Functional characterization of novel and putative Two Component Systems in *Acinetobacter baumannii*

Submitted by

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Abstract

Acinetobacter baumannii is a pathogenic bacterium responsible for various hospital-acquired infections in immunocompromised patients. It is highly resilient and can survive in harsh environmental conditions. *A. baumannii* is infamous for its ability to develop resistance against various antimicrobials. Two Component Systems (TCS) are signal transduction systems which enable bacteria to sense and adapt to changes in environmental conditions. Various studies have indicated their involvement in regulation of antibiotic susceptibility and virulence mechanisms in bacteria. In this project, we investigated the functional characteristics of novel and putative TCSs in *A. baumannii* and their role in regulation of antibiotic susceptibility and virulence phenotypes.

The first part of this study involved the functional characterization of AvnR, a conserved response regulator, in the clinical isolate - *A. baumannii* AB030. AvnR has previously been linked with regulation of various virulence-associated phenotypes including biofilm formation, motility, and nitrogen metabolism. The *avnR* gene in *A. baumannii* AB030 has been found to be naturally disrupted by an insertion sequence, rendering it non-functional. We investigated the impact of this disruption on biofilm formation, motility, and nitrogen metabolism in AB030. Complementation of *avnR* in AB030 had no effect on biofilm formation and motility phenotypes; however, changes in its nitrogen metabolism profile was observed. Complement strain, AB030:mini-Tn7:*avnR*, exhibited enhanced growth in L-serine and L-arginine compared to the wild type, whereas a decrease in growth was observed in Alanine-Histidine, Alanine-Leucine, and L-Valine.

In the second part of this study, the functional characteristics of a putative TCS, A1S_1977-78, was investigated in *A. baumannii* ATCC 17978. Previous studies have indicated a potential link between this TCS and regulation of AdeN, a Tet-R type regulator protein. AdeN is associated with expression of AdeIJK efflux pump as well as virulence-associated phenotypes. A1S_1977-78 was, therefore, predicted to be associated with antibiotic susceptibility and virulence in *A. baumannii.* Deletion of *A1S_1977-78* in ATCC 17978 genome resulted in a two-fold increase in susceptibility to ciprofloxacin. However, no change in expression was observed for *adeN* and *adeIJK* in the deletion mutant compared to the wild type strain, suggesting that A1S_1977-78 may not be involved in regulation of *adeN*/*adeIJK* expression.

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Chapter 1: Literature review

1. Introduction

1.1. *Acinetobacter baumannii*

Acinetobacter baumannii is an opportunistic Gram-negative bacterial pathogen responsible for a wide variety of hospital-acquired infections in immunocompromised patients (Peleg et al., 2008). It is a coccobacillus currently assorted to the Moraxellaceae family of Gammaproteobacteria (Vrancianu et al., 2020). Initially considered to be benign, *A. baumannii* has become increasingly important due to the severity of its infections (Mea et al., 2021). Infections caused by this bacterium include pneumonia, bacteremia, meningitis, urinary tract infections, and skin and soft tissue infections (Peleg et al., 2008). They are non-fermentative, strictly aerobic, oxidase negative and non-fastidious (Peleg et al., 2008). While originally thought to be non-motile, *A. baumannii* have been found to exhibit twitching motility and surface-associated motility (Clemmer et al., 2011).

Acinetobacter species are ubiquitous and have been isolated from various sources including soil, fresh and marine water sources, animals, and insects (Bergogne-Berezin, 2008; Peleg et al., 2008; Hammoud et al., 2021). The presence of *A. baumannii* has been mostly observed in hospitals; and infections by this bacterium outside of hospital settings have rarely been reported (Sarshar et al., 2021). *A. baumannii* is extremely adept in acquiring resistance against a broad spectrum of antibiotics drugs and therefore is classified as an ESKAPE pathogen (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* species) (Antunes et al., 2014; Imperi et al., 2011). These bacterial species are known for their ability to evade the detrimental effects of antibiotics due to increased multi-drug resistance, and thus, difficult to treat. Due to the impressive ability of *A. baumannii* to evade the action of antibiotics using a combination of intrinsic and acquired mechanisms, there has been an increase in the occurrence of multi-drug resistance (MDR) *A. baumannii*, including resistance to last resort antibiotics such as carbapenems and polymyxins (Ilsan et al., 2021). The importance of *A. baumannii* as a serious threat to modern healthcare can be emphasized by the fact that it has been listed as a top priority pathogen by the World Health Organization, for which novel therapeutics are urgently required (Tacconelli et al., 2018)(World Health Organization, 2017). Apart from their ability to resist antibiotics *A. baumannii* is also

adept at tolerating stress due to harsh environmental conditions such as oxidative stress, change in osmolarity, incubation temperatures, nutrient availability, long term desiccation and disinfection (Sarshar et al., 2021). Over the years, several mechanisms responsible for antibiotic resistance and virulence in *A. baumannii* have been identified (Vrancianu et al., 2020). However, the molecular mechanisms by which *A. baumannii* sense and respond to environmental changes and the effect of such changes on their antibiotic susceptibility and virulence remain poorly understood (De Silva, 2019).

1.2. Mechanisms of antibiotic resistance in *A. baumannii*

Antibiotic resistance in *A. baumannii* is achieved by a combination of intrinsic and acquired mechanisms (Zwama & Nishino, 2021). These mechanisms are broadly classified into four categories, which include, antibiotic inactivating enzymes, target site modification, reduced outer membrane permeability and overexpression of efflux pumps (Vrancianu et al., 2020).

1.2.1. Enzymatic inactivation of antibiotic molecules

Enzymatic inactivation of antibiotic molecules is a major feature of reduced susceptibility to a wide array of β-lactams as well as aminoglycosides and tetracyclines in *A. baumannii* (Kyriakidis et al., 2021). Resistance against β-lactams in *A. baumannii* is achieved by antibiotic hydrolyzing enzymes known as β-lactamases (Lee et al., 2017). Based on the hydrolyzing mechanisms, there are four Classes of β-lactamases expressed in *A. baumannii*. Class A β-lactamases generally mediate resistance against penicillins, however, they can also hydrolyze a broader spectrum of β-lactams including cephalosporins, ceftazidime, cefotaxime, ceftriaxone and aztreonam (Kyriakidis et al., 2021). Class B β-lactamases, also known as metallo-β-lactamases (MBLs), are broad spectrum enzymes that require zinc or other heavy metals for catalysis of β-lactamase activity (Cornaglia et al., 2011). They can hydrolyze nearly all β-lactams except monobactams (Cornaglia et al., 2011). Four types of MBLs have been identified in *A. baumannii*, which include, Imipenemase (IMP), New Delhi Metallo-β-Lactamase (NDM), Verona Integron-embedded MBL (VIM) and Seoul Imipenemase (SIM) (Cornaglia et al., 2011). Class C β-lactamases are conserved cephalosporinase enzymes in *A. baumannii* that are encoded

by a chromosomally embedded gene, *blaADC* (formerly *blaAmpC)* (Héritier et al., 2006). The insertion of resistant islands sequences ISAba1 and ISAba25 upstream of this gene results in overexpression of these β-lactamases, resulting in a broad-spectrum resistance against cephalosporins and carbapenems (Hamidian & Hall, 2013). Class D β-lactamases are carbapenem hydrolyzing enzymes and can inactivate all β-lactams (Kyriakidis et al., 2021). They are also known as oxacillinase and are serine dependent (Lee et al., 2017). Unlike Class A β-lactamases, oxacillinase enzymes are resistant to β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Poirel et al., 2010). Apart from beta-lactamases, *A. baumannii* also expresses antibiotic modifying enzymes to evade various aminoglycosides (Lee et al., 2017). These enzymes neutralize such antibiotic molecules by altering their active sites, resulting in loss of function (Nemec et al., 2004). Based on the site of modification on the aminoglycoside molecules, three enzymes have been classified, namely, acetyltransferase, nucleotidyltransferase and phosphotransferase (Nemec et al., 2004). Recent studies have also revealed a plasmid mediated *tet(X)* variant genes encoding for monooxygenase enzymes such as Tet(X3), Tet(X4) and Tet(X5), which can inactivate a broad spectrum of tetracyclines, including tigecycline, which is a last resort antibiotic (He et al., 2019).

1.2.2. Target site modification

Target site modification is another important mechanism for evading the action of antibiotics, which involves the alteration of the binding sites of antibiotic molecules. In *A. baumannii,* mutations in *gyrA,* which encodes for DNA gyrase, and *parC,* encoding for DNA topoisomerase, result in reduced affinity to fluoroquinolone binding resulting in resistance against quinolones such as ciprofloxacin (Seward & Towner, 1998). Colistin resistance in *A. baumannii* is another example of this mechanism. Colistin is a cationic antimicrobial lipopeptide which bind to the negatively charged Lipid A domain of the lipopolysaccharide layer resulting in disruption of the cell envelope (Li et al., 2006). Mutations in genes regulating PmrAB TCS results in overexpression of *pmrC*. This gene encode for phosphoethanolamine (pEtN) transferase (Ilsan et al., 2021). Increased pEtN concentrations affects Lipid A by reducing the negative charge on the cell surface, therefore preventing colistin binding (Arroyo et al., 2011). *eptA* is another gene that has been found to be overexpressed due to mutation in *pmrAB* and has been associated with

increased pEtN formation (Ilsan et al., 2021). Altered expression of *naxD,* results in differential expression of acetyl-galactosamine deacetylase, resulting in the modification of galactosamine concentration of Lipid A (Ilsan et al., 2021). Other mechanisms for evading colistin binding include mutations in Lipid A biosynthetic genes such as *lpxA, lpxC* and *lpxD* which result in defective LPS biosynthesis (Moffatt et al., 2013). Tetracycline resistance in *A. baumannii* is achieved by expression of Ribosomal Protection Proteins such as TetM, TetW,TetS and TetO, which is responsible for modification of 30S ribosomal target sites (Kyriakidis et al., 2021). TetM has been shown to be responsible for a GTP-dependent release of tetracycline molecules from 30S ribosomal binding sites, therefore, rendering it functional despite the presence of tetracycline in the cells (Kakoullis et al., 2021). Resistance to macrolides such as azithromycin is achieved by alteration of 23S rRNA and ABC-F type ribosomal protection proteins (Kyriakidis et al., 2021). Resistance to rifampin is achieved through mutation of the *rpoB* gene resulting in alterations in the RNA polymerase (RNAP) β-subunit active sites, therefore, reducing binding of the antibiotic molecules (Kyriakidis et al., 2021).

1.2.3. Reduced outer membrane permeability

Differential expression of porins have been credited as a contributor of antibiotic resistance in many drugs resistant bacteria (Uppalapati et al., 2020). Porins are outer membrane proteins present on the outer membrane of bacteria and are responsible for selective permeation of different molecules across the cell envelope. Three porins have been identified in *A. baumannii* that have been associated with antibiotic susceptibility phenotype, namely, OmpA, CarO, and 33-36 kDa porin (Uppalapati et al., 2020). OmpA (Outer Membrane Protein A) is the most well characterized porin in *A. baumannii* and has been associated with reduced susceptibility against chloramphenicol, aztreonam and nalidixic acid (Gribun et al., 2003)(Uppalapati et al., 2020). Disruption of *ompA* have demonstrated a decrease in susceptibility to these antibiotics in *A. baumannii* (Smani et al., 2013). CarO is another porin that has been associated with carbapenem resistance (Zhu et al., 2019). Conformational changes in CarO have been shown to result in reduced susceptibility to imipenem (Limansky et al., 2002)(Zhu et al., 2019). Another protein, 33-36 kDa has been shown to be associated with *A. baumannii*'s susceptibility to imipenem and meropenem (Clark, 1996)(Tomás et al., 2005).

1.2.4. Efflux pumps

Efflux pumps in bacteria are protein assemblies localized in the cytoplasmic membrane and are responsible for transportation of various toxins and xenobiotic molecules like heavy metals, dyes, and antimicrobials across the cell envelope (Du et al., 2018). Based on the amino acid composition and source of energy used, efflux pumps in bacteria are categorized into six families, namely, ATP Binding Cassette (ABC) transporters family, Major Facilitator Superfamily (MFS) transporters family, Small Multidrug Resistance (SMR) family, Multidrug and Toxin Extrusion (MATE) family, Proteobacterial Antimicrobial Compound Efflux system (PACE) family, and Resistance Nodulation Division (RND) family (Du et al., 2018). Efflux pumps belonging to the RND family have been identified as the main contributor of intrinsic resistance in MDR strains of bacteria (Zwama & Nishino, 2021). These comprise of tripartite protein assemblies consisting of an Inner Membrane Transporter (IMT), a Periplasmic Adaptor Protein (PAP) and an Outer Membrane Factor (OMF) (Du et al., 2014). Together, they form a conduit connecting the inner cytosolic region to the extracellular matrix (Du et al., 2014). They use proton motive force to extrude a broad spectrum of substrates out of the cells (Meehan et al., 1997). Overexpression of such pumps result in increased tolerance against a broad spectrum of antimicrobials in *A. baumannii*. To date, three RND efflux pumps have been characterized, namely, AdeABC, AdeFGH and AdeIJK (Kornelsen & Kumar, 2021).

AdeABC is the most widely studied RND pump in *A. baumannii* (Yoon et al., 2013). Due to its prominence in a vast majority of clinically isolated strains, it is credited to be a major contributor of intrinsic as well as acquired resistance against a broad spectrum of antibiotics in *A. baumannii* (Yoon et al., 2013). This pump is associated with resistance against aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, trimethoprim, erythromycin, and tetracycline (Kornelsen & Kumar, 2021). Overexpression of this pump has also been associated with tigecycline and netilmicin resistance (Ruzin et al., 2007). Genes encoding this pump are arranged in an operon and, therefore, are co-transcribed. The expression of this pump is regulated by the TCS AdeRS (Yoon et al., 2013).

AdeFGH is another highly conserved RND pump and is regulated by AdeL, a LysR type regulator (Coyne et al., 2010). Mutations in the *adeL* gene leading to the inactivation of AdeL can result in increased expression of this pump and result in resistance against nalidixic acid,

fluroquinolones, erythromycin, tetracycline, tigecycline, chloramphenicol and trimethoprim (Kornelsen & Kumar, 2021). However, the overexpression of this pump does not affect the antibiotic concentration of β-lactams and aminoglycosides in the cells (Coyne et al., 2010). AdeFGH can also efflux dyes such as EtBr and safranines (Coyne et al., 2010).

