin, X., Peng, X. 2009. From proteome to genome for functional characterization of pH-dependent outer membrane proteins in *Escherichia coli*. *Journal of Proteome Research*, 8:1059–70.

Xia, X. 2013. DAMBE5: a comprehensive software package for data analysis in molecular biology and evolution. *Molecular Biology and Evolution*, 30:1720–8. doi:10.1093/molbev/mst064

Xiao, Z.P., Wang, X.D., Wang, P.F., Zhou, Y., Zhang, J.W., Zhang, L., Zhou, J., Zhou, S.S., Ouyang, H., Lin, X.Y., Mustapa, M., Reyinbaike, A., Zhu, H.L. 2014. Design, synthesis, and evaluation of novel fluoroquinolone-flavonoid hybrids as potent antibiotics against drug-

resistant microorganisms. *European Journal of Medicinal Chemistry*, 80C:92–100. doi:10.1016/j.ejmech.2014.04.037

Yatsuyanagi, J., Saito, S., Konno, T., Harata, S., Suzuki, N., Kato, J., Amano, K. 2006. Nosocomial outbreak of ceftazidime-resistant *Serratia marcescens* strains that produce a chromosomal AmpC variant with N235K substitution. *Japanese Journal of Infectious Diseases*, 59:153–9.

Yoshida, T., Qin, L., Egger, L. a & Inouye, M. 2006. Transcription regulation of *ompF* and *ompC* by a single transcription factor, OmpR. *The Journal of Bioogical Chemistry*, 281: 17114–23. doi:10.1074/jbc.M602112200

Yoshida, T., Phadtare, S. & Inouye, M. 2007. Functional and structural characterization of EnvZ, an osmosensing histidine kinase of *E. coli*. *Methods in Enzymology*,. 423:184–202. doi:10.1016/S0076-6879(07)23008-3

Yoshimura, F., Nikaido, H. 1985. Diffusion of beta-lactam antibiotics through the porin channels of *Escherichia coli* K-12. *Antimicrobial Agents Chemotherapy*, 27:84–92. doi:10.1128/AAC.27.1.84.

Zamorano, L., Reeve, T. M., Deng, L., Juan, C., Moyá, B., Cabot, G., Vocadlo, D.J., Mark, B.L., Oliver, A. 2010. NagZ inactivation prevents and reverts beta-lactam resistance, driven by AmpD and PBP 4 mutations, in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*, 54:3557–63. doi:10.1128/AAC.00385-10

Zeth, K., Thein, M. 2010. Porins in prokaryotes and eukaryotes: common themes and variations. *The Biochemical Journal*, 431:13–22. doi:10.1042/BJ20100371

Zhang, Q., Weyant, R., Steigerwalt, A.G., White, L. A., Melcher, U., Bruton, B. D., Pair S.D., Mitchell, F.L., Fletcher, J. 2003. Genotyping of *Serratia marcescens* strains associated with cucurbit yellow vine disease by repetitive elements-based polymerase chain reaction and DNA-DNA hybridization. *Phytopathology*, 93:1240–6. doi:10.1094/PHYTO.2003.93.10.1240

Zhou, R., Zhang, R., Sun, Y., Platt, S., Szczotka-Flynn, L., Pearlman, E. 2012. Innate immune regulation of *Serratia marcescens*-induced corneal inflammation and infection. *Investigative Ophthalmology and Visual Science*, 53:7382–8. doi:10.1167/iovs.12-10238

Ziervogel, B. K., Roux, B. 2013. The binding of antibiotics in OmpF porin. *Structure*, 21:76–87. doi:10.1016/j.str.2012.10.014

APPENDIX

APPENDIX 1. PRIMER EFFICIENY

Purpose: To determine genes that will be used as house-keeping genes. Primers were designed and evaluated with the same cDNA to determine primer efficiency. Primer efficiency expressed in (%), refers to the percent of template that is amplified in each cycle. From all *S. marcescens* Db11 HK genes evaluated, *rpoB* was selected as internal control for qRT-PCR.

Table A1.1 Primer efficiency to select primers used as house-keeping (HK) genes in the qRT-PCR experiments.

	Primer set	Primer efficiency (%)
1	Sm *rpoB*(L)1/ Sm *rpoB*(R)1	99.5
2	Sm *rplU*(L)1/ Sm *rplU*(R)1	98
3	Sm16SHK(L)1/ Sm16SHK(R)1	96.9
4	Sm *gyrA*(L)1/ Sm *gyrA*(R)1	96
5	Sm *gyrB*(L)1/ Sm *gyrB*(R)1	101.9

* Primer sequences are in Table 2.4.

APPENDIX 2. qRT-PCR DATA GENERATED WITH *ompF* AND *ompC* MUTATIONS.

Purpose: Transcript levels of selected porins genes were evaluated to detect any compensatory effect when OmpF and OmpC were not being produced under defined conditions. The gene *rpoB* selected as internal control (Table A1.1), was used. Total RNA was purified from overnight culture. In the graph presented bellow, there is an increase in the expression of *phoE* and *lamB* in the mutants.

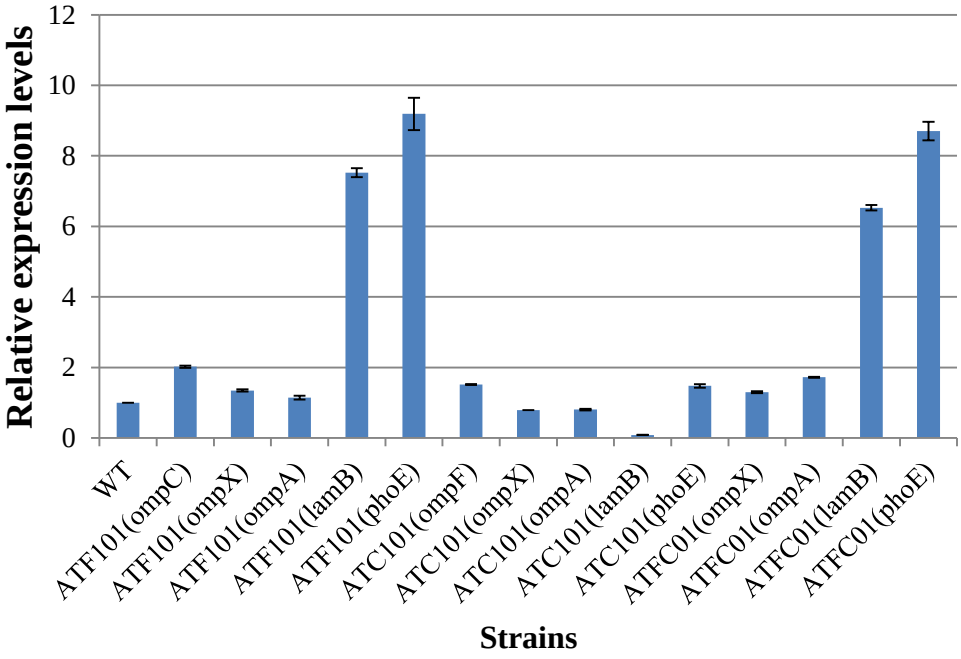


Figure A2.1. qRT-PCR of *S. marcescens* wild type and mutants at the stationary growth phase at 37 °C. Graph represents one biological replica performed by triplicate to analyze the transcriptional levels of *ompF*, *ompC*, *ompX*, *ompA*, *lamB* and *phoE* in stationary phase. The *rpoB* gene was selected as housekeeping gene.

Purpose: Transcript levels of selected porins genes were evaluated to detect any compensatory effect when OmpF and OmpC were not being produced. Gene *rpoB* was used as internal control. Total RNA was purified from cultures grown for 3 hours at 37C after sub-culturing from an overnight culture. In the graph presented, there is not increase in the expression of *phoE* and *lamB* in the mutants.

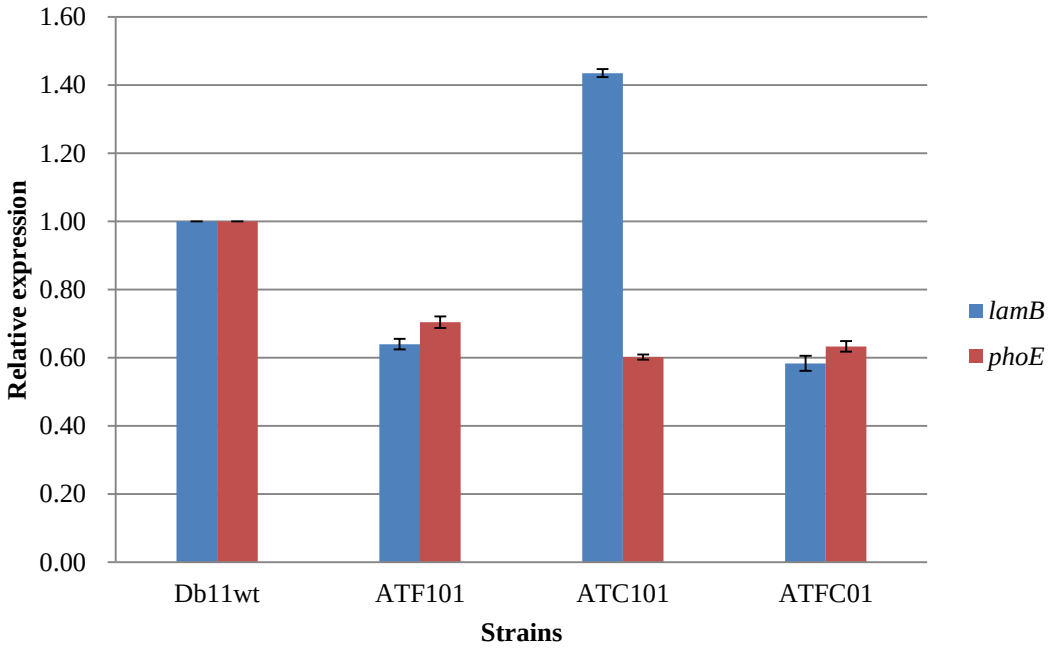
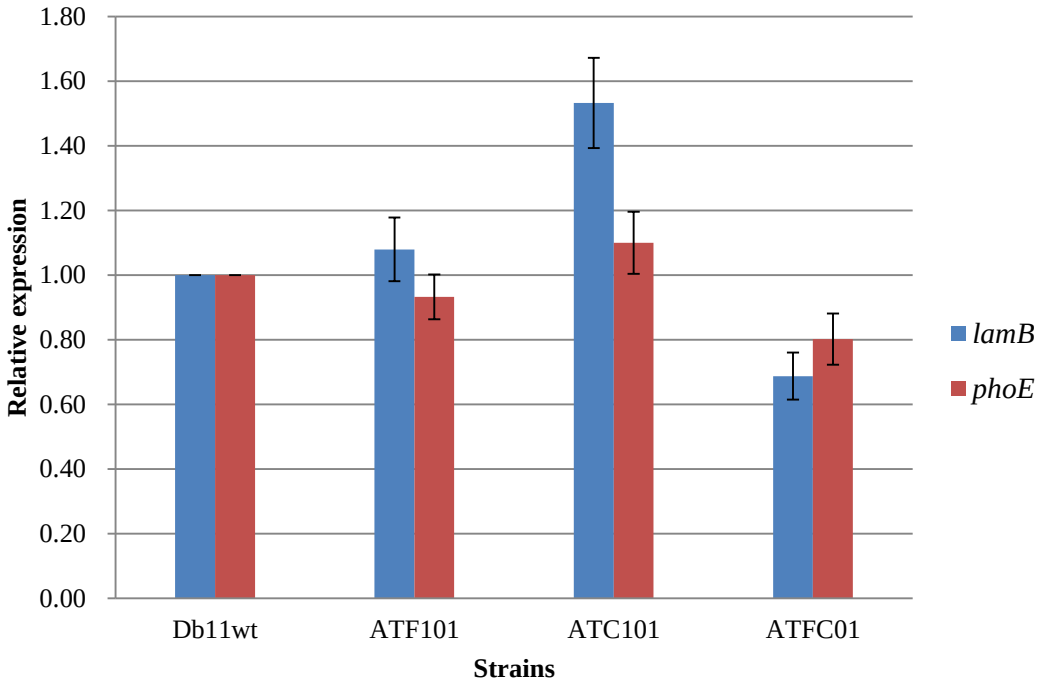