AdeIJK is an RND pump that is constitutively expressed in *A. baumannii* and works together with AdeABC in providing antibiotic resistance (Damier-Piolle et al., 2008). Unlike AdeABC, this pump is associated with intrinsic resistance only (Yoon et al., 2015). Overexpression of AdeIJK over a certain threshold has been found to be toxic to *A. baumannii* (Damier-Piolle et al., 2008). The expression of AdeIJK is regulated by AdeN, a regulator protein belonging to the Tet-R family (Rosenfeld et al., 2012). The gene encoding this regulator is located 813kb upstream of the *adeIJK* operon (Rosenfeld et al., 2012). AdeN acts as a repressor and the expression of AdeIJK is inversely associated with the expression of this pump (Rosenfeld et al., 2012). Increased expression of AdeIJK can result in resistance against a wide spectrum of antibiotics including β-lactams, tetracyclines, fluroquinolones, chloramphenicol, rifampin, trimethoprim, novobiocin, erythromycin and Sodium Dodecyl Sulfate (SDS) (Damier-Piolle et al., 2008). Recent studies have also demonstrated the role of this pump in providing resistance against triclosan, a biocide commonly used as ingredient in various household disinfectants, toothpaste, soaps, and hospital equipment (Fernando et al., 2014).

Apart from the RND family, some transporters and efflux pumps belonging to other families have also been found to be important contributors of antibiotic resistance in *A. baumannii*. AbeM, a transporter belonging to MATE (Multidrug And Toxic Compound Extrusion) family is responsible for resistance against fluoroquinolones (Su et al., 2005). MacAB-TolC, an efflux pump belonging to ABC (ATP Binding Cassette) transporter family is associated with macrolide and tigecycline resistance (Henry et al., 2012a). Some MFS (Major Facilitator Superfamily) transporters including AmvA, AbaF, AbaQ and TetA are responsible for resistance against erythromycin, fosfomycin, quinolone and tigecycline respectively (Kornelsen & Kumar, 2021). ClmA and CraA are associated with chloramphenicol resistance (Roca et al., 2009)(Fournier et al., 2006). AbeS, belonging to SMR (Small Multidrug Resistance) family, is responsible for chloramphenicol, fluoroquinolones, erythromycin and novobiocin (Srinivasan et al., 2009).

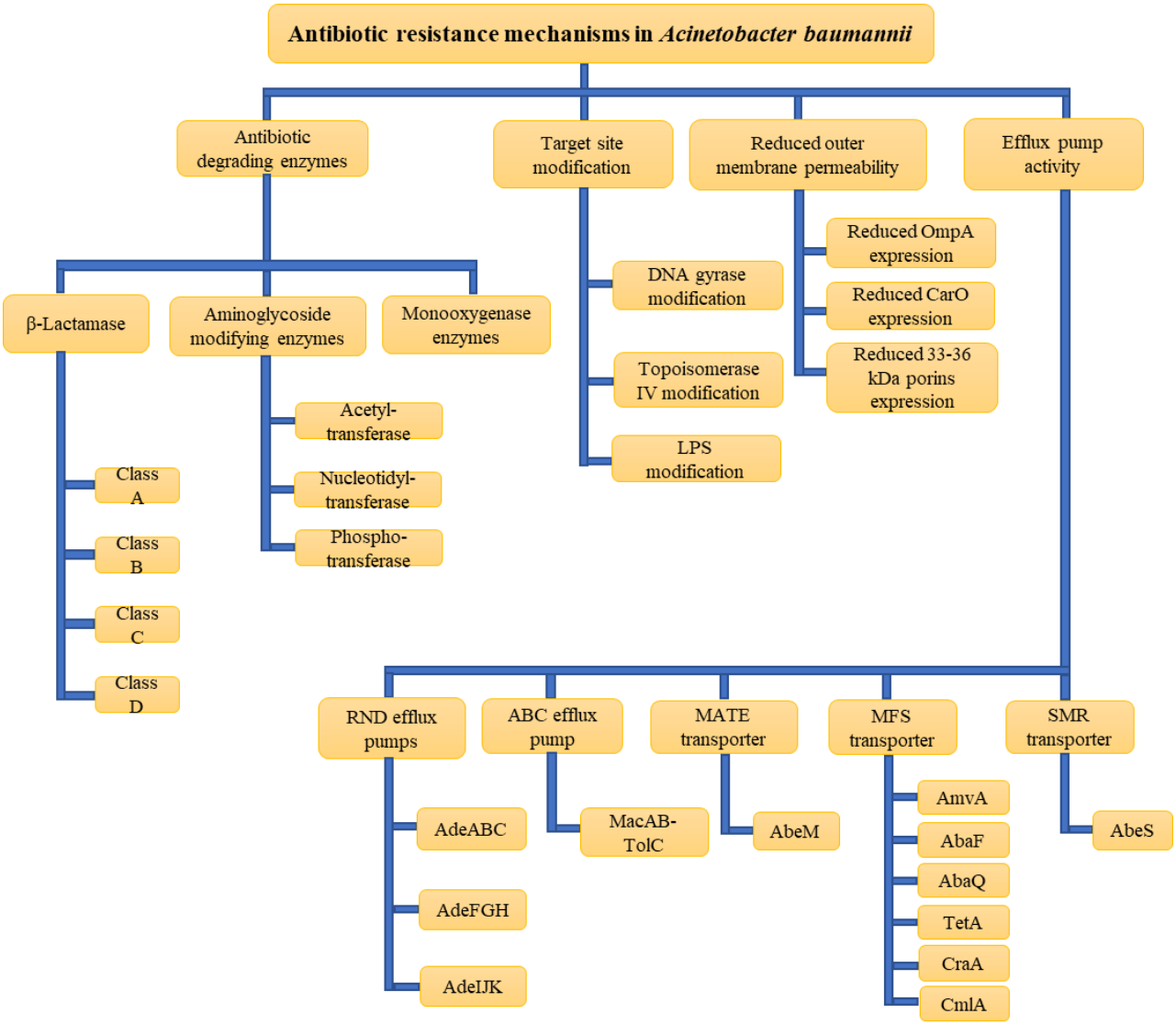


Figure 1.1. Overview of antibiotic resistance mechanisms in *Acinetobacter baumannii*

1.3. Virulence mechanisms in *A. baumannii*

Pathogenesis in *A. baumannii* involves a "persist and resist" tactic and is a result of the combined action of multiple virulence factors, which facilitate bacterial adhesion, invasion, and cytotoxicity in the host cell (Mea et al., 2021).

1.3.1. Outer Membrane Proteins

Outer Membrane proteins (OMP) play an important role in pathogenesis and are the most well studied in virulence factor in *A. baumannii* (Uppalapati et al., 2020). These are monomeric β-barrel porins present in the outer membrane and have been associated with various virulence phenotypes conferring infection and apoptosis of host epithelial cells (Smith et al., 2007). OmpA is a conserved porin and is the most well characterized OMP in *A. baumannii* (Gribun et al., 2003). It is also the most abundant porin in *A. baumannii* and is responsible for adhesion, invasion, and cytotoxicity in host epithelial cells (Uppalapati et al., 2020). During an infection, *A. baumannii* attaches to the host epithelial cell and secrete OmpA, which results in a "zipper-mechanism", involving actin rearrangement and membrane reorganization in the host cell surface (Choi et al., 2008). This results in the engulfment of the bacterial cells into the host, where they are packaged within a membrane bound vacuole (Uppalapati et al., 2020). OmpA secreted by these cells are translocated to the mitochondria and nucleus, where they engage in cytotoxic activities resulting in apoptosis of the host cell (Kim et al., 2008). OMP 33-36 kDa porin is another example of an OMP that has been associated with cell adhesion, invasion, and cytotoxicity (Smani et al., 2013). Recombinant *A. baumannii* strains with inactivated *omp33* gene showed significant reduction in these functions in human lung cells infection experiments (Smani et al., 2013). Biofilm-associated protein (Bap) is an OMP associated with regulation of hydrophobicity of the bacterial cell surface and host cell adherence, but are not involved in internalization of the bacterial cells (Brossard & Campagnari, 2012). The OMP - *Acinetobacter* trimeric autotransporter (Ata) has been shown to mediate cell adherence and contribute to host cell apoptosis (Weidensdorfer et al., 2019).

1.3.2. Phospholipase

Phospholipases are important virulence factors in *A. baumannii* and have been shown to play a significant role in host cell invasion (Lee et al., 2017). Two phospholipases have been identified in *A. baumannii,* namely Phospholipase C and D (Camarena et al., 2010)(Jacobs et al., 2010). Recombinant mutants of *A. baumannii* with disruption of Phospholipase D exhibited significant reduction in its ability to invade host cells (Jacobs et al., 2010). It has also resulted in reduced persistence in human serum. Phospholipase C disruption in *A. baumannii* has been shown to decrease in cytolytic activity in the host cells (Camarena et al., 2010).

1.3.3. Lipooligosaccharide

Lipooligosaccharide (LOS), which is an essential component of the outer membrane of *A. baumannii,* is another contributor of cytotoxicity during host cell infection (Moffatt et al., 2013). Disruption of genes *lpxA* (acyl transferase), *lpxC* (deacetylase) and *lpxD* (N-acyl transferase), which encode for essential components of the lipid A biosynthetic pathway, resulted reduced persistence in human serum (Moffatt et al., 2013).

1.3.4. Type II Secretion System

Type II secretion system in *A. baumannii* is responsible for secretion of Gamma-glutamyl transferase enzyme (GGT) (Johnson et al., 2016). High level of GGT has been associated with reactive oxygen stress in the host cells. It has also been associated with cell apoptosis, necrosis, and host cell cycle arrest (Elhosseiny et al., 2020).

1.3.5. Other virulence factors

Some virulence factors identified in *A. baumannii* have been shown to be involved in mechanisms associated with virulence such as biofilm formation and motility; however, their role in bacterial pathogenicity and the molecular interactions involved are not fully understood. Type I pili (also known as CSU pili) is one such example and is responsible for twitch motility

and biofilm formation in *A. baumannii* (Eijkelkamp et al., 2011). Type VI secretion system is used by *A. baumannii* to kill competing bacteria in a microbiome; however, its role in virulence is yet to be determined(Lopez et al., 2020). Some strains of *A. baumannii* have been shown to have a capsule, which acts as a protective shield against unfavorable conditions such as desiccation and phagocytosis, therefore, contributing to environmental persistence (Doi et al., 2015). However, its role in bacterial virulence is yet to be determined. Expression of siderophores such as acinetobactin and baumannoferrin have been shown to facilitate iron uptake in *A. baumannii* and persistence in iron deficient environments during infections (Ramirez et al., 2019).

1.4. Two Component Systems

Signal transduction is an essential mechanism by which bacteria rapidly adapt to changes in their environment and survive in stressful conditions (De Silva & Kumar, 2019). In pathogenic bacteria, it enables tolerance to sudden changes in environmental conditions, such as nutrient availability, temperature, osmolarity and innate host defense systems during infection into a host body (Bhagirath et al., 2019). Two Component Systems (TCS) are signal transduction systems ubiquitous in bacteria (Alm et al., 2006). They comprise of two domains, namely, a sensory Histidine Kinase (HK) and a Response Regulator (RR) (Nixon et al., 1986). Functionally, the HK sense stress due to change in environmental conditions and undergo conformational changes generating a phosphate molecular signal. This signal is phosphorylated to the RR and activates this domain. The activated RR binds to the appropriate genes and regulates their expression in response to the environmental stimuli (Szurmant, H.et al., 2007).

HKs are homodimer proteins present in the transmembrane of bacteria comprising of multiple conserved domains (De Silva & Kumar, 2019). These include a Dimerization and Histidine Phosphotransfer (DHp) domain and a Catalytic ATP binding (CA) domain (De Silva & Kumar, 2019). The DHp comprise of a pair of parallel alpha-helices joined by a loop. One of these helices consists of the H Box containing the conserved Histidine residue (De Silva & Kumar, 2019). The CA domain is composed of a five stranded beta-sheet flanked by three alpha-helices and has an ATP generating pocket. The DHp and CA are linked by a variable loop, and this system is linked to the extracellular sensor domain through transmembrane helices (Casino et al.,

2009). On sensing environmental stress, this system generates ATP molecules at the histidine residue, which is transferred to the RR via autophosphorylation (Zschiedrich et al., 2017). The RR consists of two parts, namely, the receiver domain (REC) and the effector domain (Casino et al., 2009). The REC is a highly conserved domain consisting of five parallelly stranded beta-sheet and two alpha-helices doubly wounded to form an alpha/beta fold (Zschiedrich et al., 2017). The effector domain is variable and is responsible for specificity of the RR towards its target genes (Casino et al., 2009). The REC domain contains a conserved aspartate residue which receives the phosphorylated stimuli from the HK and activates the RR (Yamamoto et al., 2005). This activated RR then binds to necessary genes and generate appropriate phenotypic response (Kenney, 2010). Further, the cessation of the environmental stress results in dephosphorylation in the HK resulting in reversal of the transcriptional response of the RR (Hsing, W., & Silhavy, 1997).

A fast-paced advancement in bioinformatics and Next Generation Sequencing technologies has resulted in an exponential increase in the number of HKs and RRs identified in various species of bacteria. While most HKs and RRs of various TCSs have been shown to confer high specificity (Casino et al., 2009), there have been some HKs which have been found to associate with more than one RR (López-redondo et al., 2010). Similarly, some RRs have been found to be activated from signals from multiple HKs (Laub & Goulian, 2007). Together, these systems respond to various environmental stimuli including, varying temperatures, PH, osmolarity, moisture, and nutrient availability.

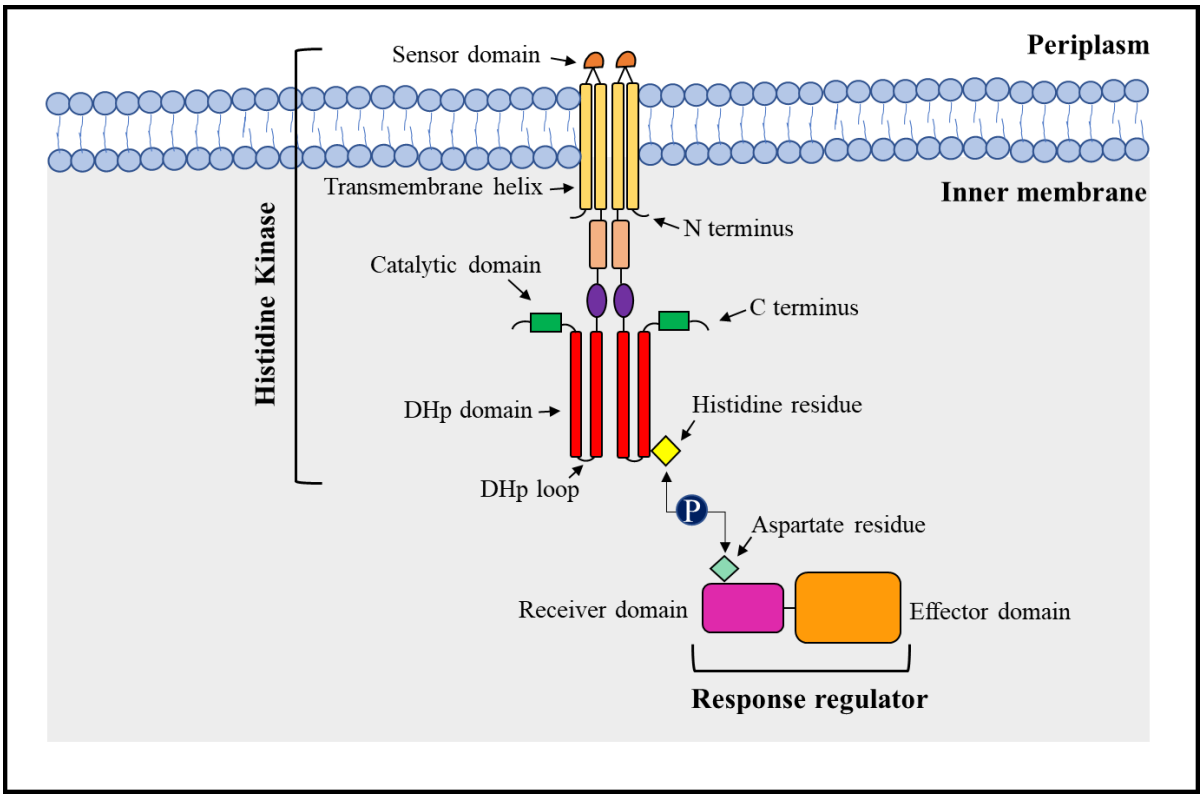


Figure 1.2. Schematic diagram of the prototypic structure of a Two Component System. Two Component System generally comprise of a sensory Histidine Kinase (HK) at the inner membrane region and a Response Regulator (RR) at the cytosolic region. The HK sense environmental signals and undergo autophosphorylation, generating a phosphate molecule at its conserved histidine residue. This phosphate is relayed to the RR, where it binds at its conserved aspartate region, resulting in activation of the RR.

1.4.1. Two Component Systems in *A. baumannii*

Genomic analyses of various well characterized clinical isolates have revealed around 20 TCS encoding genes in *A. baumannii* (De Silva & Kumar, 2019). Seven TCSs have been partially characterized so far, whereas 12 putative TCSs are yet to be functionally elucidated. A summary of the currently known TCSs are as follows.

(i) *AdeRS*

AdeRS was the first TCS to be studied in *A. baumannii* and was identified in the clinical isolate *A. baumannii* BM4454 (Marchand et al., 2004). It is also the most well characterized TCS in this species. AdeRS is best known for its role in reduced susceptibility against multiple classes of antibiotics including aminoglycosides, β-lactams, chloramphenicol and tigecycline due to its involvement in the activation of AdeABC efflux pump expression (Yoon et al., 2013). Previous studies on this TCS have shown that inactivation of *adeR* or *adeS* resulted in increased susceptibility of *A. baumannii* to aminoglycosides (Marchand et al., 2004)(Yoon et al., 2013). Further, the overexpression of AdeABC because of point mutations in the *adeRS* operon, resulting in increased antibiotic resistance, has also been observed (Yoon et al., 2015). However, some studies have demonstrated the overexpression of AdeABC in strains lacking AdeRS, therefore indicating the regulation of this pump is not exclusive to this TCS (Sun et al., 2010). The expression of AdeRS has also been linked with virulence in pulmonary infection models, therefore indicating the involvement of AdeRS in regulation of virulence in *A. baumannii* (Yoon et al., 2015). It has also been shown to regulate the expression of various genes involved in biofilm formation, motility, and virulence in *Galleria mellonella* infection models (De Silva & Kumar, 2019). However, in a study conducted by Richmond et al, deletion of *adeRS* did not affect biofilm formation in the strain *A. baumannii* S1, but disrupted this phenotype in *A. baumannii* AYE, indicating the role of AdeRS in regulation of this phenotype to be strain specific (Richmond et al., 2016).

(ii) *BaeSR*

Named after its homolog in *E.coli*, BaeSR is a TCS in *A. baumannii* which comprise of overlapping regulons with AdeRS (Leblanc et al., 2011)(Lin et al., 2014). It has been associated with tigecycline resistance in *A. baumannii* due to its involvement in AdeABC pump expression alongside AdeRS (Lin et al., 2014). Apart from this, studies have also indicated their role in the expression of AdeIJK and MacAB-TolC pumps (Henry et al., 2012b). Mutant strains of *A. baumannii* with disruption of *baeSR* showed significant reduction in these three pumps (Henry et al., 2012b). Studies on determining the binding sites for BaeR have, however, been unsuccessful, suggesting an indirect role of this TCS in regulation of these pumps (Lin et al., 2015). Deletion of *baeR* have been found to reduce tolerance of *A. baumannii* to tannic acid (Lin et al., 2015). BaeSR has been associated with modulation of antibiotic susceptibility phenotypes in response to osmotic stress when exposed to high concentration of sucrose (Lin et al., 2014).

(iii) *BfmRS*

Biofilm formation is an essential mechanism by which *A. baumannii* persist in adverse conditions and tolerate harsh environmental stresses such as desiccation, nutrient starvation, unfavorable temperatures, and disinfectants (Kröger et al., 2017). Attachment of bacterial cells on a surface is essential for biofilm formation and is achieved by the expression of pili (Gaddy and Actis, 2009). The Csu-usher chaperone assembly, which comprise the CsuA/BABCDE is responsible for formation of these pili, and therefore, bacterial attachment to the surface and biofilm formation (Gaddy and Actis, 2009). BfmRS is a TCS responsible for regulating the expression of CsuA/BABCDE and has been shown to be responsible for biofilm formation (Tomaras et al., 2008). Deletion of the RR-encoding gene *bfmR* resulted in loss of biofilm formation in *A. baumannii* (Tomaras et al., 2008). Deletion of the sensor, *bfmS*, resulted in a significant decrease in biofilm formation, but not complete elimination of the process (Tomaras et al., 2008). This indicate that BfmR may couple with unknown sensory kinases for activation (Tomaras et al., 2008). BfmRS is also involved in regulation of genes involved in production of exopolysaccharides which responsible for capsule production in *A. baumannii* (Geisinger & Isberg, 2015). The capsule play a role in serum resistance during infections and facilitate virulence in the host body (Geisinger & Isberg, 2015).

Apart from their role in virulence, BfmRS has also been associated with regulation of antibiotic susceptibility, where disruption of both individual components of this TCS resulted in variation in the Minimum Inhibitory Concentration for different antibiotics (Russo et al., 2016). Studies have shown that deletion of *bfmR* in *A. baumannii* resulted in an increased MIC for meropenem and colistin (Russo et al., 2016). In a study conducted by Liou et al., deletion of *bfmS* in strain ATCC 17978 resulted in increased susceptibility to imipenem but decreased susceptibility to ciprofloxacin (Liou et al., 2014).

(iv) *PmrAB*

The widespread occurrence of infections by MDR and XDR strains of *A. baumannii* has resulted in an increased use of last resort antibiotics such as colistin for treatment (Kaye et al, 2016). This has resulted in the emergence of colistin resistant strains of *A. baumannii* globally (Kaye et al., 2016). Investigations on the molecular mechanisms for colistin susceptibility in such strains have revealed the involvement of PmrAB (Adams et al., 2009). The role of this TCS in polymyxin resistance has been previously demonstrated in other Gram-negative pathogens including *E. coli*, *Salmonella enterica, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Quesada et al, 2015)(Cheng et al., 2010)(Moskowitz et al., 2004) (Riquet et al., 2011). Mutations in *pmrA* and *pmrB* have been shown to result in the overexpression of the *pmrCAB* operon (Da Silva & Domingues, 2017). Consequently, the increase in *pmrC* expression results in modifications in the LPS layer due to the addition of phosphoethanolamine in the Lipid A (Da Silva & Domingues, 2017). The resulting decrease in negative charge on the bacterial cell surface prevents colistin from binding to the outer membrane (Arroyo et al., 2011). While a complete loss of the LPS layer due to mutations in *lpxA, LpxC* and *lpxD* has also been recorded as a mechanism for colistin resistance in *A. baumannii,* the role of PmrAB in this is not yet known (Moffatt et al., 2010). The environmental stimuli involved in the activation of PmrAB in *A. baumannii* is yet to be determined. However, previous studies have indicated that low pH may contribute to activation of this TCS (Adams et al., 2009).

(v) *GacSA*

GacSA was first identified in a transposon mutant library screen of the *A. baumannii* ATCC19606 genome (Dorsey et al., 2002). It has been found to be involved in regulation of the citrate metabolism pathway of *A. baumannii,* where disruption of the *gacS* sensor kinase gene inhibited its ability to utilize citrate as sole carbon source (Dorsey et al., 2002). GacSA has also been found to be involved in regulating the gene cluster of the *paa* operon, encoding for phenylacetic acid which is responsible for catabolism of aromatic compounds (Bhuiyan et al., 2016). Further, it is involved in regulation of various virulence factors including biofilm formation, motility, serum resistance and pili formation (Kröger et al., 2017). Deletion of *gacS* in *A. baumannii* also resulted in a significant decrease in virulence, as observed in infection models in mouse, zebra fish and *Candida albicans* (Bhuiyan et al., 2016) (Kröger et al., 2017). Studies on GacS in *A. baumannii* ATCC17978 revealed that a REC domain is present along with the Histidine residue containing HisKA domain, suggesting that it may be a hybrid HK (De Silva & Kumar, 2019).

(vi) *A1S_2811*

First characterized in 2017, A1S_2811 is a hybrid Sensor Kinase which is associated with regulation of biofilm formation and motility (Chen et al., 2017). It consists of a histidine residue containing domain and a CheA/Y like domain (Chen et al., 2017). The gene encoding for A1S_2811 is part of a five gene operon comprising of the *pilG, pilH, pilI* and *pilJ* genes (De Silva & Kumar, 2019). Deletion of the *A1S_2811* gene resulted in significant reduction in motility and biofilm formation in *A. baumannii* (De Silva & Kumar, 2019). This deletion also resulted in downregulation of the *abaI* gene, which encodes for N-acylhomoserine lactone synthase. *abaI* is also associated with quorum sensing (De Silva & Kumar, 2019). The biofilm formation and motility were revived when this deletion mutant was grown in synthetic homoserine lactone, therefore, confirming that it regulates the *abaI* expression (De Silva & Kumar, 2019).

(vii) *AvnR*

AvnR is an orphan response regulator recently characterized in *A. baumannii* ATCC17978 (De Silva et al., 2020). The sensor kinase responsible for activation of this RR is currently unknown. It has been characterized as a CheY like protein (De Silva et al., 2020). Preliminary studies showed that deletion of *avnR* in ATCC17978 resulted in a significant reduction in motility and biofilm formation (De Silva et al., 2020). Further, deletion of AvnR also resulted in decreased virulence in *Galleria mellonella* infection model (De Silva et al., 2020). However, this deletion resulted in increased cell adhesion on A549 lung epithelial cells. Complementation of *avnR* reversed all observed phenotypic changes. Apart from virulence, AvnR was also found to regulate nitrogen metabolism, since disruption of AvnR resulted in significant changes in the ability of this mutant to utilize different nitrogen sources(De Silva et al., 2020).

1.5. TCS as potential targets for novel therapeutics.

The rapid emergence of *A. baumannii* strains with the ability to tolerate high concentrations of clinically relevant antibiotics has resulted in a fast decline in treatment options for infections by such strains (Bhagirath et al., 2019). The emergence of pan-drug resistant (PDR) *A. baumannii* strains, with the ability to tolerate last resort antibiotics such as carbapenem, colistin and tigecycline has resulted in an urgent demand to development of novel antibiotic molecules (Bhagirath et al., 2019). However, due to a slow progress in development of new therapeutic options and no new antibiotics in the developmental pipeline, alternate treatment strategies for treatment of MDR *A. baumannii* are required. Due to their involvement in regulating important pathogenic phenotypes in this bacterium, TCS have been regarded as potent candidate for the development of novel therapeutics to tackle MDR infections (De Silva & Kumar, 2019). Further, TCS are conserved in bacterial cells and absent in mammalian cells, making it an ideal target for designing novel antimicrobials (Bhagirath et al., 2019)(De Silva & Kumar, 2019). It is, therefore, essential to have a better understanding of the characteristics of these systems.

1.6. Hypothesis

The working hypothesis of this project is that Two Component Systems regulate antibiotic susceptibility and virulence phenotypes in *A. baumannii* in response to environmental signals.

Therefore, the objectives of this project are:

1) Characterization of the orphan response regulator AvnR in the clinical isolate *Acinetobacter baumannii* AB030

2) Characterization of the putative two component system A1S_1977 and A1S_1978 in *Acinetobacter baumannii* ATCC 17978.

Chapter 2: Characterization of the Response Regulator, AvnR, in a clinical isolate *Acinetobacter baumannii* AB030

2.1. Introduction

Acinetobacter baumannii is notorious for causing hospital-acquired infections in immunocompromised patients (Peleg et al., 2008). Due to the plasticity of its genome, *A. baumannii* has an impressive ability to acquire antibiotic resistance and virulence phenotypes through intrinsic mechanisms and horizontal gene transfer (Imperi et al., 2011). Due to this, there has been an increased occurrence of Multidrug Resistant (MDR) strains of *A. baumannii* globally (Vrancianu et al., 2020). These strains have been found to resist a broad spectrum of clinically used antibiotics (Vrancianu et al., 2020). The emergence of Extremely Drug Resistant (XDR) and Pan Drug Resistant (PDR) strains of *A. baumannii*, which can resist last resort antibiotics such as carbapenems and colistin, has posed a serious problem in modern healthcare (Thomas et al., 2019). A rapid depletion of treatment options, slow progress in development of novel antibiotics, and the lack of new antibiotics in the developmental pipeline has resulted in increased morbidity and mortality by such strains globally (Thomas et al., 2019).

Apart from its ability to resist antibiotics, *A. baumannii* has an impressive ability to survive in harsh environmental conditions such as unfavorable temperatures, oxidative stress, nutrient scarcity, desiccation, and osmolarity (Sarshar et al., 2021). A major attribute enabling *A. baumannii* to thrive in such conditions is its ability to sense and adapt to environmental stress. Two Component Systems (TCS) are signal transduction systems ubiquitous in bacteria, which enable them to sense and adapt to changes in environmental conditions (De Silva & Kumar, 2019). They comprise of two parts, namely a membrane bound sensory Histidine Kinase (HK) and a cytosolic Response Regulator (RR) (Nixon et al, 1986). Generally, the HK sense stress due to change in environmental conditions and generate ATP based information at its conserved histidine residue (Szurmant et al., 2007). This information is phosphorylated to the RR, where it is received at its conserved aspartate residue (Szurmant et al., 2007). The activated RR, on receiving the phosphoryl group, bind to the necessary genes, generating adaptive phenotypic responses to these stimuli (De Silva & Kumar, 2019).