Figure A2.2. qRT-PCR of *S. marcescens* grown for 3 hours at 37 °C. The *lamB* and *phoE* transcriptional levels of cells grown on LB media for 3 hours. The graph represents one biological replica performed by triplicate. The *rpoB* gene was selected as housekeeping gene. ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*)

Purpose: Transcript levels of selected porins genes were evaluated to detect any compensatory effect when OmpF and OmpC were not being produced. Gene *rpoB* was used as internal control. Total RNA was purified from 5 hours grown cultures. In the graph presented, there is not increase in the expression of *phoE* and *lamB* in the mutants.



FigureA2.3. qRT-PCR of *S. marcescens* grown for 5 hours at 37 °C. Gene expression of *phoE* and *lamB* in *S. marcescens* on LB medium after 5 hour. The graph represents one biological replica performed by triplicate. The *rpoB* gene was selected as housekeeping gene. ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*)

APPENDIX 3. *S. marcescens* PHENOTYPES ASSOCIATED WITH *C. elegans*.

Purpose: Motility assay to assess production of serrawettin. *S. marcescens* Db11 produces serrawettin, which is a biosurfactant that enhances bacterial motility on surfaces. The assay was performed to evaluate if the absence of porins affected serrawettin production following the swarming motility.

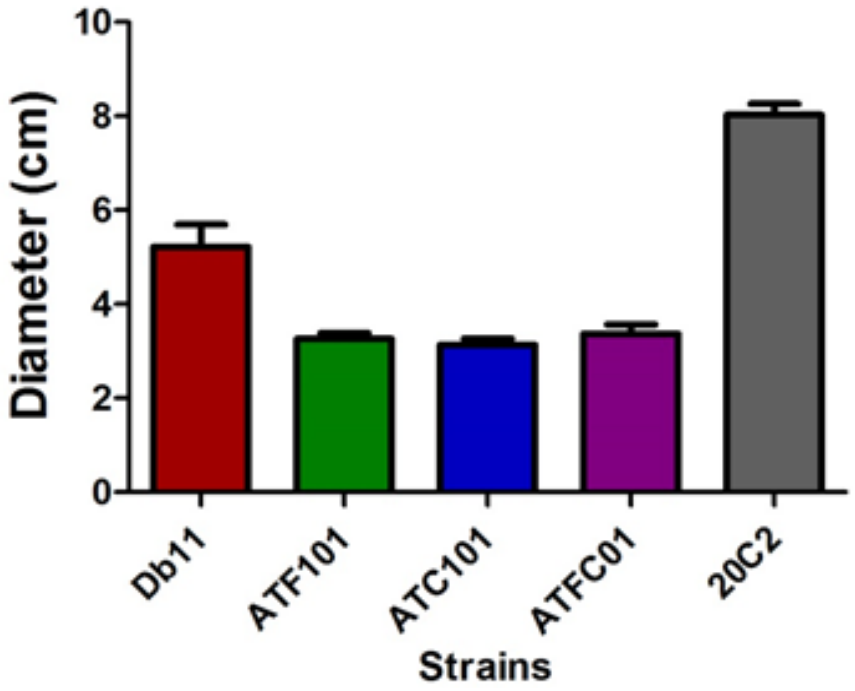


Figure A3.1 Swarming motility at 25 °C after 16 hours. Five microliters of an overnight culture was added to the center of an LB 0.5 % agar plate. 20C2 shows the highest swarming motility. $P < 0.0001$. ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*)

APPENDIX 4. FOOD PREFERENCE EXPERIMENT.

Purpose: Use an assay to evaluate if *S. marcescens* porin mutants produced bacterial lawn avoidance in the nematode. *C. elegans* localizes nutrients by olfactory stimuli and move toward the potential food. In this experiment *E. coli* OP50 and *S. marcescens* mutants were used as food sources. In the presence of biosurfactant serrawettin produced by *S. marcescens* Db11, *C. elegans* move away to avoid to be in contact with the biosurfactant.

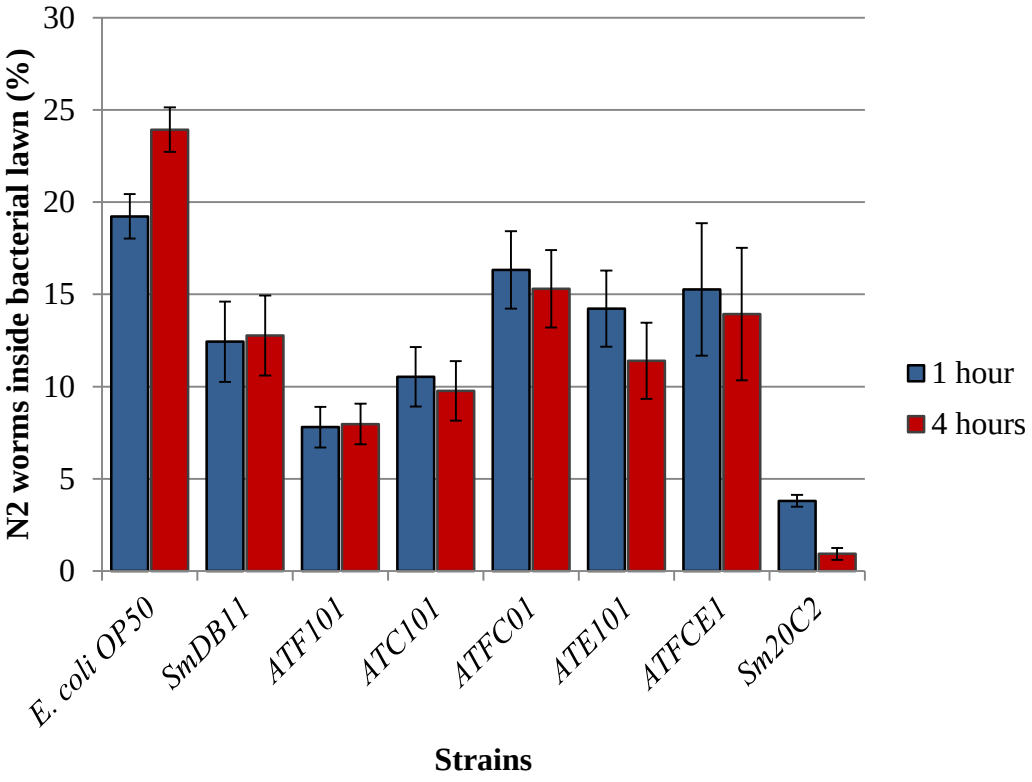


Figure A4.1. Food preference experiment results. Synchronized L4 stage *C. elegans* N2 worms were spotted in the center of NGM plate (Petri dish 10 cm) that contained previously prepared bacterial lawns. Worms moved by olfactory stimulus to the different strains and remain longer in those bacterial lawn that were the best as food sources. The bars represent one representative experiment performed three times. Figure shows the percent of the number of *C. elegans* N2 nematodes in each bacterial lawn. *P*= 0.0003 ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*), ATE101 (Δ*phoE*), ATFCE1 (Δ*ompF*Δ*ompC*Δ*phoE*).

APPENDIX 5. Minimal inhibitory concentrations of selected antibiotics in *S. marcescens* Db11 and isogenic mutant strains.

Purpose: Complete evaluation of antimicrobial resistance of *ompF* and *ompC* mutant strains as supplemental information to Moya-Torres *et al.*, 2014. ATF101 (Δ*ompF*), ATC101 (Δ*ompC*), ATFC01 (Δ*ompF*Δ*ompC*), ATF242 (Δ*ompF*Δ*ompC*) *ompF* complemented, ATC50 *ompC* complemented.

Table A5.1	Db11	ATF101	ATC101	ATFC01	ATF242	ATC50
	MIC (µg/ml)					
Amoxicillin/Clavulanic acid	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32	≥ 32
Ampicillin	16	≥ 32	16	≥ 32	≥ 32	≥ 32
Cefazolin	≥ 64	≥64	≥ 64	≥ 64	≥ 64	≥ 64
Cefotaxime	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Cefoxitin	8	>= 64	8	≥ 64	32	≥ 64
Ceftazidime	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Tobramycin	8	8	8	8	8	8
Tigecycline	2	1	2	1	2	2
Ceftriaxone	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1
Amikacin	8	8	8	8	8	8
Ciprofloxacin	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25
Gentamicin	2	2	2	2	≥ 16	≥ 16

Ertapenem	≤ 0.5	≤ 0.5	≤ 0.5	1	≤ 0.5	≤ 0.5
Meropenem	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25	≤ 0.25
Piperacillin/Tazobactam	≤ 4	≤ 4	≤4	≤ 4	≤ 4	16
Nitrofurantoin	128	256	128	256	128	256
Trimethoprim/Sulfamethoxazole	≤20	≤20	≤20	≤20	≤20	≤20

APPENDIX 6. Generation of *ampD* mutant.

Purpose: Strategy to delete *ampD* in the *ampDE* operon. Gene *ampE*, forms an operon with *ampD*. AmpE is involved in the regulation of β-lactamase expression but the mechanism is unknown. The *ampE* gene encodes a cytoplasmic membrane protein that act as a sensory transducer involved in *ampC* expression as part of the *ampDE* operon.

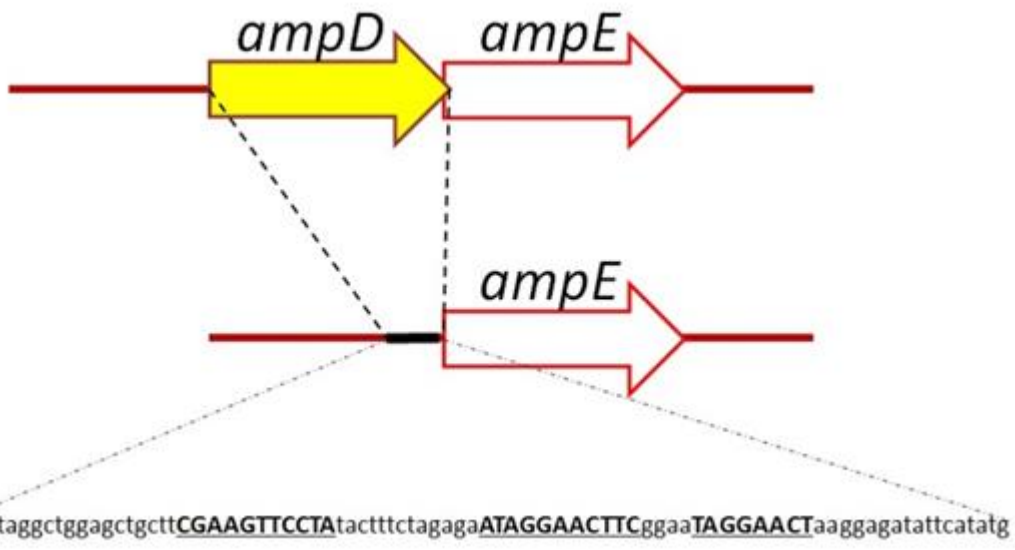


Figure A6.1. After allelic gene exchange, the FRT sequence is left in place replacing the excised *ampD* upstream of *ampE*. The 85 nt sequence replacing *ampD* in the ATD101 mutant is shown. The FRT sites sequences are underlined.

APPENDIX 7. Alignment of AmpD amidases using PRALINE

Purpose: Evaluation of amino acid conserved sequence in AmpD amidase of *S. marcescens* Db11 and *P. aeruginosa*.

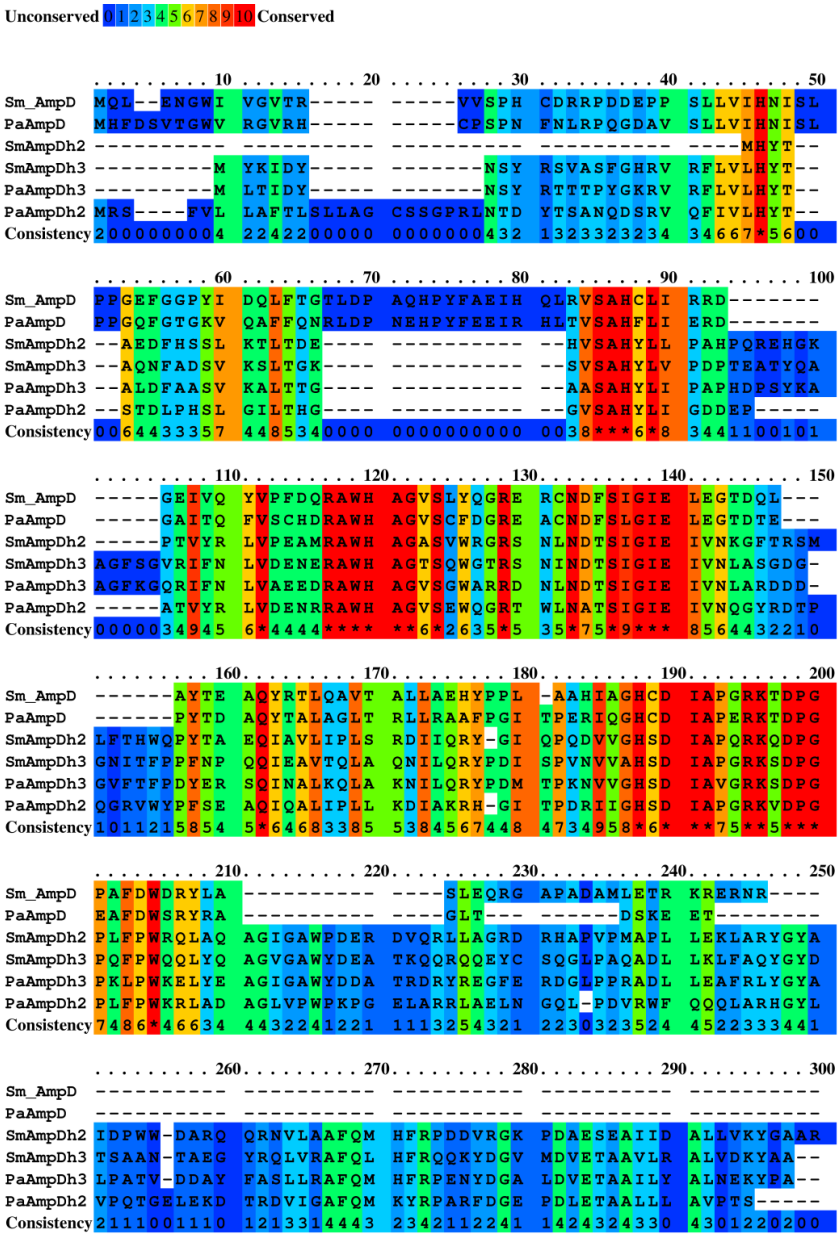


Figure A7.1. Alignment of *S. marcescens* Db11 AmpD and *P. aeruginosa* PAO1 AmpDh2 and AmpDh3. The alignment was generated using the Praline program (Simossis & Heringa, 2005). In red are presented the sites of higher homologies of all these sequences. Three putative amidase genes (*ampDh1*, *ampDh2*, *ampDh3*) were found in *S. marcescens* Db11.

APPENDIX 8. DNA sequences used to generate ATF101 (Δ*ompF*) mutant. The gentamicin cassette is shown in colour green.

Serratia marcescens Db11 *ompF*

3’-
ACCAAGCCTTCGTCAGGGCCCTGCGCAAAGCCTTCGATCTGCCGCTGGTGAAGAAA
GCCAGCTGAACCTCCTCTATATAGAAAGAAAGCCGGCCTGGTTCACGCGCCGGCTTT
TTTGTTTTTATCCCGACCGCCCTCTTTTCAGTCAGCCGCAATAAATGTTGCGCCAATC
CGATGATTTCTGCTTGTGCTCTGCACCGCGGCAAGTAACATAGCCATTACCCCCATA
TTCTTGGGTTGTAAGCTATTTCTAAACCAAATTATTAAAGAGAGAAATCTATGAGCG
TAGTGCCTGTAGTCGACGTACTGCAAGGTCGTGCTGCGGTTGACAGTGAAGTCACCG
TACGCGGCTGGGTACGTACCCGGAGAGATTCAAAAGCTGGTATCTCCTTCCTGGCCG
TCTATGACGGTTCCTGCTTTGATCCGTTACAGGCCGTCGTTAATAATTCTCTGCCGAA
TTATCAGGATGAAGTGCTGCATCTGACTACCGGTTGTTCGGTTGAAGTCACCGGCAA
AGTCGTCGCTTCACCGGGCGAAGGCCAGAGCTTCGAACTGCAGGCTACCGCGATCA
AAGTGGTCGGCTGGGTCGACGATCCGGACACTTACCCGATGGCGGCCAAACGTCAC
AGCATCGAATACCTGCGCGAAGTGGCTCACCTGCGCCCGCGTACCAACCTGATCGG
CGCCGTGGCGCGCGTGCGTCATACGCTGGCGCAGGCGATTCACCGCTTCTTCCATGA
AAATGGCTATTTCTGGGTGTCCACCCCGCTGATCACCGCCTCCGATACCGAAGGCGC
CGGCGAGATGTTCCGCGTGTCCACGCTGGACCTGGAAAACCTGCCGCGCACCGACA
AGGGCGCCGTCGACTTCAGCGAAGACTTCTTCGGTAAGGAAGCCTTCCTTACCGTTT
CCGGCCAGCTGAACGGCGAAACCTACGCTTGCGCGCTGTCGAAGGTTTACACCTTCG
GCCCAACCTTCCGCGCCGAAAACTCCAACACCAGCCGCCACCTGGCGGAATTCTGG
ATGATCGAGCCGGAAGTGGCCTTCGCGACGCTGGACGACGTTGCCGGCCTGGCCGA
AAGCATGCTGAAGTATGTGTTCCAGGCGGTGCTGGATGAACGCGCCGACGATCTGA
AATTCTTCGCCGAGCGCGTGGATAAAGACGCTATCGCTCGTCTGGAGCGTTTCGTTT
CTTCCGATTTCGCCCAGGTGGACTACACCGACGCCATTGAGATCCTGCTGGCCTCGG
GCCAAACCTTCGAAAACCCGGTTTCCTGGGGTATCGATCTCTCTTCCGAACATGAAC
GTTACCTGGCTGAGAAACACTTCCAAGCGCCGGTGGTGGTGAAAAACTACCCGAAA
GACATCAAAGCGTTCTATATGCGCATGAACGAAGACGGCAAAACCGTTGCGGCCAT
GGACGTGCTGGCACCGGGCATCGGCGAAATCATCGGCGGCTCGCAGCGTGAAGAAC
GCCTCGACGTGCTCGATCAGCGTCTGGAAGAGATGGGTCTGAATAAAGAAGACTAC
TGGTGGTATCGCGATCTGCGCCGCTACGGCACCGTGCCGCATTCCGGTTTCGGCCTG
GGCTTTGAACGTTTAATCGCCTATGTCACCGGCGTGCAAAACGTGCGCGATGTGATT
CCATTCCCACGCACGCCACGTAACGCAAGCTTCTAATTTATACGATCACTCGTTTTTT
AAACGATTTCGTTACAAAACAAAGGCCAGCCTCGCTGGCCTTTCGCATTTTTTAATA
TTGACGCACGTCACAAAGTTCCCGAAAAATTACATTTGGTTACACATACTTTCCTTTT
GCAACCTGATTGGGACATTGGTATCATTTTCGTCCTAGATTAACCCGCCTGTGAATG
GAACACTGCGTTCAGACACAGGACGACACCAATCTATCAACAATAGTTCCCAAAGA