AvnR (*Acinetobacter* virulence and nitrogen utilization Regulator) is a RR, first characterized in *A. baumannii* ATCC 17978 (De Silva et al., 2020). The gene encoding this protein does not have a HK encoding gene in its vicinity, and the HK with which AvnR interact with is currently not known (De Silva et al., 2020). It has, therefore been described as an orphan response regulator. It

is predicted to be a 22kDa protein encoded by a 591 bp open reading frame and is well conserved in *A. baumannii* species (De Silva et al., 2020). Preliminary studies on this RR have described it as a CheY like regulator comprising of a RNA binding ANTAR (AmiR and NasR transcription anti-termination regulators) domain (De Silva et al., 2020). It has been associated with regulation of various virulence phenotypes and nitrogen metabolism in *A. baumannii* ATCC 17978. Deletion of *avnR* in a lab strain ATCC 17978 resulted in significant decrease in motility, biofilm formation and virulence in *Galleria mellonella* infection model (De Silva et al., 2020). The complementation of this gene resulted in reversion to the wild-type phenotype. Further, deletion of this gene also severely affected the expression of various genes associated with nitrogen metabolism (De Silva et al., 2020). These observations have demonstrated a link between AvnR and expression of these phenotypes. However, since these studies were conducted in a lab strain of *A. baumannii,* the clinical relevance of these findings is yet to be determined.

In this study, the role of AvnR was further investigated in a clinical isolate *A. baumannii* AB030. This is an XDR strain of *A. baumannii* which was isolated from a hospital in Canada (Loewen et al., 2014). It can resist virtually all clinically used antibiotics except colistin (Loewen et al., 2014). It has also been characterized to be a hypervirulent strain (Singh et al., 2020). Previous analysis of the genome of AB030 had revealed that the *avnR* gene in this strain is naturally disrupted by a 3902bp transposon insertion, rendering it non-functional (Alsaadi, 2014). Comparative phenotypic analyses of AB030 using ATCC 17978 as reference strain had revealed AB030 to be non-motile (Singh et al., 2020). Further, AB030 also produced significantly less biofilm compared to ATCC 17978 (Singh et al., 2020). Since AvnR has previously been demonstrated to play a role in regulation of these phenotypes, we were curious if the observed difference in these phenotypes were attributed to the disruption of *avnR* in AB030. We, therefore, investigated the effect of AvnR expression in the regulatory network of virulence associated phenotypes and nitrogen metabolism in AB030.

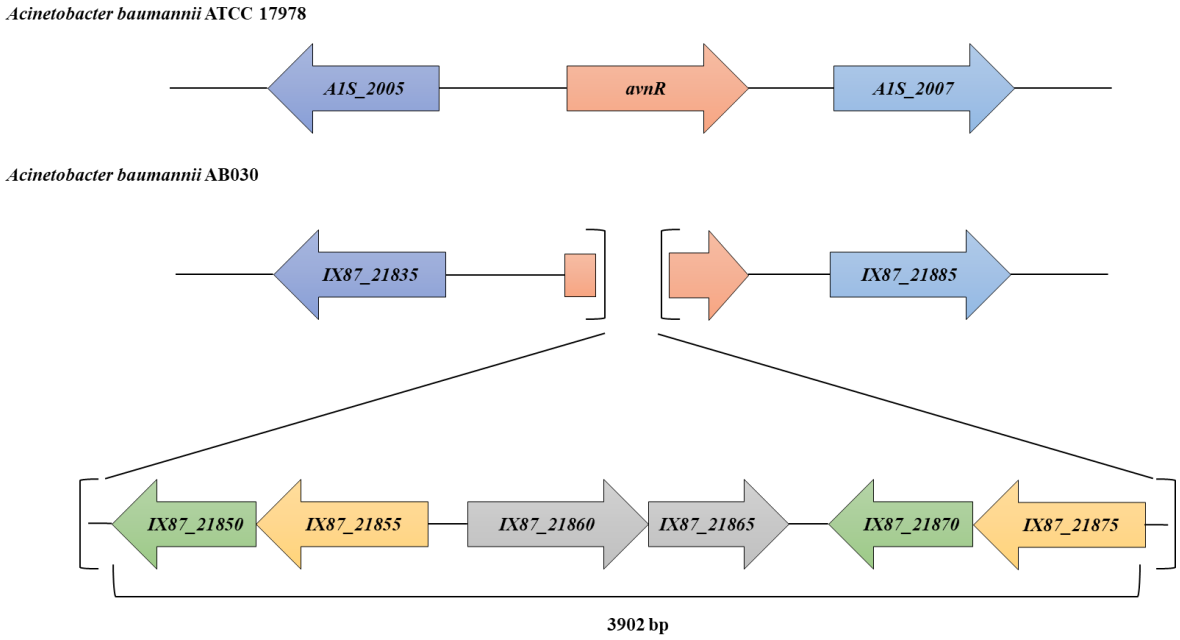


Figure 2.1. Schematic representation of the genetic organization of *avnR* in *A. baumannii* ATCC 17978 and AB030. *avnR* in AB030 is disrupted by a 3902bp insertion which renders it non-functional. The insertion is comprised of four transposase encoding genes, *IX87_21850*, *IX87_2885*, *IX87_21870* and *IX87_21875*, flanking the genes *IX87_21860*, and *IX87_21865*. *IX87_21860* has been annotated as a β-lactamase encoding gene and *IX87_21865* is annotated a hypothetical protein (GenBank: CP009257.1).

2.2. Materials and Methods

2.2.1. Bacterial strains, plasmids, and oligonucleotides

All bacterial strains used in this study have been listed in Table 1. All plasmids used in this study have been listed in Table 2. All oligonucleotides used in this study have been listed in Table 3.

2.2.2. Preparation of Experimental strains

(i) *Cloning of avnR in a single copy complementation plasmid vector*

AvnR was chromosomally complemented in *A. baumannii* AB030 using the single copy complementation vector pUC18T-mini-Tn7T-Apr (Ducas-Mowchun et al., 2019). Briefly, DH5α/pPLS256 and DH5α/pPLS294 were grown in 3mL LB broth supplemented with appropriate antibiotics. DH5α/pPLS256 was selected in 100µg/mL ampicillin and DH5α/pPLS294 was selected in 30µg/mL apramycin. Plasmids were extracted using BioBasic Plasmid Miniprep Kit (BioBasic Inc. Markham, ON, Canada) following manufacturer guidelines. The *avnR* gene along with its native promoter, sourced from ATCC 17978, was digested from pUC18T-mini-Tn7T-Gm:*avnR* (pPLS256) using the restriction enzymes, BamHI-HF and HindIII-HF (New England Biolabs, Ontario, Canada). The *avnR* gene fragment was gel purified using E.Z.N.A.® gel extraction kit (Omega Bio-tek, GA, USA) following manufacturer guidelines. The purified *avnR* fragment was then ligated into pUC18T-mini-Tn7T-Apr using T4 DNA ligase (ThermoFisher Scientific) and transformed in *E. coli* DH5α competent cells. The resulting recombinant plasmid was named pPLS393. Ligation was carried out following manufacturer guidelines and the reaction mixture was incubated at 16^{o}C for 16-18 hours.

Table 2.1. Bacterial strains and plasmids used in this study

Strain	Characteristics	Source
Acinetobacter baumannii		
ATCC 17978	Lab strain	ATCC
AB104	ATCC 17978:Δ*avnR*	(De Silva et al., 2020)
AB030	Wild Type	(Singh et al., 2020)
AB408	AB030:mini-Tn7T:*avnR*	This study
AB409	AB030:mini-Tn7T (Empty vector)	This study
Escherichia coli		
DH5 α	F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	Invitrogen

Table 2.2. List of plasmids used in this study (All plasmids were transformed in *E. coli* DH5α)

***E.coli* strain/Plasmids**	**Characteristics**	**Source**
DH5α/pPLS256	pUC18T-mini-Tn7T-Gm:*avnR*, Gentamicin resistance	(De Silva et al., 2020)
DH5α/pPLS294	pUC18T-mini-Tn7T-Apr, Apramycin resistance	(Ducas-Mowchun et al., 2019)
DH5α/pPLS393	pUC18T-mini-Tn7T-Apr:*avnR*, Apramycin resistance	This study
DH5α/pTNS2	*tnsABCD.* Helper plasmid for Tn7 transposon integration into *attn7* insertion site. Ampicillin resistance.	(Choi et al., 2005)
HB101/pRK2013	Helper plasmid for conjugation. Kanamycin resistance	(Figurski & Helinski, 1979)
DH5α/pFLPZ	*flp* recombinase. Zeocin resistance	(Ducas-Mowchun et al., 2019)

Table 2.3. List of oligonucleotides used in this study

Primer name	Sequence	Source
A1S_2006 Full F	CGCGTCGAATTGGCGCATTT	(De Silva et al., 2020)
A1S_2006 Full R	GCCGAGTTGCAATTGAGTC	(De Silva et al., 2020)
Ab glmS F	TATGGAAGAAGTTCAGGCTC	(Choi et al., 2005)
Tn7R	CACAGCATAACTGGACTGATTTC	(Choi et al., 2005)
16S qRT F	CTTCGGACCTTGCGCTAATA	Kumar Lab
16S qRT R	ATCCTCTCAGACCCGCTACA	Kumar Lab
A1S_2006 qRT F	GCCCGCTTACATACCATTTT	(De Silva et al., 2020)
A1S_2006 qRT R	TGCCAGTTTGGTTTGAGCTT	(De Silva et al., 2020)

(ii) *Complementation of avnR in A. baumannii AB030*

Complementation of *avnR* in AB030 was done using four-parental conjugation method (Ducas-Mowchun et al., 2019). Briefly, *A. baumannii* AB030 (recipient strain), DH5α/pPLS393 (pUC18T-mini-Tn7T-Apr:*avnR*), HB101/pRK2013 (conjugation helper plasmid) and DH5α/pTNS2 (helper plasmid for Tn7 insertion) were inoculated in 3mL of LB broth supplemented with appropriate antibiotics (Table2.2), and incubated at 37ºC and shaking at 250rpm for 16-18 hours. DH5α/pPLS393 was selected in LB media supplemented with 30µg/mL of apramycin. DH5α/pTNS2 was selected in LB media supplemented with 100µg/mL of ampicillin and pRK2013 was selected in 35µg/mL of kanamycin. 100µL of these cultures were aliquoted into a sterile 1.5mL centrifuge tube and centrifuged at 12,000 x g for 2 minutes. The supernatant was carefully removed using a micropipette, and the pellet was resuspended in 1mL LB and washed three times. The pellet was resuspended in 10µL of LB and spotted on a sterile nitrocellulose paper placed inside an LB agar plate and incubated at 30ºC for 24 hours. The nitrocellulose paper with the conjugation mix was then transferred into a sterile centrifuge tube and the bacterial cells were resuspended in 0.85% saline. 400µL of this mixture was plated on Simmon Citrate agar media supplemented with 100µg/mL apramycin and incubated at 37ºC for 48 hours. The marked mutants obtained from this was electroporated with pFLPZ (*flp* recombinase plasmid) to remove the apramycin marker and was selected in LB media supplemented with 1500µg/mL zeocin. This was then counter selected on LB plates supplemented with 10% sucrose to obtain an unmarked complement strain. An empty vector strain was created along with the complemented strain to be used as a control.

2.2.3. Gene expression analysis using RT-qPCR assay.

The expression of *avnR* in the complement strain AB408 (*A. baumannii* AB030:mini-Tn7T:*avnR*) was screened using RT-qPCR.

(i) *RNA extraction, purification, and cDNA synthesis*

Briefly, the experimental strains ATCC 17978, AB104 (ATCC 17978:Δ*avnR*), AB030 and AB408 (AB030:mini-Tn7T:*avnR*) were inoculated in 3mL LB broth and incubated at 37ºC and shaking at 250rpm for 16 – 18 hours. Following this, the strains were sub-cultured in 1:100 ratio in fresh LB broth and incubated at 37ºC and shaking at 250rpm until mid-log phase (A_{600} value between 0.5 – 0.8). 1mL of each culture was transferred into sterile 1.5mL microcentrifuge tubes and centrifuged at 12,000 X g for 2 minutes. Using a micropipette, the supernatant was carefully removed from each microcentrifuge tube without dislodging the pellets and these tubes were kept at -72ºC for 18 hours. RNA extraction was performed using RNeasy mini kit (Life Technologies) and following manufacturer instructions. Briefly, the bacterial cell pellets, that were frozen overnight, were resuspended in 100µL of 1mg/mL lysozyme. 600µL of lysis buffer and 3.5µL of β-mercaptoethanol was added to the mixture, followed by 700µL of 95% ethanol. The tubes containing these mixtures were mixed thoroughly by vortex. The mixtures were then transferred into spin cartridges provided in the kit and centrifuged at 12,000 X g for 15 seconds. Following this the spin cartridges were washed using the wash buffer provided in the kit and dried by centrifugation of the cartridges at 12,000 X g for 15 seconds. Elution of RNA was done using 60µL of RNAse free water and centrifugation at 12000 X g for 2 minutes.

Following elution, the concentration of the RNA extracts was determined using a NanoDrop lite spectrophotometer (Thermo Scientific). 1000ng of the RNA was treated with 1µL of (2.73U/µL) DNAseI (Qiagen) to remove traces of carryover DNA and obtain purified RNA, to be used as template for cDNA synthesis. Reverse transcription of the RNA templates was carried out using SuperScript VILO cDNA synthesis kit (Invitrogen) and following manufacturer guidelines.

(ii) *RT-qPCR assay*

Expression analysis of *avnR* in AB408 (AB030:mini-Tn7T:*avnR*) was performed using SYBR select Master mix (Life technologies) following manufacturer protocol. Briefly, using sterile MilliQ water, cDNA templates of strains ATCC 17978, AB104, AB030 and AB408 were diluted to 1:5 ratio. A master mix comprising of SYBR select and 100nM of forward primer (A1S_2006 qRT F) and reverse primer (A1S_2006 qRT R) (Table 2.2) was prepared. All primers were

standardized to confirm 95-100% efficiency by obtaining standard curves using 10-fold dilutions of gDNA from ATCC 17978. Reaction mixtures were setup at final volume of 8µL per reaction, each rection containing 2.3µL of cDNA template and 5.3µL of master mix. A No Reverse Transcriptase Control (NRT) was prepared by mixing 2µL of DNAse treated RNA templates of all test strains and adjusting final volume with MilliQ water to obtain 1:5 dilution. A No Template Control (NTC) was setup, containing only mastermix. Final reaction mixtures for NRT and NTC was setup identical to the test samples, where 2.3µL of diluted RNA was added to 5.3µL of master mix for the NRT and equivalent amount of MilliQ water was added for NTC. All reactions were carried out in a StepOnePlus (Applied Biosystems) machine with the following reaction conditions: Initial denaturation at 95ºC for 2 minutes, 40 cycles comprising of 95ºC for 15 seconds and by 60ºC for 1 minutes per cycle. Product efficiency was determined by obtaining melt curves with the following reaction conditions: 95ºC for 10 seconds, 60ºC for 5 seconds and 95ºC for 10 seconds. Relative fold change for gene expression was calculated by Pfaffl's method using 16S rRNA as internal reference. Statistical analysis was performed using Microsoft Excel. Graphs were plotted using GraphPad Prism v7.0. P values were determined using 1 way ANOVA and Turkey's multi-comparison analysis in Graphpad Prism v7.0. The experiment was performed with three independent bioreplicates, and three technical replicate per strains.