ATTATTGGCGGCAGTGGCAAAGGTGTCCGAATAACACCAATGAGGGTAATAATGAT
GAAGCGCAACATTCTTGCAGTGGTTATCCCGGCTCTGTTGGCTGCTGGTGCAGCAAA
CGCAGCTGAAATCTACAACAAAGACGGCAACAAGCTGGATCTGTACGGCAAAGTTG
ACGGTCTGCACTACTTCTCCGACGACAAAGGTAACGACGGCGATCAGACCTATGTTC
GTTTCGGCTTCAAAGGTGAAACTCAGATTACTGACCAACTGACCGGTTACGGCCAGT
GGGAATACAACGTCCAGGCTAACCACTCCGAATCTCAGGGCACCGAAGGCACCAAA
ACCCGTCTGGGCTTCGCCGGTCTGAAATTCGCTGACTACGGCTCCTTCGACTACGGC
CGTAACTACGGCGTACTGTACGACGTGGAAGGCTGGACCGATATGCTGCCAGAGTT
CGGTGGCGACACCTACACCTACACCGACAACTTCATGACCGGCCGTACCAACGGCG
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GCAATACCAGGGCAAAAACCAGAACGACGGTCGTAACGTCAAGAAACAAAACGGC
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GCATACGCTTCCTCCAACCGTACCGACGATCAGAAACTGCGTTCCAACGAGCGTGGC
GACAAGGCTGATGCATGGACCGTGGGTGCGAAATACGACGCCAACAACGTTTACCT
GGCGGCGATGTACGCAGAAACCCGTAACATGACCCCGTTCGGCGGCGGTAACTTCG
GTCCAGGCTGTGCAGCTACCGATGACAAGTGCGGTGGCTTCGCGAGCAAAACTCAG
AACTTCGAAGTGACTGCTCAGTACCAGTTCGACTTCGGTCTGCGTCCAGAAGTGTCT
TACCTGCAGTCTAAAGGTAAAAACCTGAACGTCCCAGGCGTAGGTTCTGACCAAGA
TCTGGTTAAATATGTTTCTGTTGGTACCACTTACTACTTCAACAAAAACATGTCCACC
TACGTTGATTACAAAATCAACCTGCTGGATGACAACGAGTTCACCAAAGCAACCGG
CACCGCTACCGACGATATCGTAGCTGTTGGTCTGGTGTACCAGTTCTAAGTTGTCTC
GCTTAACGGCGGTTTACCGTCGGCTAAGTTAAAAAACAGGGCTTCGGCCCTGTTTTT
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GTCGAATCCTTCCCGTTCACAAGATCGCGGTGTTTTTATCGCAAACGGTTGGCAAAG
CCGATAACTGGCGCTACCCTGATGCTTCTGTCTGCTTAACTCCGAGATTTGGAAGCA
CCTGACATGTTTGAAAAAATCACCGCCGCACCCGCCGACCCGATCCTGGGCCTGACC
GACATTTTCCGCGCCGACGCCCGCCCGAATAAAATCAATTTAGGGATTGGCGTTTAT
AAAGACGAAACCGGTAAAACCCCGGTGTTAACCAGCGTAAAGAAAGCTGAACAGTA
TCTGCTGGAAAATGAGACCACCAAAAACTATCTCGGCATTGAAGGCATCCCGGCGTT
TGCCAGCTGCACGCAAGTGCTGCTGTTCGGTAAAGAGAGCCCGATCGTCGCCGATC
GTCGCGCACGTACCGCACAGACGCCAGGTGGCACCGGTGGCCTGCGCGTGGCGGCA
GACTTTATCGCCAACCAGACCAGCGCCAAGCGCATCTGGATCAGCAACCCAAGCTG
GCCGAACCACAAGAACGTCTTTAGCGCCGTGGGCCTGGAAGTCCTGGAGTATGCCT
ACTACGACGCCGCCAACCACGCATTGGATTTCGACGGCCTGCTGAACAGCCTGAAA
CAGGCCCAGGCCGGTGATGTGGTGCTGTTTCACGGCTGCTGCCATAACCCGACCGGT
ATCGATCCAACGGCGGAACAGTGGGCACAGCTGGCTGAGCTTTCCGTGACCAATGG
CTGGCTGCCTCTGTTCGACTTCGCCTACCAGGGCTTCGCCAAGGGTCTGGAAGAGGA
TGCGCAGGGCCTGCGTATTTTCGCCGCCAAACATCAAGAGCTGATTGTCGCCAGCTC
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TGCCGATGCCGAAACCGCCGATCGCGCCTTCAGCCAGGTAAAAGCGGCTATTCGCG

CCAACTACTCCAACCCGCCGTCACACGGCGCCGCCGTTGTCGCCACCATTTTGGGTA
ACGATGCGCTGCGCGCGATGTGGGAGCAGGAGCTGACCGACATGCGCCAGCGCATT
CACCGCATGCGTCAGCTGTTCGTGAACACCCTGCAGGAAAAAGGCGCCCAACAGGA
CTTCAGCTTCATCATCCAGCAGAATGGCATGTTCTCCTTCAGCGGTCTGACCAAAGA
ACAGGTGCTGCGTCTGCGCGAAGAGTTTGGCGTCTACGCCGTGAACTCAGGCCGGGT
GAACGTGGCCGGCATGACGCCGGACAACATGGCACCGCTGTGTGAAGCCATCGTCG
CCGTGCTCTAAATGCATCGACACAGGTC

Gentamycin cassette from pMB838 (pR6Kfrt-gent-frt)(exact match to pUCGM)

TGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGG
AATAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCCACCGGCTTGAAC
GAATTGTTAGGTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGTAT
GCCCAACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTA
GATCACATAAGCACCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGG
TGGCAATGCCCTGCCTCCGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCT
CACTACGCGGCTGCTCAAACCTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACC
GCTTCTTGGTCGAAGGCAGCAAGCGCGATGAATGTCTTACTACGGAGCAAGTTCCCG
AGGTAATCGGAGTCCGGCTGATGTTGGGAGTAGGTGGCTACGTCTCCGAACTCACG
ACCGAAAAGATCAAGAGCAGCCCGCATGGATTTGACTTGGTCAGGGCCGAGCCTAC
ATGTGCGAATGATGCCCATACTTGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCT
GCTGCGTAACATCGTTGCTGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACAT
CGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACAAA
AAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTT
CGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTAC
GAACCGAACAGGCTTATGTCAACTGGGTTCGTGCCTTCATCTCTGCGAAGTGATCTT
CCGTCACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGA
ATAGGAACTAAGGAGGATATTCATATG

OmpF knock-out constructs

3’-
ACCAAGCCTTCGTCAGGGCCCTGCGCAAAGCCTTCGATCTGCCGCTGGTGAAGAAA
GCCAGCTGAACCTCCTCTATATAGAAAGAAAGCCGGCCTGGTTCACGCGCCGGCTTT
TTTGTTTTTATCCCGACCGCCCTCTTTTCAGTCAGCCGCAATAAATGTTGCGCCAATC
CGATGATTTCTGCTTGTGCTCTGCACCGCGGCAAGTAACATAGCCATTACCCCCATA
TTCTTGGGTTGTAAGCTATTTCTAAACCAAATTATTAAAGAGAGAAATCTATGAGCG
TAGTGCCTGTAGTCGACGTACTGCAAGGTCGTGCTGCGGTTGACAGTGAAGTCACCG
TACGCGGCTGGGTACGTACCCGGAGAGATTCAAAAGCTGGTATCTCCTTCCTGGCCG

TCTATGACGGTTCCTGCTTTGATCCGTTACAGGCCGTCGTTAATAATTCTCTGCCGAA
TTATCAGGATGAAGTGCTGCATCTGACTACCGGTTGTTCGGTTGAAGTCACCGGCAA
AGTCGTCGCTTCACCGGGCGAAGGCCAGAGCTTCGAACTGCAGGCTACCGCGATCA
AAGTGGTCGGCTGGGTCGACGATCCGGACACTTACCCGATGGCGGCCAAACGTCAC
AGCATCGAATACCTGCGCGAAGTGGCTCACCTGCGCCCGCGTACCAACCTGATCGG
CGCCGTGGCGCGCGTGCGTCATACGCTGGCGCAGGCGATTCACCGCTTCTTCCATGA
AAATGGCTATTTCTGGGTGTCCACCCCGCTGATCACCGCCTCCGATACCGAAGGCGC
CGGCGAGATGTTCCGCGTGTCCACGCTGGACCTGGAAAACCTGCCGCGCACCGACA
AGGGCGCCGTCGACTTCAGCGAAGACTTCTTCGGTAAGGAAGCCTTCCTTACCGTTT
CCGGCCAGCTGAACGGCGAAACCTACGCTTGCGCGCTGTCGAAGGTTTACACCTTCG
GCCCAACCTTCCGCGCCGAAAACTCCAACACCAGCCGCCACCTGGCGGAATTCTGG
ATGATCGAGCCGGAAGTGGCCTTCGCGACGCTGGACGACGTTGCCGGCCTGGCCGA
AAGCATGCTGAAGTATGTGTTCCAGGCGGTGCTGGATGAACGCGCCGACGATCTGA
AATTCTTCGCCGAGCGCGTGGATAAAGACGCTATCGCTCGTCTGGAGCGTTTCGTTT
CTTCCGATTTCGCCCAGGTGGACTACACCGACGCCATTGAGATCCTGCTGGCCTCGG
GCCAAACCTTCGAAAACCCGGTTTCCTGGGGTATCGATCTCTCTTCCGAACATGAAC
GTTACCTGGCTGAGAAACACTTCCAAGCGCCGGTGGTGGTGAAAAACTACCCGAAA
GACATCAAAGCGTTCTATATGCGCATGAACGAAGACGGCAAAACCGTTGCGGCCAT
GGACGTGCTGGCACCGGGCATCGGCGAAATCATCGGCGGCTCGCAGCGTGAAGAAC
GCCTCGACGTGCTCGATCAGCGTCTGGAAGAGATGGGTCTGAATAAAGAAGACTAC
TGGTGGTATCGCGATCTGCGCCGCTACGGCACCGTGCCGCATTCCGGTTTCGGCCTG
GGCTTTGAACGTTTAATCGCCTATGTCACCGGCGTGCAAAACGTGCGCGATGTGATT
CCATTCCCACGCACGCCACGTAACGCAAGCTTCTAATTTATACGATCACTCGTTTTTT
AAACGATTTCGTTACAAAACAAAGGCCAGCCTCGCTGGCCTTTCGCATTTTTTAATA
TTGACGCACGTCACAAAGTTCCCGAAAAATTACATTTGGTTACACATACTTTCCTTTT
GCAACCTGATTGGGACATTGGTATCATTTTCGTCCTAGATTAACCCGCCTGTGAATG
GAACACTGCGTTCAGACACAGGACGACACCAATCTATCAACAATAGTTCCCAAAGA
ATTATTGGCGGCAGTGGCAAAGGTGTCCGAATAACACCAATGAGGGTAATATGTGT
AGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG
GAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCCACCGGCTTGAACGAATT
GTTAGGTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGTATGCCCA
ACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTAGATCA
CATAAGCACCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGGTGGCA
ATGCCCTGCCTCCGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCTCACTA
CGCGGCTGCTCAAACCTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACCGCTTC
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AATCGGAGTCCGGCTGATGTTGGGAGTAGGTGGCTACGTCTCCGAACTCACGACCG
AAAAGATCAAGAGCAGCCCGCATGGATTTGACTTGGTCAGGGCCGAGCCTACATGT
GCGAATGATGCCCATACTTGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCTGCTG
CGTAACATCGTTGCTGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACATCGAC

CCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACAAAAAAA
CAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTC
AAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTACGAACC
GAACAGGCTTATGTCAACTGGGTTCGTGCCTTCATCTCTGCGAAGTGATCTTCCGTC
ACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG
GAACTAAGGAGGATATTCATATGGTTGTCTCGCTTAACGGCGGTTTACCGTCGGCTA
AGTTAAAAAACAGGGCTTCGGCCCTGTTTTTATTTGTGTCTTCCGGTAACGTTTTATC
CTGCAGATTTCCCCTTCCCCATCATTTTTTCTGTCGAATCCTTCCCGTTCACAAGATC
GCGGTGTTTTTATCGCAAACGGTTGGCAAAGCCGATAACTGGCGCTACCCTGATGCT
TCTGTCTGCTTAACTCCGAGATTTGGAAGCACCTGACATGTTTGAAAAAATCACCGC
CGCACCCGCCGACCCGATCCTGGGCCTGACCGACATTTTCCGCGCCGACGCCCGCCC
GAATAAAATCAATTTAGGGATTGGCGTTTATAAAGACGAAACCGGTAAAACCCCGG
TGTTAACCAGCGTAAAGAAAGCTGAACAGTATCTGCTGGAAAATGAGACCACCAAA
AACTATCTCGGCATTGAAGGCATCCCGGCGTTTGCCAGCTGCACGCAAGTGCTGCTG
TTCGGTAAAGAGAGCCCGATCGTCGCCGATCGTCGCGCACGTACCGCACAGACGCC
AGGTGGCACCGGTGGCCTGCGCGTGGCGGCAGACTTTATCGCCAACCAGACCAGCG
CCAAGCGCATCTGGATCAGCAACCCAAGCTGGCCGAACCACAAGAACGTCTTTAGC
GCCGTGGGCCTGGAAGTCCTGGAGTATGCCTACTACGACGCCGCCAACCACGCATT
GGATTTCGACGGCCTGCTGAACAGCCTGAAACAGGCCCAGGCCGGTGATGTGGTGC
TGTTTCACGGCTGCTGCCATAACCCGACCGGTATCGATCCAACGGCGGAACAGTGG
GCACAGCTGGCTGAGCTTTCCGTGACCAATGGCTGGCTGCCTCTGTTCGACTTCGCC
TACCAGGGCTTCGCCAAGGGTCTGGAAGAGGATGCGCAGGGCCTGCGTATTTTCGCC
GCCAAACATCAAGAGCTGATTGTCGCCAGCTCCTACTCAAAAAACTTCGGTCTGTAC
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CGCCTTCAGCCAGGTAAAAGCGGCTATTCGCGCCAACTACTCCAACCCGCCGTCACA
CGGCGCCGCCGTTGTCGCCACCATTTTGGGTAACGATGCGCTGCGCGCGATGTGGGA
GCAGGAGCTGACCGACATGCGCCAGCGCATTCACCGCATGCGTCAGCTGTTCGTGA
ACACCCTGCAGGAAAAAGGCGCCCAACAGGACTTCAGCTTCATCATCCAGCAGAAT
GGCATGTTCTCCTTCAGCGGTCTGACCAAAGAACAGGTGCTGCGTCTGCGCGAAGAG
TTTGGCGTCTACGCCGTGAACTCAGGCCGGGTGAACGTGGCCGGCATGACGCCGGA
CAACATGGCACCGCTGTGTGAAGCCATCGTCGCCGTGCTCTAAATGCATCGACACAG
GTC

APPENDIX 9. DNA sequences used to generate ATC101 (Δ*ompC*) mutant. The gentamicin cassette is shown in colour green.

Serratia marcescens Db11 ompC

In red: 1161 nucleotides downstream OmpC (ORF)

In blue: 1062 nucleotides upstream OmpC (ORF)

3’-
AACGCACCGCGATGCGCGCGCCGTCAAACGCCCACAGCTCCTTAATCAGCCGATAC
TCCAGTTCTTTCGCCCACTTGCGTTGCAAAAAGCCGACCACCGCCGCGCGCCCATCG
ACAAATTCCGCGCGGTTACGCCAGTGGGTATCGAGGGAATACACCTGCGATACACG
TTCGGGATCGCGGCTATTCCAGGCATCCTCCGCCAGCCGCACCTTCTCGATAGCGGA
TTCGCGGGTAAAAGGCGGCAATGGGGGTTTAATGCTCATGTTTATCTCCTAAGTGAA
GGCGTGTAGACAACTCTGTCTACATCATTACGCTAGCGCATGTAGACAGAGTTGTCT
ACAATGACTCGGAAATAGCCTGGGAGAAAATGATGACCGCCGATCTCAACGCCTTA
CCCGCCCGCCAGCGCATCCTGCTGACGGCGCACGATCTGTTTTATCAAGAAGGGATC
CGCGCCACCGGTATCGATCGCATCATCAAAGAATCGGGCGTGACCAAGGTGACCTT
CTACCGCCACTTTCCCAGCAAGAATGATTTAATTACGGCGTTTTTGACCTATCGCCAT
CAGCAGTGGCTCACCTGGTTCAGTACAGCGTTGGATCGACATGTGGCACAGAGCGG
CGGGTTACTGCCGGCGCTGGCGCCCTGCCTGGCGGAATGGTTCGACGATCCGCGTTT
TCGCGGCTGCGCGTTTATCAATACGGCGGTGGAAATCGCCGATCTGCTGCCGGAAAG
CCTGCACATCGCCAGCCGGCATAAGCGGCAAATGGCGGAGGAACTGGCACGTCACC
TGCCCGCCGGCCCACAGCAGGAACAGCAGGCAGCAATGCTGGCGATGCTGATCGAC
GGCGCCATCGTCAGAGTGCAGATAGAGCGGCAACCGCAGGCGGCGCTGCAGGTGCT
GAATGCCGCTCTGGCGTTGCTGGCACAAGGCGGGTTCGATCAGTAATTGGCCCAGAT
GCCCGGCGTCACCAGTTCTAACAGGTGGGCGTCGGGATCGCGAAAATAAATACTCT
CCCCGCCGTGTTCCCAGCGCATCCGCCCCTCAATCTCCACGCCGTTGGCCGTCAGAT
GCCGTTCCCAGTGTGGCAGTTGCGCTTTCGCCACCGCCAGCCCGATATGCGCCGGCC
CCACGCCATCGTGCGCAGGAATAAACCCGGTAGGATAGTGCGCCCCGCGCAGCGAG
TCGCCCTCGATAAACAGCAACAGTACGCTTCGATCGCCGACGTTATAGGCGCGAAA
CCGTTCGTTAGCGACCATCGCCGGCAGTTTCAGCACCTCCCGGTAAAAAGCGTCCGC
GCGTTCAATATCGCTGACGTAGAGCACCGTTTCGATCACCTTGTCGATCTTGAGTTCC
ATATCATCCCCCTCTTTTTCTGTACTGACAGCGTAGGCTTAGAGGAGGAAAACCACG
GTGCTTCCCGTGCCGATAGCGGATCAATCCGTGCTTTTTTACCGGCTTACAGGCAAA
AAAAACGCGGCCCGAAGGCCGCGCGATGACGTTCACACTGAGGTGCTTAGAACTGG
TAAACCAGGCCCACGGCAACCACGTTGTCGGTGTTGATACCGGCAGCGTCGGTGAA
GTCGTTGTTGTCCACCAGGTTGATTTTGTAATCAACATAGGTGGACATGTTTTTGTTG
AAGAAGTAGGTCGCACCCAGGTCGACGAATTTGACCAGATCTTGATCGCCGTAGTCC

TTGCCGTTGCCGGCGCGGCCCAAGTCTTTACCTTTGGTCTGGTTATAACCCACGAAC
GGACGCAGACCGAAGTCGAACTGGTAGTGCGCGTAGGCTTCGAAGCTCTGCGCCTT
GTTGGCGTAGCCATAAACGCTGCTGTCAGAACTACCGAAACGCGCCGCATTGTAGG
ACTGGGTGAACATCACCGCCAGGTAGACGTCGTTGGCGTCATATTTCAGACCGCCGG
AGTACCCTTCGGCCTTGTCGCCGCGACCCATGATGTTCTGATGACCGTTACCGCCGT
TCTGCTCGCTGGTACGACGCGAGTTGAAGAAGGCACCTGCGGCGCTGATGCCGTAG
CCCATGTCATAGCTCATGGACATACCGTAGCCTTCGCCGTTTTGACCCAGAACGTCA
CGACCGTTGTTGGTCTCTTCGCCGTTGCCGTTTTTACCCTGGTACTGCAGGGCGAAGT
TCAGGCCGTCAACCAGACCGAAGAAGTCGTTGTTACGGTAGGTCGCCAGGCCGCTG
GAGCGCTGGAACATGAACTGGTCGGCGCCGTAGGTCATACCGTCGAACTCAGGCTG
CAAGTCGGTATACGCCGCGACGTCGTACAGCACGCCGGTGTTACGGCCGTAGTCGA
AGGAGCCATAGTCGCCGAATTTCAGACCGGCGAAGCCGTAACGGGTGAAGTTCTTG
TTGTCCTGGCTTTCAGCGTGATTCAGGTTCGCCTGATATTCCCACTGGCCGTAACCGG
TCAGCTGATCGCTGATTTGCGTTTCGCCGCGCAGGCCAAAACGCATATAAGACTGAT
CGCCGTCCACGCCGTTGTTGCTGGAGAAATAGTGAAGACCGTCGACTTTACCGTACA
AATCCAGTTTATTACCGTCTTTGTTGTAGATTTCCGCCGCGCCTGCTGTGCCTGCAAC
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TAATTATGTCGAGCTACGGCCACTGCCCTTTTCGAGCATTATTAGCACGACTGTGCA
TTACCATTCTGGTAACAAGGGTTATATTAATCCAGAAAGAAAATGATACCAAGTATC
CGATAGTGTTTCAGTGTAATTAATAAAGGTTTCCAAATGTAAATACGCGCGAACTTT
CACAAAACATTACAGACAAACAAAATAAAGCACTTAAAGACAAAGAATAGATTAAT
TGATAAAAAATCATGTAGTTATACATATCTTTAAGAAAAAAAAGCCAACCATACAA
TTGAGCCAGACAATAAGCACATTGTGCCAAAACGCCGTCCGCACGCAGCCGCTATA
ATCACCTCGCTATCATCATTATTTTCATTATTACCTTCATTATCCGAGATCCGAGGAT
AATTTCTGCACTCCTTTAACCGGCTTCTGGCCGGTTTTTTATTTTTCTCCATACTCTCC
TCACAATTCCGACTCGTACAAATAGCACGCAAATAAATAACCTAATGATATAGTTTC
ATTAATTTACTTTCGCACTAATTGCTAATCTTTTCCCTTATTTTAGTCTACAAATTGAA
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CCGCAGAGTTTCAATTCTGATAACCTGGAAAAAGTGCACCTCCAGCGAGTAAAATTC
ATGATGCCCTTTCAACCCGACGTGCTGTGGCGGCGGCTACAAACCTCCCCCTTCCGC
GCTCGCTTCCGTTTAAATGCCAAAGACCAAACTTATCTCGACGGCAAGGGATTGCCG
CTGATACTCAGCCATGCCCGCGATTTTATCGACCGCCGCCTGGCCGCCGCCCATCCT
AAGAATGACGGCAAGCAAACACCGATGCGCGGCCACCCGGTGTTCGTCGCCCAGCA
TGCCACCGCTACCTGCTGCCGCAGCTGTCTGGAAAAGTGGCACGGCATACCGCAAG
GCGTCGCACTGAACGAGCAGCAAAAGGAGTATATTGTCCAAGCTATCGCGCTGTGG
CTGGTGCGCAGAGGCGGTGCCACTTTTCATTTCGTGCTTTATCCCATCGATTATTTCC
CGCCGCAGGCACAATTAGCCGCAGAGTTTCAATTCTGATAACCTGGAAAAAGTGCA
CCTCCAGCGAGTAAAATTCATGATGCCCTTTCAACCCGACGTGCTGTGGCGGCGGCT
ACAAACCTCCCCCTTCCGCGCTCGCTTCCGTTTAAATGCCAAAGACCAAACTTATCT
CGACGGCAAGGGATTGCCGCTGATACTCAGCCATGCCCGCGATTTTATCGACCGCCG