2.2.4. Surface associated motility assay

Surface associated motility assay was conducted using a method previously described by (Harding et al., 2013) with some modifications. The strains ATCC 17978 (reference control), AB030 (wild-type strain), AB408 (AB030:mini-Tn7T:*avnR*) and AB409 (empty vector control) were grown in LB broth at 37ºC and 250rpm shaking for 16-18 hours. A_{600nm} values of each strain were recorded using a spectrophotometer (Thermo Scientific) and the OD was adjusted to A_{600} of 1. 3µl of culture was inoculated in 0.3% agarose plates by stabbing halfway at the center of the plates using the micropipette tip and slowly dispensing the culture into the resulting well. The plates were incubated at 37ºC for 16-18 hours. The diameter of the displaced bacterial cultures was measured. 0.3% agarose plates were prepared using the following recipe: 5g/L of tryptone, 2.5g/L of sodium chloride and 3g/L of agarose in 1L of MilliQ water. The experiment

was conducted in at least 3 independent bio-replicates with 3 technical replicates per bio-replicates.

2.2.5. Biofilm assay

Biofilm assays were conducted using a previously described method (Iwashkiw et al., 2012) with the following modifications. Briefly, cultures of ATCC 17978 (reference control), AB030 (wild type strain), AB408 (AB030:mini-Tn7T:*avnR*) and AB409 (empty vector control) were grown in LB broth at 37^{o}C and shaking at 250rpm for 16-18 hours. The cultures were centrifuged at 12000 X g for 1 minute in a 1.5mL microcentrifuge tube. The supernatant was carefully discarded by pipetting and the pellet was resuspended in 50% (w/v) Muller Hilton Media (Oxoid, Basingstoke, Hants, UK). The cells were washed three times in 50% MHB by centrifugation at 12,000 X g for 2 minutes. The cultures were diluted to 10% (v/v) in 50% MHB and 100 µL of these cultures were aliquoted in a flat clear-bottomed 96-well plate and incubated at a static temperature of 37^{o}C for 48 hours. Following incubation, A_{600} values were recorded using a Spectramax M2 plate reader. (Molecular Devices). The loose planktonic cells and broth were carefully removed by pipetting and the wells were carefully washed with sterile distilled water. Once the wells were dried, each well was stained with 150µL of 0.1% (w/v) Crystal violet solution and washed carefully to remove excess stain using distilled water. 200µL of 30% acetic acid was added to each well and incubated for 30 minutes. 100µl of the solution was transferred to a fresh 96 well clear flat-bottomed plate and OD550 values were recorded using Spectramax M2 plate reader (Molecular Devices). OD550/OD600 values were calculated. Statistical analysis was performed using Microsoft excel and Graphpad Prism 7.0 with 2-way ANOVA (Turkey multi analysis). The experiment was conducted in 3 bioreplicates, with 10 technical replicates for each experimental strains.

2.2.6. BIOLOG™ Microplate Assay

BIOLOG™ PM3B Microplate™ and Inoculation Fluid (IF) was purchased from Biolog Inc, Hayward, CA, USA. Assays were conducted as per manufacturer instructions with minor

modifications. Experimental strains AB030 and AB408 were grown in LB broth at 37^{o}C and 250rpm in a shaker incubator for 16-18 hours. The cultures were transferred into sterile 1.5mL microcentrifuge tubes and centrifuged at 13000rpm for 2 minutes. The supernatant was discarded carefully by pipetting without dislodging the pellet. The pellet was resuspended in IF and centrifuged at 13000rpm for 2 minutes. The cells were washed two times in IF and suspended in 1mL IF. Using a Densicheck^{TM} (Biomérieux, Montréal, Canada), cells were standardized to 0.5 MacFarland. The standardized cells were diluted to 1:100(v/v) ratio in IF. 100µL of this inoculum was transferred to each well in a BIOLOG^{TM} PM3B plate. Growth curves were obtained using Spectramax M2 plate reader. Absorbance readings were obtained for 24 hours with 1-hour intervals per reading. Readings were taken at OD of 595nm. Raw data was generated in Microsoft Excel, graphs were generated and analyzed using dPhenome module of DuctApe following the manual provided in the program. (Galardini et al., 2014)(https://combogenomics.github.io/DuctApe/howto.htmL#dphenome). The experiment was conducted in two independent bioreplicates.

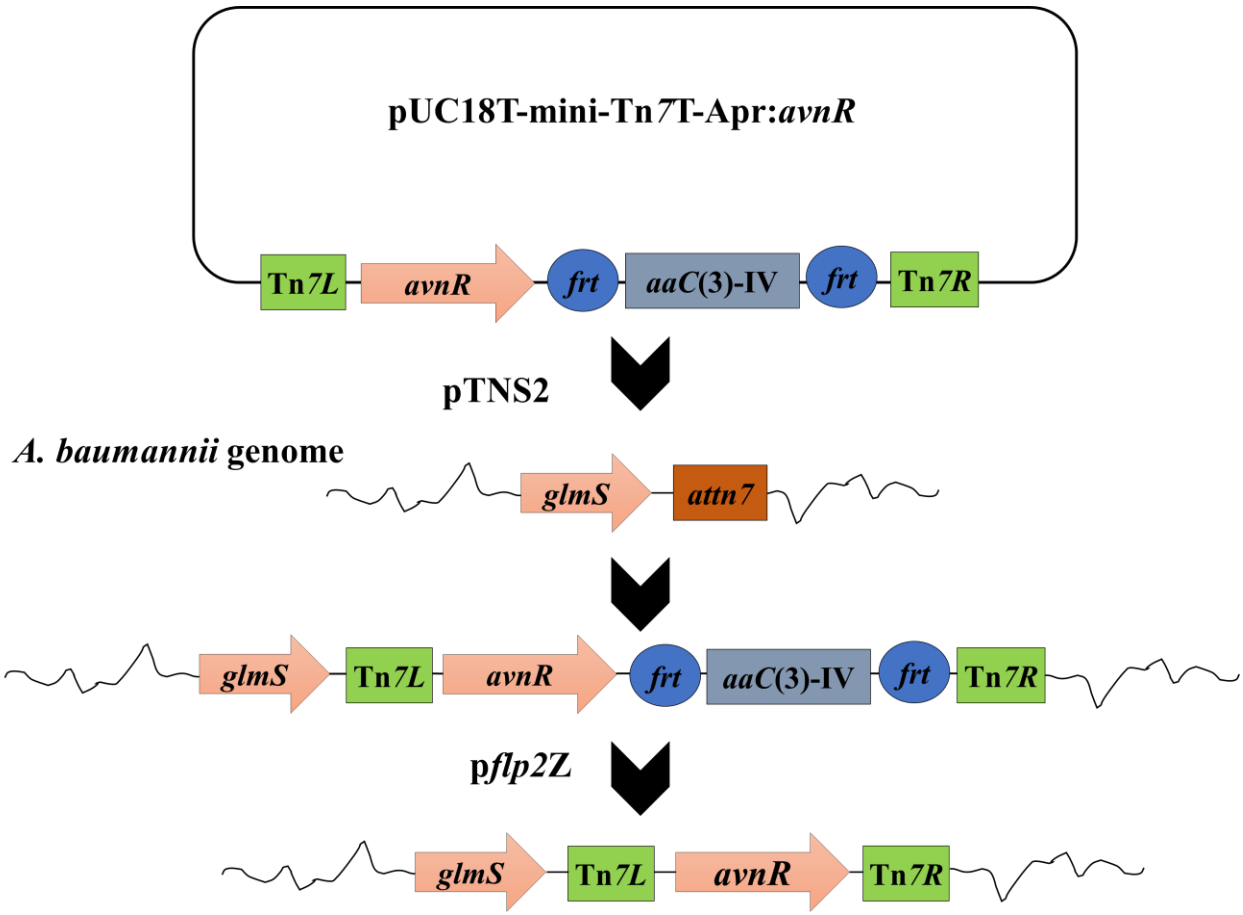


Figure 2.2. Creation of complement strain AB030:mini-Tn7T:*avnR* (AB408) using single copy complementation vector. pUC18T-mini-Tn7T-Apr was used for single copy complementation of *avnR* in strain AB030. It comprises of a Tn7 transposon sequence flanked upstream and downstream of the multiple cloning site, and an apramycin resistance cassette flanked with frt sites. The genome of *A. baumannii* contains a single Tn7 insertion site, *attn7*. The plasmid vector cloned with *avnR* gene was introduced into the recipient cell along with a helper plasmid pTNS2 resulting in formation of marked mutants of avnR complement in AB030. A pFLP2Z plasmid was then introduced into the marked mutant which removed the apramycin marker, resulting in formation of an unmarked complement.

2.3. Results

2.3.1. Expression analysis using RT-qPCR

Expression analysis using RT-qPCR was conducted for Wild type strain AB030 and complement strain AB408 (AB030:mini-Tn7T:*avnR*). The lab strain ATCC 17978 was used as reference control and AB104 (ATCC 17978:Δ*avnR*) was used as a negative control. Strain AB408 showed significantly higher expression of *avnR* gene compared to the reference control, whereas no Ct values were obtained from AB030 for *avnR* expression (Figure 2.2).

2.3.2. Motility Assay

Surface associated motility assay was conducted using lab strain ATCC 17978 as reference control. AB030 was used as wild-type control, and AB409 was used as empty vector control. We found that AB408 did not have any observable change in motility compared to wildtype AB030 and empty vector control. This indicates that complementation of *avnR* in AB030 did not have any effect on the motility in AB408 despite higher expression of *avnR* (Figure 2.3).

2.3.3. Biofilm assay

Biofilm assays were conducted using lab strain ATCC 17978 as reference control, AB030 as wild type and AB409 as an empty vector control. Experiments were conducted in 3 biological replicates with 10 technical replicates for each strain. Statistical analysis showed that there was a significant increase in biofilm formation in AB408 compared to AB030 and AB409(Figure 2.4). However, a considerable variation was observed for AB408, therefore, making our observation inconclusive. Further trials, therefore, need to be conducted.

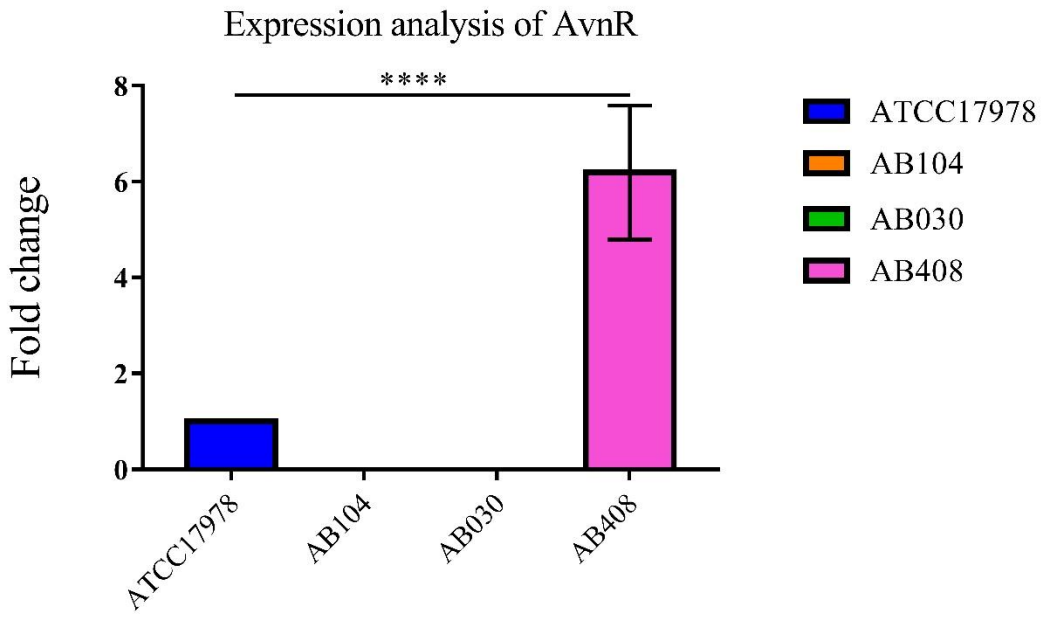
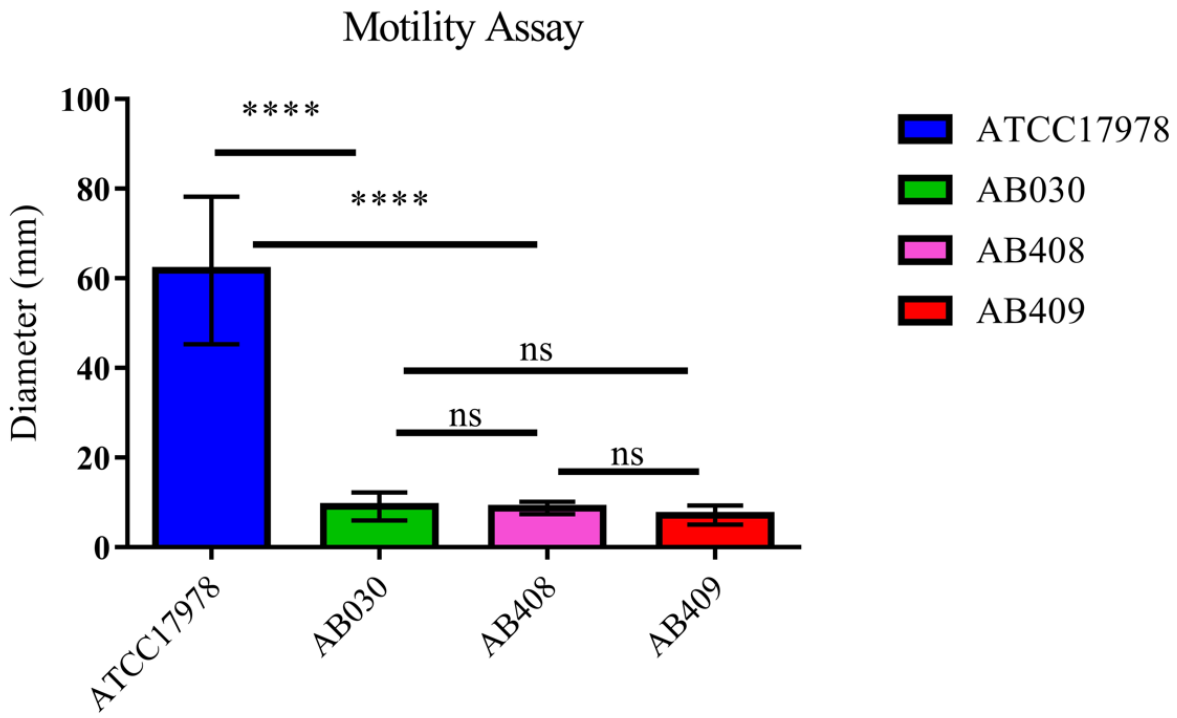


Figure 2.3. Expression analysis using RT-qPCR: The lab strain ATCC 17978 has been used as a reference control. *avnR* deletion mutant, AB104 (ATCC 17978:Δ*avnR*), has been used as negative control. Strain AB030 is the wild type and AB408 (AB030:mini-Tn7T:*avnR*) is the AB030 complemented with *avnR.* AB408 showed significantly higher expression of *avnR* compared to ATCC 17978. Deletion mutant, AB104 and wild type strain, AB030 did not express *avnR*, as expected.

(A)



(B)

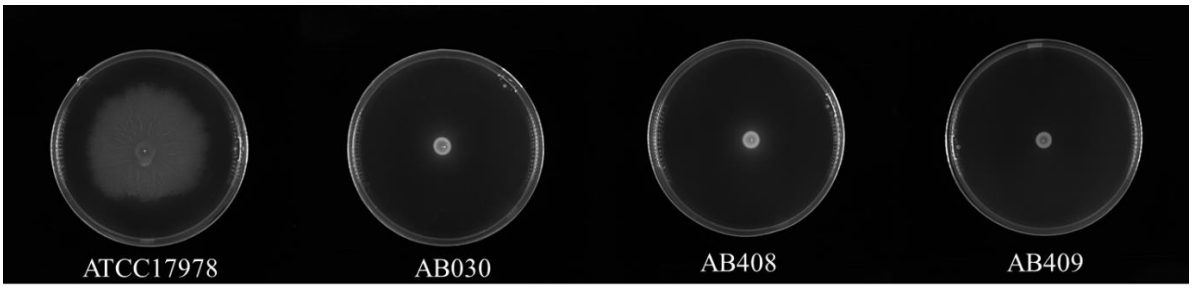


Figure 2.4. Motility Assay: (A) Surface associated motility assay was conducted using 0.3% Agarose plates. Plates were incubated at 37ºC for 16-18 hours. No significant difference in motility was observed between AB030, AB408 and AB409. (B) Representative images of motility plates of reference control - ATCC 17978, wild type – AB030, experimental strain – AB408 and empty vector control – AB409.

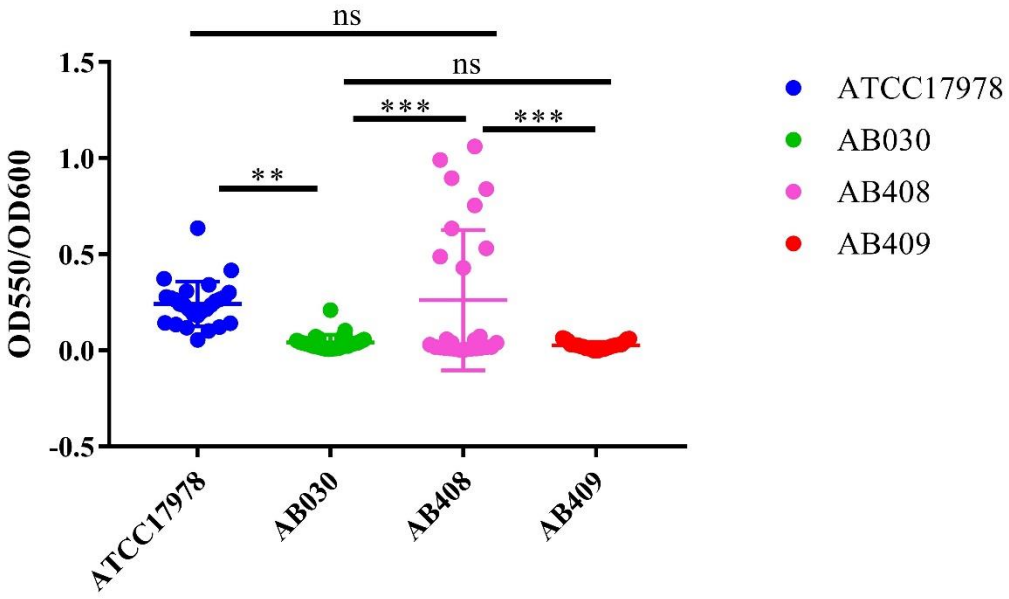
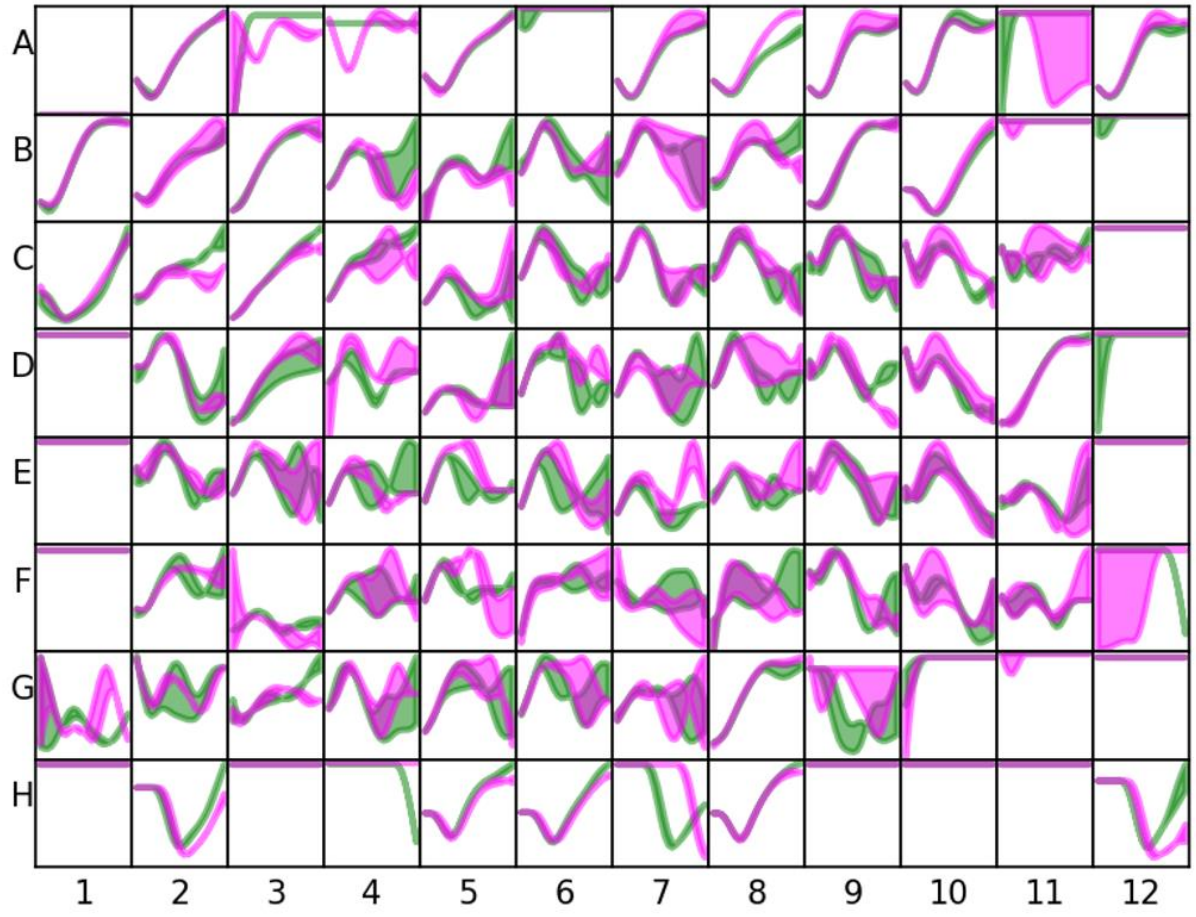


Figure 2.5. Biofilm assay: Lab strain ATCC 17978 was used as reference control, AB030 is the Wild type control, AB409 (AB030:mini-Tn7T) was used as empty vector control. AB408 (AB030:mini-Tn7T:*avnR*) is the experimental strain. AB408 showed a significant increase in biofilm formation compared to AB030 and AB408. ATCC 17978 exhibited higher biofilm formation compared to AB030 and AB409. No significant difference in biofilm formation was seen between AB030 and AB409. No significant difference in biofilm formation was between ATCC 17978 and AB408.

2.3.4. BIOLOG™ plate assay

BIOLOG™ microplate assays revealed an increased growth in AB408 in presence of L-Arginine and L-serine compared to Wild type strain AB030. In contrast, AB408 showed decreased growth in the presence of L-Valine. Further, decreased growth was also observed in wells containing Alanine-Histidine, and Alanine-Leucine combinations. No significant difference in growth rate was observed when L-Histidine and L-Leucine was used as sole nitrogen sources. Also, no significant changes in growth were observed in presence of D-Alanine and L-Alanine as sole nitrogen sources. Further investigation is required to understand the reason behind this observation.

A) **BIOLOG™ PM3B Microplate**

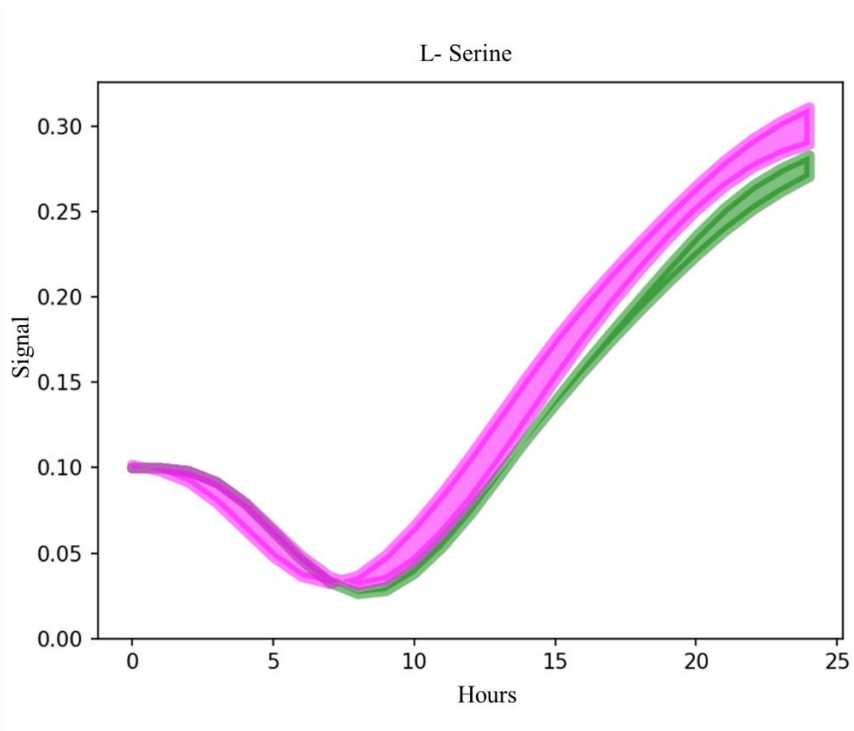


(B)

A1 Negative control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic acid	A11 L-Cysteine	A12 L-Glutamic acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D- Alanine	C4 D-Asparagine	C5 D-Aspartic acid	C6 D-Glutamic acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D1 N-Acetyl-L-Glutamic acid	D2 N-Phthaloyl-L-Glutamic acid	D3 L-Pyroglutamic acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 B-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric acid	G4 Alloxan	G5 Allantoin	G6 Parabanic acid	G7 D,L-α-Amino-N-Butyric acid	G8 γ-Amino-N-Butyric acid	G9 ε-Amino-N-Caproic acid	G10 D,L-α-Amino-Caprylic acid	G11 δ-Amino-N-Valeric acid	G12 α-Amino-N-Valeric acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

Figure 2.6. Growth kinetics assay using BIOLOG™ PM3B Microplate™. (A) Growth curves of AB030 (Green) and AB408 (Magenta) in different nitrogen sources. (B) Template of a BIOLOG™ PM3B Microplate™ Nitrogen sources plate. Normalization of data and generation of graph was done using DuctApe (Galardini et al., 2014). Raw data is presented in Appendix II.

(A)


L- Serine
Signal
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
5
10
15
20
25
Hours

(B)