CCTGGCCGCCGCCCATCCTAAGAATGACGGCAAGCAAACACCGATGCGCGGCCACC
CGGTGTTCGTCGCCCAGCATGCCACCGCTACCTGCTGCCGCAGCTGTCTGGAAAAGT
GGCACGGCATACCGCAAGGCGTCGCACTGAACGAGCAGCAAAAGGAGTATATTGTC
CAAGCTATCGCGCTGTGGCTGGTGCGCAGAGGCGGTGCC-5’

Gentamycin cassette from pMB838 (pR6Kfrt-gent-frt)(exact match to pUCGM)

TGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGG
AATAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCCACCGGCTTGAAC
GAATTGTTAGGTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGTAT
GCCCAACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTA
GATCACATAAGCACCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGG
TGGCAATGCCCTGCCTCCGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCT
CACTACGCGGCTGCTCAAACCTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACC
GCTTCTTGGTCGAAGGCAGCAAGCGCGATGAATGTCTTACTACGGAGCAAGTTCCCG
AGGTAATCGGAGTCCGGCTGATGTTGGGAGTAGGTGGCTACGTCTCCGAACTCACG
ACCGAAAAGATCAAGAGCAGCCCGCATGGATTTGACTTGGTCAGGGCCGAGCCTAC
ATGTGCGAATGATGCCCATACTTGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCT
GCTGCGTAACATCGTTGCTGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACAT
CGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACAAA
AAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTT
CGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTAC
GAACCGAACAGGCTTATGTCAACTGGGTTCGTGCCTTCATCTCTGCGAAGTGATCTT
CCGTCACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGA
ATAGGAACTAAGGAGGATATTCATATG

OmpC knock-out construct

3’-
AACGCACCGCGATGCGCGCGCCGTCAAACGCCCACAGCTCCTTAATCAGCCGATAC
TCCAGTTCTTTCGCCCACTTGCGTTGCAAAAAGCCGACCACCGCCGCGCGCCCATCG
ACAAATTCCGCGCGGTTACGCCAGTGGGTATCGAGGGAATACACCTGCGATACACG
TTCGGGATCGCGGCTATTCCAGGCATCCTCCGCCAGCCGCACCTTCTCGATAGCGGA
TTCGCGGGTAAAAGGCGGCAATGGGGGTTTAATGCTCATGTTTATCTCCTAAGTGAA
GGCGTGTAGACAACTCTGTCTACATCATTACGCTAGCGCATGTAGACAGAGTTGTCT
ACAATGACTCGGAAATAGCCTGGGAGAAAATGATGACCGCCGATCTCAACGCCTTA
CCCGCCCGCCAGCGCATCCTGCTGACGGCGCACGATCTGTTTTATCAAGAAGGGATC
CGCGCCACCGGTATCGATCGCATCATCAAAGAATCGGGCGTGACCAAGGTGACCTT
CTACCGCCACTTTCCCAGCAAGAATGATTTAATTACGGCGTTTTTGACCTATCGCCAT
CAGCAGTGGCTCACCTGGTTCAGTACAGCGTTGGATCGACATGTGGCACAGAGCGG

CGGGTTACTGCCGGCGCTGGCGCCCTGCCTGGCGGAATGGTTCGACGATCCGCGTTT
TCGCGGCTGCGCGTTTATCAATACGGCGGTGGAAATCGCCGATCTGCTGCCGGAAAG
CCTGCACATCGCCAGCCGGCATAAGCGGCAAATGGCGGAGGAACTGGCACGTCACC
TGCCCGCCGGCCCACAGCAGGAACAGCAGGCAGCAATGCTGGCGATGCTGATCGAC
GGCGCCATCGTCAGAGTGCAGATAGAGCGGCAACCGCAGGCGGCGCTGCAGGTGCT
GAATGCCGCTCTGGCGTTGCTGGCACAAGGCGGGTTCGATCAGTAATTGGCCCAGAT
GCCCGGCGTCACCAGTTCTAACAGGTGGGCGTCGGGATCGCGAAAATAAATACTCT
CCCCGCCGTGTTCCCAGCGCATCCGCCCCTCAATCTCCACGCCGTTGGCCGTCAGAT
GCCGTTCCCAGTGTGGCAGTTGCGCTTTCGCCACCGCCAGCCCGATATGCGCCGGCC
CCACGCCATCGTGCGCAGGAATAAACCCGGTAGGATAGTGCGCCCCGCGCAGCGAG
TCGCCCTCGATAAACAGCAACAGTACGCTTCGATCGCCGACGTTATAGGCGCGAAA
CCGTTCGTTAGCGACCATCGCCGGCAGTTTCAGCACCTCCCGGTAAAAAGCGTCCGC
GCGTTCAATATCGCTGACGTAGAGCACCGTTTCGATCACCTTGTCGATCTTGAGTTCC
ATATCATCCCCCTCTTTTTCTGTACTGACAGCGTAGGCTTAGAGGAGGAAAACCACG
GTGCTTCCCGTGCCGATAGCGGATCAATCCGTGCTTTTTTACCGGCTTACAGGCAAA
AAAAACGCGGCCCGAAGGCCGCGCGATGACGTTCACACTGAGGTGCTGTGTAGGCT
GGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACT
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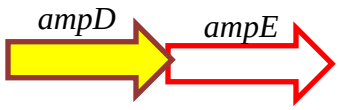
GTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGTATGCCCAACTTT
GTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTAGATCACATAA
GCACCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGGTGGCAATGCC
CTGCCTCCGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCTCACTACGCGG
CTGCTCAAACCTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACCGCTTCTTGGT
CGAAGGCAGCAAGCGCGATGAATGTCTTACTACGGAGCAAGTTCCCGAGGTAATCG
GAGTCCGGCTGATGTTGGGAGTAGGTGGCTACGTCTCCGAACTCACGACCGAAAAG
ATCAAGAGCAGCCCGCATGGATTTGACTTGGTCAGGGCCGAGCCTACATGTGCGAA
TGATGCCCATACTTGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAA
CATCGTTGCTGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACATCGACCCACG
GCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACAAAAAAACAGTC
ATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGG
TTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTACGAACCGAAC
AGGCTTATGTCAACTGGGTTCGTGCCTTCATCTCTGCGAAGTGATCTTCCGTCACAG
GTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACT
AAGGAGGATATTCATATGCGTTATTATCCTCGTTAATTATGTCGAGCTACGGCCACT
GCCCTTTTCGAGCATTATTAGCACGACTGTGCATTACCATTCTGGTAACAAGGGTTA
TATTAATCCAGAAAGAAAATGATACCAAGTATCCGATAGTGTTTCAGTGTAATTAAT
AAAGGTTTCCAAATGTAAATACGCGCGAACTTTCACAAAACATTACAGACAAACAA
AATAAAGCACTTAAAGACAAAGAATAGATTAATTGATAAAAAATCATGTAGTTATA
CATATCTTTAAGAAAAAAAAGCCAACCATACAATTGAGCCAGACAATAAGCACATT
GTGCCAAAACGCCGTCCGCACGCAGCCGCTATAATCACCTCGCTATCATCATTATTT

TCATTATTACCTTCATTATCCGAGATCCGAGGATAATTTCTGCACTCCTTTAACCGGC
TTCTGGCCGGTTTTTTATTTTTCTCCATACTCTCCTCACAATTCCGACTCGTACAAATA
GCACGCAAATAAATAACCTAATGATATAGTTTCATTAATTTACTTTCGCACTAATTG
CTAATCTTTTCCCTTATTTTAGTCTACAAATTGAAATTACTTTTCATTTCGTGCTTTAT
CCCATCGATTATTTCCCGCCGCAGGCACAATTAGCCGCAGAGTTTCAATTCTGATAA
CCTGGAAAAAGTGCACCTCCAGCGAGTAAAATTCATGATGCCCTTTCAACCCGACGT
GCTGTGGCGGCGGCTACAAACCTCCCCCTTCCGCGCTCGCTTCCGTTTAAATGCCAA
AGACCAAACTTATCTCGACGGCAAGGGATTGCCGCTGATACTCAGCCATGCCCGCG
ATTTTATCGACCGCCGCCTGGCCGCCGCCCATCCTAAGAATGACGGCAAGCAAACA
CCGATGCGCGGCCACCCGGTGTTCGTCGCCCAGCATGCCACCGCTACCTGCTGCCGC
AGCTGTCTGGAAAAGTGGCACGGCATACCGCAAGGCGTCGCACTGAACGAGCAGCA
AAAGGAGTATATTGTCCAAGCTATCGCGCTGTGGCTGGTGCGCAGAGGCGGTGCCA
CTTTTCATTTCGTGCTTTATCCCATCGATTATTTCCCGCCGCAGGCACAATTAGCCGC
AGAGTTTCAATTCTGATAACCTGGAAAAAGTGCACCTCCAGCGAGTAAAATTCATGA
TGCCCTTTCAACCCGACGTGCTGTGGCGGCGGCTACAAACCTCCCCCTTCCGCGCTC
GCTTCCGTTTAAATGCCAAAGACCAAACTTATCTCGACGGCAAGGGATTGCCGCTGA
TACTCAGCCATGCCCGCGATTTTATCGACCGCCGCCTGGCCGCCGCCCATCCTAAGA
ATGACGGCAAGCAAACACCGATGCGCGGCCACCCGGTGTTCGTCGCCCAGCATGCC
ACCGCTACCTGCTGCCGCAGCTGTCTGGAAAAGTGGCACGGCATACCGCAAGGCGT
CGCACTGAACGAGCAGCAAAAGGAGTATATTGTCCAAGCTATCGCGCTGTGGCTGG
TGCGCAGAGGCGGTGCC-5’

APPENDIX 10. DNA sequences used to generate ATD101 (Δ*ampD*) mutant. The gentamicin cassette is shown in colour green.