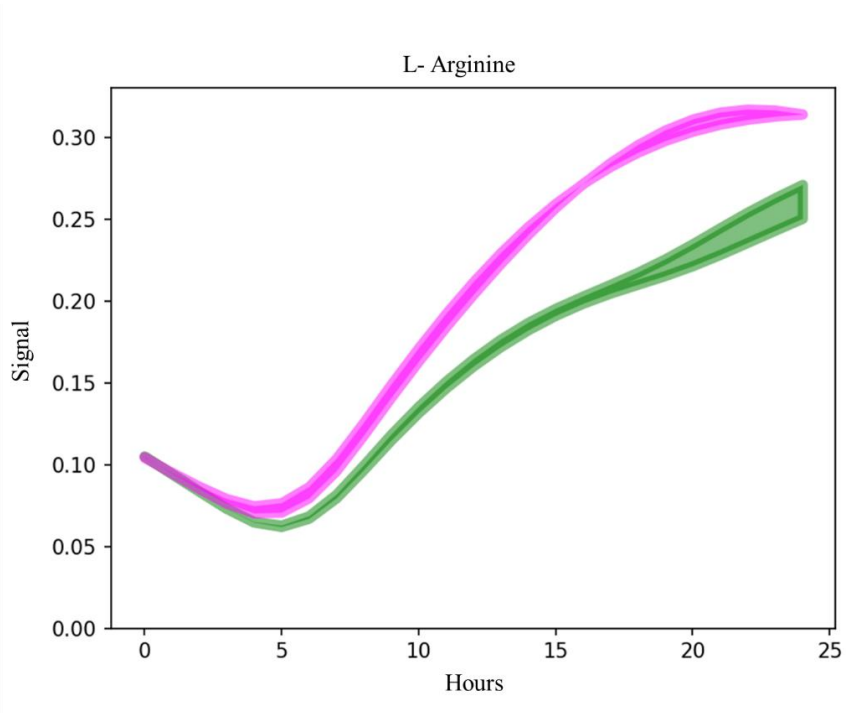
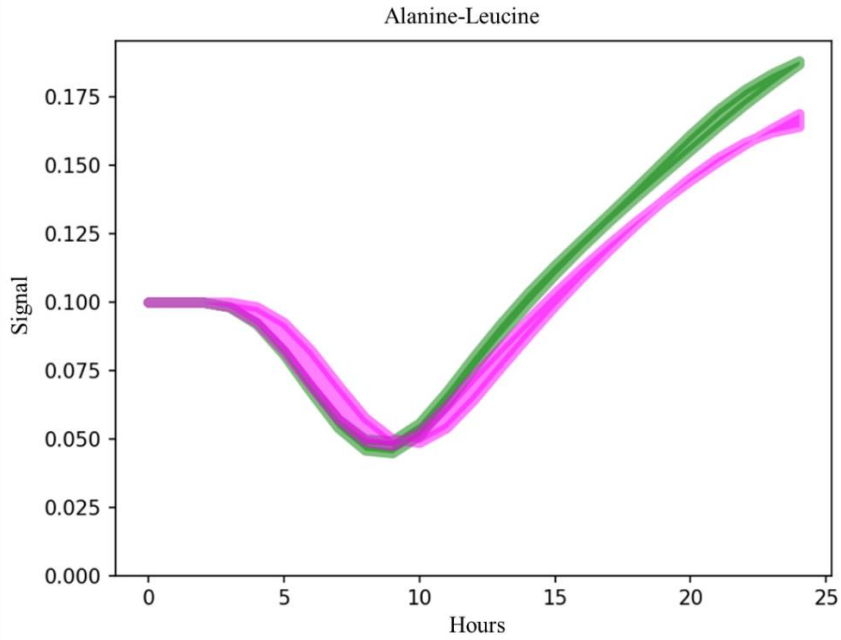
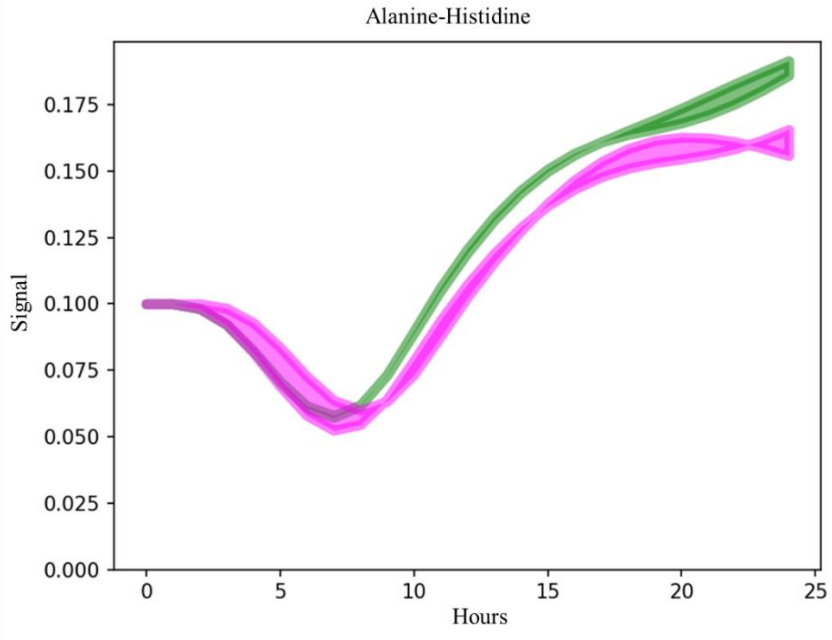

L- Arginine
Signal
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0
5
10
15
20
25
Hours

Figure 2.7. Growth kinetics assay using BIOLOG™ PM3B Nitrogen sources. The following graphs represent growth curve assays where expression of *avnR* resulted in increase in growth rate in the experimental mutant AB408 compared to wild type strain AB030. (A) Growth curve of AB030 and AB408 with L-serine as sole nitrogen source. (B) Growth curve of AB030 and AB408 with L-Arginine as sole nitrogen source. AB030 has been plotted in GREEN and AB408 has been plotted in MAGENTA.

(A)


Alanine-Leucine
Signal
0.000
0.025
0.050
0.075
0.100
0.125
0.150
0.175
0
5
10
15
20
25
Hours

(B)


Alanine-Histidine
Signal
0.000
0.025
0.050
0.075
0.100
0.125
0.150
0.175
0
5
10
15
20
25
Hours

(C)

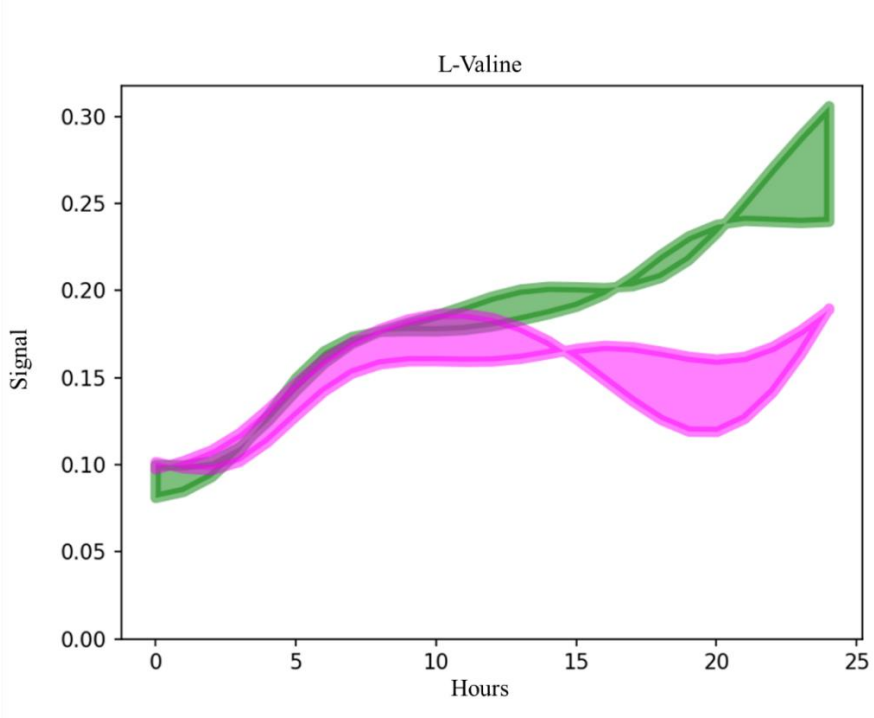


Figure 2.8. Growth kinetics assay using BIOLOG™ PM3B Nitrogen sources. The following graphs present growth represent growth curve assays where expression of *avnR* resulted in decrease in growth rate in the experimental mutant AB408 compared to Wild type strain AB030. (A) Growth curve of AB030 and AB408 with Alanine-Leucine combination as nitrogen source. (B) Growth curve of AB030 and AB408 with Alanine-Histidine as sole nitrogen source. (C) Growth curve of AB030 and AB408 with L-Valine as sole nitrogen source. AB030 has been plotted in GREEN and AB408 has been plotted in MAGENTA.

2.4. Discussion

A. baumannii has been regarded as a serious threat due to its ability to resist high concentrations of various antibiotics (Vrancianu et al., 2020). Extensive research has been conducted to study various antibiotic resistance phenotypes in this bacterium. Unlike antibiotic resistance, studies of virulence phenotypes in *A. baumannii* have been limited. Moreover, studies of the molecular mechanisms by which *A. baumannii* interact with its environment and the effect of such interactions on antibiotic resistance and virulence phenotypes has been limited (De Silva & Kumar, 2019). Two Component Systems have been found to be an essential factor responsible for sensing environmental stress and generating adaptive responses to ensure survival of the bacterium (Nixon et. al, 1986). AvnR is a recently characterized response regulator that has been associated with regulation of virulence phenotypes and nitrogen metabolism in the lab strain *A. baumannii* ATCC 17978 (De Silva et al., 2020). To determine the clinical significance of these observations, in this study, the role of AvnR was further studied in a clinical isolate of *A. baumannii.* For this, we selected *A. baumannii* AB030, an XDR strain which was isolated from a hospital in Canada (Loewen et al., 2014). Multi-sequence alignment of nucleotide sequences between AB030 and ATCC 17978 genomes on NCBI showed a percentage identity score >98%, indicating that AB030 is closely related to ATCC 17978. Previous studies on AB030 had shown that the *avnR* in this strain was naturally disrupted and non-functional (Alsaadi, 2014). Further, this strain also exhibited a lack of motility and low biofilm formation compared to reference control ATCC 17978 (Singh et al., 2020). Since, AvnR has been previously associated with regulation of these phenotypes, this strain was chosen as a candidate for our study. Previous studies have also shown that the nucleotide sequence of *avnR* in AB030 and ATCC 17978 share a percentage identity >99% (Alsaadi, 2014). We, therefore, complemented *avnR* with its native promoter sourced from ATCC 17978 in AB030. The expression of *avnR* in the resulting strain, AB408 (AB030:mini-Tn7T:*avnR*), was checked using RT-qPCR. Our results showed a significantly higher expression of *avnR* in the complement strain AB408 compared to its expression in its parent strain ATCC 17978. Statistical analysis of our biofilm assays showed significant increase in biofilm formation in AB408 compared to AB030. However, a significantly high variability in biofilm formation was observed during our trials in AB408. Further investigation is, therefore, required. Further, the higher expression of *avnR* in AB408 did not seem to have any effect on motility of AB408 since no change in motility was observed in

AB408 compared to AB030. *csuA/BABCDE* operon is known to be involved in regulation of biofilm formation and motility in *A. baumannii* (Tomaras et al., 2003). A previous study on the genome of AB030 has described that the promoter region of this operon is disrupted by a transposon, which has been cited as the reason for the non-motile nature of AB030 (Singh et al., 2020). However, RNA-seq results from previous studies on ATCC 17978 have shown that disruption of *avnR* did not affect the expression of this operon, therefore, demonstrating the involvement of AvnR in regulation of motility independent of CsuA/BABCDE (De Silva et al., 2020). In our study, however, complementation of *avnR* in AB030 did not affect motility despite high level of gene expression in AB408. This is in contradiction to the observations made in ATCC 17978. This might, however, be due to possible disruption of other pathways involved in regulating surface associated motility in AB030. It should also be noted that AB030's genome contains a number of accessory elements and is thus quite different from that of ATCC 17978 (Singh et al., 2020). Thus, the genetic elements involved in the motility and biofilm formation in AB030 can be distinct from those in involved in ATCC 17978.

The effect of AvnR expression on nitrogen metabolism of AB030 was investigated using BIOLOG^{TM} PM3B microplates. These are commercially available 96-well plates in which each well comprise of a single nitrogen source. Our studies have shown that expression of AvnR in AB408 resulted in higher growth in wells containing L- Arginine. This is consistent with previous findings where deletion of *avnR* in ATCC 17978 result in a significant decrease L-Arginine metabolism (De Silva et al., 2020). Arginine metabolism pathway has been directly associated with biofilm formation in various Gram-positive and Gram-negative pathogens including *Pseudomonas aerugionosa, Shigella flexneri, and Streptococcus mutans* (Chiang et al., 2021) (Liu et al., 2021)(He et al., 2016). L-Cysteine metabolism is another pathway that has been linked with biofilm formation. In our study, we have observed a small but reproducible increase in L-cysteine metabolism in complement strain AB408 compared to wild type AB030 (Appendix I), which is consistent with previous findings in ATCC 17978 (De Silva et al., 2020).This further supports previous observations on ATCC 17978 where AvnR is associated with biofilm formation. A small, but reproducible increase in growth was also observed in AB408 in L-serine compared to AB030. Previous studies on Uropathogenic *E. coli* (UPEC) have directly linked L-serine metabolism with colonization of urinary tract in murine infection model (Anfora et al., 2007). Further, it has been established that L-serine metabolism results in generation of acetyl

phosphate molecules which are responsible for signal transduction in *E. coli* (Klein et al., 2007). In *Pseudomonas aeruginosa* PAO1, L-serine metabolism has been linked with motility, adherence to epithelial cells, cell invasion and virulence (Yasuda et al., 2017). Disruption of the gene encoding for SerA protein resulted significant attenuation in a fly infection model, reduction of swarming and motility, bacterial adherence to Caco-2 epithelial cells, and expression of ExoS secretion system, although, deletion of *serA*, did not have any effect on twitching motility (Yasuda et al., 2017). Further, administration of L-serine on wild-type *P. aeruginosa* PAO1 resulted in significant rection in motility, virulence, and cell invasion in Caco-2 epithelial cells, therefore, demonstrating a link between L-serine biosynthesis pathway and virulence in this bacterium (Yasuda et al., 2017). In our study, nucleotide BLAST of the *serA* gene with *A. baumannii* ATCC 17978 and AB030 revealed 67.81% similarities with *A1S_3512* (in ATCC 17978) and *IX87_12540* (in AB030) respectively. Previous studies had reported that deletion of *avnR* had resulted in increase in cell adherence and reduction in L-serine metabolism in AB104 (ATCC17978:Δ*avnR*), however, no change in expression was observed for these gene when in RNA sequencing data for *avnR* mutants in ATCC 17978 (De Silva et al., 2020). L-serine metabolism has also been linked with intracellular replication in *Brucella abortis*, where disruption of necessary genes had resulted in inhibition of its proliferation inside the host cell (Révora et al., 2020). Further, disruption of L-serine biosynthesis also resulted in reduced *B. abortis* virulence in BALB/c mice infection model (Révora et al., 2020).The expression of AvnR in AB408 did not seem to have any significant effect on its growth in the presence of D-Alanine, which is contradictory to previous findings where, deletion of *avnR* in ATCC 17978 resulted in decreased growth (De Silva et al., 2020). However, the presence of Alanine seemed to result in decreased growth in wells containing Alanine-Histidine, and Alanine-Leucine, although, no significant change in growth was observed in wells containing only L-Histidine and L-Leucine respectively. D-Alanine metabolism has been linked with peptidoglycan biosynthesis in bacterial cells (Vollmer & Bertsche, 2008). Studies on *Enterococcus faecalis* has shown that disruption of alanine racemase and D-alanine-D-alanine ligase, which are essential components of this pathway, resulted in inhibition of growth of planktonic cells, biofilm formation, and cell wall damage (Jiang et al., 2021). Expression of AvnR also resulted in decreased growth of AB408 in L-Valine, which is another amino acid essential for growth and virulence in bacteria (Kaiser et al., 2016). In *A. baumannii*, valine metabolism has been linked with quorum sensing and

bacterial pathogenicity (Sun et al., 2021). A study has showed that deletion of *abaR* gene, which encode for autoinducers responsible for quorum sensing in *A. baumannii* ATCC 17978, resulted in upregulation of genes regulating valine degradation pathway (Sun et al., 2021). The Δ*abaR* mutant of ATCC 17978 also exhibited loss of surface associated motility, and biofilm formation (Sun et al., 2021). Previous studies on AvnR in ATCC 17978 have shown that deletion of *avnR* resulted in increased growth in L-valine and complementation of this gene restored this phenotype (De Silva et al., 2020). This is consistent with our results. Overall, these findings indicate that AvnR may be involved in regulation of L-valine metabolism in *A. baumannii* and may be involved in regulation of AbaI/R expression. However, further investigation is required to decipher the potential link between AvnR and AbaI/R expression.

Chapter 3: Characterization of the putative Two Component System, A1S_1977 and A1S_1978, in *Acinetobacter baumannii* ATCC 17978.