SmDb11 Sanger Inst: DNA seq 111306 -- 111901)
***The RBS of ampD is used to drive the transcription of *ampE* after the mutation be completed!!!

GGAATGCCTTTGCTGCCGGCTTCACAGCCGGCGGGCGCGCCTTCATCCAGCACGCAC
AGTTCGACCGCCAGTTTATACGGCGCCATGGTTTGCAGCATGTCGGTCATCGCCGCT
TTCTGGATGTAGCGCTGGTAGGCGGGAATGCCGATGGCGCTGAGGATGGCGATGAT
GGCGATCACCACCATCAGTTCGATCAGGCTAAAGCCGCGTTGCGTTTCCATGCGTTG
TTTCCTCCATAAGGGTGTGGAAAACAACGGTAGCGGTTTGCGCGGGGGAACGGCGA
GCGGGTTTTACGCGTTTCTCGGCGTGCTTCCATCAGTAATGATGCCGTTTGCAGCGG
AGGGAAAAACGGTGCGAGGGGCTTCGCAAATCTGCGGTAACGCCCCCGGCGGCGGC
CGGGGGGCGAGGGGATCATTTGAAGCGCATCGACAGATCGAGCGCGGTCACATGTTT
GGTCAGCGCGCCGACCGAAATGTAATCCACGCCGGTCTCGGCGAAGGTGCGCAGCG
TCTCGCTGGTGACGTTGCCGGATACCTCCAACTGGGCGCGGCCCTGGGTTTGGGCGA
CCGCCTGACGCATCATCTCGACGCTGAAGTTGTCCAGCATGATGATGTCGGCGCCGG
CGTCCAGCGCCTGCTGCAGTTCATCAAGCGATTCGACTTCCACTTCCACCGGCACGT
CGGCGTGCAGCCAGAAGGCTTTTTCCACCGCGTTTTTGATCGAGCCGGAGGCGATGA
TGTGGTTTTCCTTGATCAGGAAGGCATCGGACAGCCCCAGCCGGTGGTTGCTGCCGC
CCCCGCACAGTACGGCATATTTCAACGCGGTGCGCAGGCCGGGCAAGGTCTTGCGG
GTATCGAGCAGGCGAGTCTGGGTGCCTTCCAGCAGAGCCACATAGCGGCTGACCTC
GGTCGCCACGCCGGAGAGCATCTGCACGAAGTTCAGCGCGGTGCGCTCGCCGGTCA
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TCGCCGTCTGCCACCAGCCACTCCACCTGCACCTGGTTGCCCAACTGAATAAAGACT
TCGTTCAGCCAGCGGCGGCCACAGAACACGCCGGCTTCGCGGGTAATGACGGTGGC
TTCGGCCTGCTTGTCCGCCGGTAACAATTGCGCGGTAATATCGCGCCCGGCGTCAAC
TTCACCTCCCAGGTCTTCGCGCAGCGCCTGGGCGACGGAATAGGGAATATCACTTTC
AATACGTTCAAGAAGCTCGGTACGGCGACGGTCGGCACTGTAGCGGCGTGTCGGCA
TGGAAAACTCCGAAATGGACGGTTAGATCGTAGGGGAACATGCTACTCTGTTGCAG
GAATAAGTACCACCGCAGAGAGGTGTCTGATGCAGTTGGAAAATGGCTGGATTGTC
GGGGTGACGCGAGTGGTTTCGCCGCACTGCGATCGTCGCCCGGATGACGAGCCGCC
CTCGCTGCTGGTCATCCATAACATCAGCCTGCCGCCCGGCGAATTCGGTGGCCCCTA
TATCGACCAGCTGTTCACCGGCACGCTCGATCCCGCGCAGCACCCTTATTTCGCCGA
GATCCATCAGCTGCGAGTGTCTGCGCACTGCCTGATCCGCCGCGATGGGGAGATCGT
GCAGTATGTGCCCTTCGACCAGCGCGCGTGGCACGCGGGCGTTTCTCTGTATCAGGG
GCGCGAACGGTGCAACGATTTCTCCATCGGCATCGAGCTGGAAGGCACCGACCAGT

TAGCCTACACCGAGGCGCAATACCGCACGCTGCAGGCGGTCACCGCGCTGCTGGCC
GAACATTATCCGCCGTTGGCGGCGCATATCGCCGGGCATTGCGATATCGCCCCCGGC
CGCAAAACCGATCCCGGGCCGGCGTTCGATTGGGATCGCTATCTGGCATCTCTGGAA
CAACGCGGCGCGCCTGCCGACGCTATGCTTGAGACAAGAAAAAGGGAGAGGAATC
GCTAATGACGCTGTTTACGCTGTTGCTGGTTTTGGCCTGGGAGCGCCTGTTCAAGCT
GGGGGAACACTGGCAGCTGGATCATCGCCTCGAAGTGGTATTTCAGCGGCTGCATC
GGGTTTCGCTGGCGCAGACGCTGGCGATGACCGCCGCCTGGATGGTTGTCGTCTGGG
GCGTTCTGTGGTTGTCGCACGGGTTATTCTTTGGCGTGGTGACGCTGCTGCTGTGGAT
CGTCATCGATCTGCTGTGCGTCGGCGCCGGCATCAAGCGCAAACACTACCGCGCCTA
CCTGAAAGCGGCGCGACAGGGCGATACCCACGCCAGCGACCAGATGGCGGAAGAG
CTGGCGTTGATCCACGGTTTGCCGGTGGACTGCAGTGAGGAGCTGCGCCTGCGCGAG
CTGCAAAACGCGCTGCTGTGGATCAACTTCCGCTATTACCTGGCGCCGCTGTTCTGG
TTCGTGGTCTTTGGGCCGTACGGGCCGATCGCGCTGGCGGGATATGCCTTCCTGCGC
GCCTATCAAACCTGGCTGGCGCGCCACAATACGCCGCTGGAGCGTTCGCAGTCGGG
CATCGATCATCTGCTGAACTGGCTGGACTGGATCCCGGTGCGTTTGGCCGGGGTGGC
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GGATCGGCATTCGTCGTCGTATCAGGTGTTGACGCGGCTGGCGCAGTTCTCGCTGGC
GCGCGATCCGCATATGGATCCGGTGCAAACGCCGCGCGCGGCGGTGACGCTGGCGC
GCAAGGTGACGATGATCATCGTGGTGGTGGTGGCGCTGTTGACCATTTACGGCACGC
TGCTTTAAGCCGCAGCGAAAAACAAAACGGGCGCCGAGGCGCCCATTTTTTTGGCC
ATCCGGCCGGGATTACAGCTTGGCGAAGCAGCGACGCGCGGCGTCGATGGTGCGCT
GGATGTCTTCCTTGCTGTGCGCCACGGACATGAAGCCCGCTTCGAAGGCGGACGGTG
CCAGATACACCCCTTCTTCCAACATCAGGTGGAAGAAGCGCTTGAAGCGTTCCACGT
CGCACTGCATCACGTCCTGGTAGCAGGTGACCGCCGGCGCGTCGGTGAAGAACAGG
CCGAACATGCCGCCGACGTTGTTGACCACCAACGGGATATTCTCTTCCTGCGCCGCA
TGCAGCAGGCCTGCGGCCAGCATCTCGGTCAGTTCGGTCAGCGTCTGGTGCACGCCA
ACCTGCGACACTTCGGTCAGGCAAGCGTAGCCCGCCGCCATGGCGATCGGGTTGCC
GGACAGGGTGCCCGCCTGGTAGACCGGACCGGTCGGCGCCAGCGCCTCCATCACAT
CGCGACGGCCGCCGAAGGCGCCCACCGGCATACCCCCGCCGATGATTTTGCCGAGG
CAGGTCAGATCCGGCTCAACGCCGTAAT

Gentamycin cassette from pMB838 (pR6Kfrt-gent-frt)(exact match to pUCGM)

TGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGG
AATAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCCACCGGCTTGAAC
GAATTGTTAGGTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGTAT
GCCCAACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTA
GATCACATAAGCACCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGG
TGGCAATGCCCTGCCTCCGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCT
CACTACGCGGCTGCTCAAACCTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACC
GCTTCTTGGTCGAAGGCAGCAAGCGCGATGAATGTCTTACTACGGAGCAAGTTCCCG
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ACCGAAAAGATCAAGAGCAGCCCGCATGGATTTGACTTGGTCAGGGCCGAGCCTAC
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GCTGCGTAACATCGTTGCTGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACAT
CGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAGACTGTACAAA

AAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTT
CGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTAC
GAACCGAACAGGCTTATGTCAACTGGGTTCGTGCCTTCATCTCTGCGAAGTGATCTT
CCGTCACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGA
ATAGGAACTAAGGAGGATATTCATATG

AFTER GENE REPLACEMENT:

GGAATGCCTTTGCTGCCGGCTTCACAGCCGGCGGGCGCGCCTTCATCCAGCACGCAC
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TTTCCTCCATAAGGGTGTGGAAAACAACGGTAGCGGTTTGCGCGGGGGAACGGCGA
GCGGGTTTTACGCGTTTCTCGGCGTGCTTCCATCAGTAATGATGCCGTTTGCAGCGG
AGGGAAAAACGGTGCGAGGGGCTTCGCAAATCTGCGGTAACGCCCCCGGCGGCGGC
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CCGCCTGACGCATCATCTCGACGCTGAAGTTGTCCAGCATGATGATGTCGGCGCCGG
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CGGCGTGCAGCCAGAAGGCTTTTTCCACCGCGTTTTTGATCGAGCCGGAGGCGATGA
TGTGGTTTTCCTTGATCAGGAAGGCATCGGACAGCCCCAGCCGGTGGTTGCTGCCGC
CCCCGCACAGTACGGCATATTTCAACGCGGTGCGCAGGCCGGGCAAGGTCTTGCGG
GTATCGAGCAGGCGAGTCTGGGTGCCTTCCAGCAGAGCCACATAGCGGCTGACCTC
GGTCGCCACGCCGGAGAGCATCTGCACGAAGTTCAGCGCGGTGCGCTCGCCGGTCA
ACAGCACCCGTGCAGGGCCGCGCAGCCGGCAAAGCGGTTGGTTCGGTATCAGACGG
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GAATAAGTACCACCGCAGAGAGGTGTCTGTGTGTAGGCTGGAGCTGCTTCGAAGTTC
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TTACGCCCCGCCCTGCCACCGGCTTGAACGAATTGTTAGGTGGCGGTACTTGGGTCG
ATATCAAAGTGCATCACTTCTTCCCGTATGCCCAACTTTGTATAGAGAGCCACTGCG
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CCTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTGCGTAACA
TCGTTGCTGCTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTG
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TGCCTTCATCTCTGCGAAGTGATCTTCCGTCACAGGTAGGCGCGCCGAAGTTCCTAT
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ACGCTGTTTACGCTGTTGCTGGTTTTGGCCTGGGAGCGCCTGTTCAAGCTGGGGGAA
CACTGGCAGCTGGATCATCGCCTCGAAGTGGTATTTCAGCGGCTGCATCGGGTTTCG
CTGGCGCAGACGCTGGCGATGACCGCCGCCTGGATGGTTGTCGTCTGGGGCGTTCTG
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ACGCGCTGCTGTGGATCAACTTCCGCTATTACCTGGCGCCGCTGTTCTGGTTCGTGGT
CTTTGGGCCGTACGGGCCGATCGCGCTGGCGGGATATGCCTTCCTGCGCGCCTATCA
AACCTGGCTGGCGCGCCACAATACGCCGCTGGAGCGTTCGCAGTCGGGCATCGATC
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CGCATATGGATCCGGTGCAAACGCCGCGCGCGGCGGTGACGCTGGCGCGCAAGGTG
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GGGATTACAGCTTGGCGAAGCAGCGACGCGCGGCGTCGATGGTGCGCTGGATGTCT
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GGCCTGCGGCCAGCATCTCGGTCAGTTCGGTCAGCGTCTGGTGCACGCCAACCTGCG
ACACTTCGGTCAGGCAAGCGTAGCCCGCCGCCATGGCGATCGGGTTGCCGGACAGG
GTGCCCGCCTGGTAGACCGGACCGGTCGGCGCCAGCGCCTCCATCACATCGCGACG
GCCGCCGAAGGCGCCCACCGGCATACCCCCGCCGATGATTTTGCCGAGGCAGGTCA
GATCCGGCTCAACGCCGTAAT

APPENDIX 11. DNA sequences used to generate ATE101 (Δ*phoE*) mutant. The gentamicin cassette is shown in colour green.

Serratia marcescens Db11 PhoE

1078 bases in yellow (found in SmDb11 Sanger Inst: DNA seq 2223019 ….. 2224039) a.a seq length= 359 a.a.)

TTATTGCAGAGGACAAAAAAAAGCCCGGTACGCAACCGGGCTATGGCAGACCTACG
CGTTATTGCGGGATACGCAATACCTGCCCCGGATAAATCTTGTCCGGGCTCGACAGC
ATCGGCTTGTTGGCTTCAAAAATCTTGTTGTACAGATTGGCGTTGCCGTACATCTCTT
TAGAGATCGCGCTCAGCGTATCGCCCTTCTTCACGGTATAGAAGCGGCTCTCGGCGT
CGGGTTGCGTCACCGCCACCTTGTCTTCCACCCCGCTGATACCCGCCACGTTGCCGA
TAGCCACCAGGATCTTCTCTTTTAACTCCTGGCTGACGGCATCGCCGGTCACTACGG
CTTTACCATCGACCACCTGCACATCGACTTTGTCGGTGCCCGGCAAGCCGCTCTTAT
CGAGATGCTCCTTGAGTTTGGCGCCTTGATCTTCGGCGGAGGCATTGCCGGTGACTG
TGTCCCACAGTTTTTCTCCCGCTTCTTTGACGAAGTTAAACAAGCCCATATTCACTCC
TTTTGATGGTTGCATTAATGCGTTTAAGTAAGGCTTGTTAAGCTTAGCATCGTCAAA
AGGATATGCCATCCGCTCACGGCGCCAGAAACGGGTAGAGTATTCCGAAAGTGTCG
TCAGCGGCGTTTCAACGGTGATTAATTGCGCAATGATGCTGAAAAATCGTGCTTTTT
TACCTACACTGTTGCAGACAAGGCAACTCGACGAAGGAGGCGTGCAACGATGTACG
CATTGGTGATGTTTGTCTGTTATCTGGATGGTGGCTGCAGTGAAATGGTGGTCGACA
TATTGCGCGACGAGCCACAATGCCTGGTCGCGATGAAGGAACAGAACTTGCGACAC
GCCGGTTGTTATCCCATGGAAGAGTTCATTGACGGCTTCTGGTTGCCAGCCAGCGAA
TATTCGGATTTTTAATTCCCTCCCCGCGCGATGCTGAGGAATTTCTGATTTCATCAGA
AACTTCGCCTTCCTCCGAGGCATAAGGCCGCTGTTTTGCTAAATTGCGGCTCCCGAA
GAAGAGAGAGACAGAGAAAGGTCATGCCAGCATCAGCTCATTCCAACGAGCAATAT
GAAACTCTGCTGCGCGACGTCAGCCTGGCGTTAGGCGACGCAGTGTTGCAGTTAATC
AAAAACCATAAAAAAGTATCCGGCGGCAATATTCTTTCACAGCTGGTGACCGAAAT
AGAGCGGGAGCAGGATCAGCAACGTTTCGCCGCGCTGCGTTCCGCCATTGAGCTGG
TCGGGTTGGCACCCAAAGGCTGAGTTCCCGATAAAACAAAGGCAGGGCTTGCGCCC
TGCCTGAGGTTAATGACAAACCTGATGACATTACCAGGTTTACCGAAGACAACCTTT
AAAATAAGGAAAATCAATTCATTGATTTTCGACTGCTCACCACAAAGGAGGAAAAA
CCACGCTTTTTCCTCCTTTGTCAGCAAGCTAAGGCAGGGCTTACGCCCTGCCTGATTA
CCACTCCCGACACAGGATGAAGGTAATGAAATTGCCGCTTAGAAGCGATACGTCAA
GCTGACGGCAACGATATCATCGTTGGCCAGTTTCAGTTTGTTATTGTCGCTCAGCTG
GTTGATTTGATAATCAACGTAGGTGTACATATTTTTGTTGAAGTAGTAAGTCGCCCC
CACTTCCACATATTTCACCAGGTCGGCATCACCGATCCCTTCGATATCCTTGCCTTTG
GACTGCACGTAGCCCAAGGAAGGACGCAGGCCGTTTTCGAACTGATACTGGGCCAC
CACTTCGAAGTTTTGCGTTTTATTGGCGAAGCCGGAGGTGCCATTGACGCTGATCGG
CGTCATGTTACGGGTTTCGGCATACATCGCGGCCAGGTAAACCTGGTTGGCGTCGTA
TTTCACTGCGGTGGTCCATGCAGTGGCGCTGTCGCCCTTACCATACTGCAGCTCTTTT
TGCTTGCTGGTGCGGTTAGAATCAGAGAACGCCGCCGCTACGCCGATGCCGGAACC
GCCGATGTCCTGGTAGTCCAGGGACAGGCCGTAACCGTCGCCGTTTTGCTTGGTCAC
GCTGCGACCGTCGCCGTCGTTTTTACCCTGATACTGCACCGCCAGATTCAGGCCTTC
CACCAAACCGAAGAAATCACGGTTACGGTAGGTAGCCAAACCGTTGGAACGGGCGG
TCATAAAGTTATCGGTATAGCTGTAGGAGTCGCCGCCGAATTCTGGCAGCATGTCGG

TGTAAGCCTCGGCGTCATACACCACACCGTAGTTACGGCCGTAGTCGAAAGAACCG
TAGTTGCCGAATTTCAGCCCCGCGAAGCCCAGACGCGTTTTGGTGCCGGCGGTGCCC
TGCGCTTCGTCGTGATTGGCCTGGACGTTGTATTCCCACTGACCGTAACCGGTCAAC
TGGTCATTGATTTGCGTTTCGCCTTTGAAGCCCAGGCGAACATAGGTCATATCGCCG
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AGTTTATTACCATCCTTATTGTAGATTTCAGCCGCGTTGGCGAAGGAGGTGATCGCC
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TATAATTAGAACCCTGCCCGTCACGCCCAATTTATAGTTTGGGCTGTGATTTTATCTT
TTGGTATTTTATGAGCCCCCCTAATGTGGGGTGATGTGACATTAGCATCATGAATCA
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CGACCTGATACAGCCTTTTATTCACGCGTATAAAAGGAGGTATTAGGAACGAATAA
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AATAACGGCGGCGCGCTGACGGCGCCGCCGAGGATAAATCAATAGAGCGTAGAAC
GGGAAACTTCGCTCAACAGCATGCCAGCGCGCAGGCCCAGCGCCACAGTGGGGTTA
GGGAACAGTATCCACGCGCCCTCTTGGCCAACGCGATAGTCCTCCTGCGGCTGCTCG
CACAGCAACATCCCCGGTTTCAACTCGGTGAAGTTGGCCGTGTCATCATCCAGGTGC
AGCTTGAAATCCTCGCTGTGTTTGATCAACGAATGCATCACACGGAATACCCGGATC
GCCTCGCCCTCGCGCTCCGGCAACGGCCCCTCGCTGACCGCCGCCCGCAAAGCGCG
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CTCGAGCGTACAGCTCGCCGCATTGAGATGCTCGCTGGAATAGTGGCTAAACGTCCC
GCCTGGTGCGCTGTGCACCACCAGCGCATCCAAATCGGCGGCATCCAGCAGCTTCA
GCATCGGCTCACTGTACGGGCGTTTCTGGAAAGGCAGAATGCCGAAACGTGGCAAC
CGCGATTCACGGATGGCGGTGTGCAGATCGTAATGGAAACGCGCCGCCAGTTCCCC
ATCGAAAAAGGCGGCGAGCGCACGCTCCAACTGCTGCGCACGCACG

Gentamycin cassette from pMB838 (pR6KFRT-gent-FRT)(exact match to pUCGM)

TGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGG
AATAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCCACCGGCTTGAAC
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GCCCAACTTTGTATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTA
GATCACATAAGCACCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGG
TGGCAATGCCCTGCCTCCGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCT
CACTACGCGGCTGCTCAAACCTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACC
GCTTCTTGGTCGAAGGCAGCAAGCGCGATGAATGTCTTACTACGGAGCAAGTTCCCG
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ATGTGCGAATGATGCCCATACTTGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCT
GCTGCGTAACATCGTTGCTGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACAT
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AAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGTT
CGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAGTTTAC
GAACCGAACAGGCTTATGTCAACTGGGTTCGTGCCTTCATCTCTGCGAAGTGATCTT

CCGTCACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGA
ATAGGAACTAAGGAGGATATTCATATG

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GCGTTATTGCGGGATACGCAATACCTGCCCCGGATAAATCTTGTCCGGGCTCGACA
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