3.1. Introduction

Acinetobacter baumannii is a Gram-negative bacterial pathogen responsible for nosocomial infections including pneumonia, meningitis, urinary tract infections, and burn wound infections (Peleg et al., 2008). It is notorious for its ability to resist a broad spectrum of clinically used antibiotics through various intrinsic and acquired mechanisms (Doi et al., 2015). Consequently, treatment of infections by *A. baumannii* has become increasingly difficult (Doi et al., 2015). Due to an increase in emergence of Multidrug resistant *A. baumannii* strains, a rapid decline in treatment options, and slow progress in development of new antibiotics, the World Health Organization has categorized this bacterium as a top priority pathogen for which development of new therapeutics is urgently required (World Health Organization, 2017).

Resistance against antibiotics is mainly achieved by four mechanisms, which include, antibiotic degrading enzymes, target site modification, reduced outer membrane permeability and efflux pump activity (Kyriakidis et al., 2021). Among these, overexpression of tripartite efflux pumps belonging to the Resistance Nodulation Division (RND) family is the main contributor of multidrug resistance in *A. baumannii* (Verma et al., 2021). These are tripartite systems comprising of an Inner Membrane Transporter (IMT), a Periplasmic Adaptor Protein (PAP) and an Outer Membrane Factor (OMF) (Du et al., 2014). These proteins together form a conduit connecting the cytoplasmic region to the extracellular matrix (Du et al., 2014). Through proton motive forces, these systems extrude different classes of antibiotics, antimicrobials, dyes, and various xenobiotics from the cell body (Meehan et al., 1997). Three RND efflux pumps have been characterized in *A. baumannii*, namely, AdeABC, AdeFGH and AdeIJK (Kornelsen & Kumar, 2021).

AdeIJK is a RND efflux pump that is constitutively present in all currently known strains of *A. baumannii* (Yoon et al., 2015). It is responsible for reduced susceptibility to a wide range of antibiotics, including β-lactams, chloramphenicol, tetracycline, erythromycin, fluroquinolone and novobiocin (Damier-Piolle et al., 2008). Unlike other efflux pump-encoding operons in *A. baumannii*, *adeIJK* does not have a linked regulator (Rosenfeld et al., 2012). Rather, the expression of this efflux pump is regulated by AdeN, a regulator protein belonging to the TetR family (Rosenfeld et al., 2012). The gene encoding this regulator is located ~813kb upstream of the *adeIJK* operon (Rosenfeld et al., 2012). This regulator acts as a repressor of this pump, where

downregulation of AdeN has been found to increase *adeIJK* expression (Rosenfeld et al., 2012). Apart from AdeIJK expression, AdeN has also been associated with regulation of virulence associated phenotypes including biofilm formation and surface associated motility (Saranathan et al., 2017). A previous study has shown that disruption of *adeN* expression resulted in decreased biofilm formation and motility in *A. baumannii* (Saranathan et al., 2017). While the correlation between AdeN/AdeIJK expression and virulence associated phenotypes have been established, the molecular mechanism regulating the expression of AdeN in response to environmental stress and the environmental signals involved are yet to be determined.

Two Component Systems (TCS) are signal transduction proteins which enable bacteria to sense and respond to environmental signals (Alm et al., 2006). They are the most abundant signal transduction system in a bacterial genome and are responsible for regulating phenotypic responses to various environmental stress such as osmotic stress, unfavorable temperatures, nutrient scarcity, and oxidative stress (De Silva & Kumar, 2019)(Bhagirath et al., 2019). They also regulate antibiotic resistance and virulence phenotypes in bacteria (Bhagirath et al., 2019). Typically, a TCS comprises of two parts, which include a sensory Histidine Kinase (HK) and a Response Regulator (RR) (Nixon et al., 1986). The HK is located at the transmembrane and is responsible for sensing changes in the extra-cytoplasmic environment. Upon sensing the environmental stimulus, these proteins undergo conformational changes and transfer this information to the RR, located in the cytoplasmic region, through auto-phosphorylation. The signal is received at the conserved aspartate residue of the RR, resulting in its activation. The activated RR binds to target genes and modulate their expression (Szurmant et al., 2007).

A1S_1977 and A1S_1978 comprise a putative TCS in *A. baumannii*, where A1S_1977 has been predicted to be the sensory HK and A1S_1978 is the RR. A previous study involving comparative genotypic analyses of clinical isolates *A. baumannii* AB030 and AB031 showed significant variations in expression of *A1S_1978* gene between these strains as well ATCC 17978, which was used as a reference control (Alsaadi, 2014). *A. baumannii* AB030, an extremely drug resistant strain with high resistance to nearly all clinically used antibiotics, showed significantly lower expression of *A1S_1978* compared to drug sensitive strains AB031 and ATCC 17978 (Alsaadi, 2014). The genes encoding for A1S_1977 and A1S_1978 are directly oriented and contiguous and has been described to constitute an operon (Alsaadi, 2014).

This operon is located 276bp upstream of *adeN* and has been described to have the same orientation, therefore indicating a potential link between A1S_1977-78, AdeIJK expression and AdeN regulated virulence phenotypes (Figure3.1). This therefore suggests that this TCS may be involved in regulating antibiotic resistance and virulence in *A. baumannii.* Multi-sequence alignment of the nucleotide sequences between the ATCC 17978, AB030 and AB031 genomes showed a percentage identity >97% on NCBI, and multi-sequence alignment of the amino acid sequence for A1S_1977 and A1S _1978 between these three strains showed >99% similarity (Alsaadi, 2014)(Fernando, 2015). Therefore, in this study, we investigated the role of A1S_1977-78 in expression of AdeN associated phenotypes in *A. baumannii* ATCC 17978, and its potential link with AdeIJK mediated antibiotic resistance.

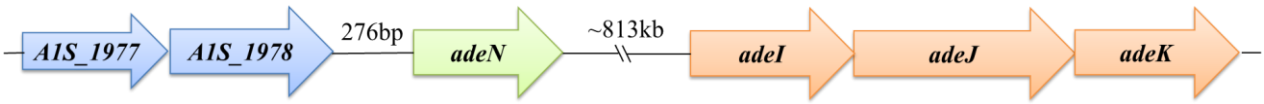


Figure 3.1. Schematic representation of the genetic arrangement of *A1S_1977-78, adeN* and *adeIJK* operon in *A. baumannii.*

3.2. Materials and Methods

3.2.1. Bacterial strains, plasmids, and oligonucleotides

All bacterial strains and plasmids used in this study have been listed in Table 3.1. All primers used have been listed in Table 3.2.

3.2.2. Creation of deletion mutant for *A1S_1977-78* in *A. baumannii* ATCC 17978

A mutant of *A. baumannii* ATCC 17978 with the *A1S_1977* and *A1S_1978* genes deleted (ATCC 17978:Δ*A1S_1977-78*) was already available and has been annotated as AB199 (Table 3.1). This strain was prepared using a previously described technique (Amin et al., 2013).

(i) *Construction of gene knockout cassette*

A knockout cassette was prepared for *A1S_1977-78* using Splice Overlap Extension (SOEing) PCR method (Choi & Schweizer, 2005). Briefly, using general PCR technique, the region ~1000bp upstream of *A1S_1977* was amplified from the genomic DNA template of ATCC 17978, using the primers: A1S_1978_DNF-GM and A1S_1978_F-SOEing. Similarly, ~1000bp downstream region of *A1S_1978* was amplified using the primers: A1S_1978_UPR-GM and A1S_1978_R-SOEing. In another PCR reaction, the gentamicin resistance marker (Gm^r) gene (*aacC1*) flanked on either side with FRT sites, was amplified using the primers, Gm_F and Gm_R. Following this, a SOEing PCR reaction was setup using the amplicons from the first three PCR reactions as templates and Taq DNA polymerase. No primers were added for this step. The reaction was run for three cycles on a BIORAD thermocycler and paused. The SOEing primers, A1S_1978_F-SOeing and A1S_1978_R-SOeing were added to this reaction mixture and further run for 30 cycles. The resulting PCR product lacking 2301bp from the operon was purified using agarose gel electrophoresis followed by gel extraction using the E.Z.N.A.® gel extraction kit (Omega Bio-tek, GA, USA) to obtain a 3136 bp knockout cassette fragment. This was further ligated in a PMO130 suicide vector and transformed in *E. coli* SM10 competent cells.

Table 3.1. List of bacterial strains and plasmids used in this study

Strain/Plasmids	Characteristics	Source
Acinetobacter baumannii		
ATCC 17978	Wild Type	ATCC
AB199	ATCC 17978:Δ*A1S_1977-78*	Kumar lab
AB451	AB199:mini-Tn7T-LAC-Gm:*A1S_1977-78*	This study
AB452	AB199:mini-Tn7T-LAC-Gm (Empty vector)	This study
Escherichia coli		
pMO130 (JM109)	Suicide vector, Kanamycin resistant	(Hamad et al., 2010)
pPLS233 (SM10)	pMO130:Δ*A1S_1977-78*:Gm:FRT	Kumar Lab
pPLS400 (DH5α)	pCR-XL-2-TOPO:A1S_1977-78	This study
pPLS403 (DH5α)	pUC18T-mini-Tn7T-LAC-Gm:*A1S_1977-78*	This study
	pUC18T-mini-Tn7T-LAC-Gm	(Choi et al., 2005)
pTNS2 (DH5α)	*tnsABCD,* Ampicillin resistance	(Choi et al., 2005)
pRK2013 (HB101)	Kanamycin resistance	(Figurski & Helinski, 1979)
pFLP2 (DH5α)	*flp* recombinase, Ampicillin resistance, Carbenicillin resistance	(Hoang et al., 1998)

Table 3.2. List of primers used in this study.

Primer name	Sequence	Characteristics	Source
A1S_1977_1978_R	CGGCTAAATTAAATTAGCCGC	Full length amplification of *A1S_1977-78*	Kumar lab
A1S_1977_1978_F	CAATGAAGATGCCATTGCG	Full length amplification of *A1S_1977-78*	Kumar Lab
A1S_1978_RT_F	CGCCCGATGTCATCTTAACT	*A1S_1978* gene expression	Kumar Lab
A1S_1978_RT_R	GTCGGGGTGAGAGTTTTTCA	*A1S_1978* gene expression	Kumar Lab
A1S_1978_DNF-GM	AGGAACTTCAAGATCCCCAATTCGGCTACTCCAATGTTTGAGCGC	*A1S_1977-78* disruption cassette	Kumar Lab
A1S_1978_UPR-GM	TCAGAGCGCTTTTGAAGCTAATTCGAAACCGGTTGTCCAGAAGCGC	*A1S_1977-78* disruption cassette	Kumar Lab
A1S_1978_R-SOeing	GCATTCCCATAATCATTCGCCATTC	Downstream amplification of *A1S_1977-78* disruption cassette	Kumar Lab
A1S_1978_F-SOeing	CACCTGCACGTTTTAACGCAGCAGC	Upstream amplification of *A1S_1977-78* disruption cassette	Kumar Lab
A1S_1977_SacI-F	CGAGCTCCAATGAAGATGCCATTG	Upstream amplification of *A1S_1977-78* with SacI cut site	This study
A1S_1978_BamHI-R	TTGGATCCGTTTTAAGCTTCTGAC	Downstream amplification of *A1S_1977-78* with BamHI cut site	This study
Gm_F	TGGAGCAGCAACGATGTTAC	FRT site amplification	Kumar Lab

Gm_R	TGTTAGGTGGCGGTACTTGG	FRT site amplification	Kumar Lab
16s qRT F	CTTCGGACCTTGCGCTAATA	16S gene expression	Kumar Lab
16s qRT R	ATCCTCTCAGACCCGCTACA	16S gene expression	Kumar Lab
adeN17978_RT_Fwd1	CAACCTGAACACATTGCCTTT	*adeN* gene expression	Kumar Lab
adeN17978_RT_Rev1	TTTTGGACATCCAGAGCACA	*adeN* gene expression	Kumar Lab
adeJ_F1_RT	CATCGGCTGAAACAGTTGAA	*adeJ* gene expression	Kumar Lab
adeJ_R1_RT	GCCTGACCATTACCAGCACT	*adeJ* gene expression	Kumar Lab
AdeI_RT_Fwd_2	GCAGCAGCTAAGGCTCAAGT	*adeI* gene expression	Kumar Lab
AdeI_RT_Rev_2	TGCAGTAACCAAAGCACCAG	*adeI* gene expression	Kumar Lab
AdeK_RT_Fwd_1	GCGTTTGCTTGAAGTGATTG	*adeK* gene expression	Kumar Lab
AdeK_RT_Rev_1	TGGAAGCTGGTTGTTCTGTG	*adeK* gene expression	Kumar Lab
Ab_glmS_F	TATGGAAGAAGTTCAGGCTC	Upstream amplification of *glmS* gene	(Choi et al., 2005)
Tn7R	CACAGCATAACTGGACTGATTTC	stream amplification of *tn7* transposon	(Choi et al., 2005)

(ii) *Deletion of A1S_1977-78 in A. baumannii ATCC 17978*

Deletion of *A1S_1977-78* gene from the *A. baumannii* ATCC 17978 genome was done using three parental conjugation method (Amin et al., 2013). Briefly, overnight cultures of *A. baumannii* ATCC 17978, *E.coli* DH5α/pPLS233 and *E.coli* HB101/pRK2013 were grown in LB supplemented with their respective antibiotics (Table 3.1) at 37^{o}C and shaking at 250rpm. 100µL of each culture were aliquoted in a sterile 1.5mL centrifuge tube, mixed by vortex, and centrifuged at 12,000 X g for 2 minutes. Using a micropipette, the supernatant was carefully discarded, and the pellet was resuspended in 1mL of fresh LB and centrifuged for washing. The cells were washed 2 to 3 times and the final pellet was resuspended in 10µL of LB. The resuspended cell mixture was carefully spotted at the center of a sterile nitrocellulose paper placed in an LB agar plate and incubated for 16-18 hours at 30^{o}C. Following the incubation, the nitrocellulose paper containing the conjugation mix was transferred into a sterile centrifuge tube and the cells were resuspended in 0.85% saline solution. The resuspended cells were plated in Simmons citrate agar plates supplemented with 50µg/mL gentamicin and incubated at 37^{o}C for 48 hours. The colonies obtained were patched on LB agar supplemented with 50µg/mL gentamicin to obtain marked knockout mutants. To remove the gentamicin cassette, the marked mutants were electroporated with pFLP2 plasmid and counter selected on 10% sucrose plates to obtain unmarked deletion mutants.

3.2.3. Complementation of *A1S_1977-78* in AB199.

(i) *Cloning of A1S_1977-78 in single copy complementation vector*

A1S_1977-78 was complemented in AB199 using a single copy complementation vector pUC18T-mini-Tn7T-LAC-Gm (Kumar et al., 2010). Briefly, *A1S_1977-78* fragments with restriction sites for SacI and BamHI at the upstream and downstream region respectively, was amplified using the primers: A1S_1977_SacI-F and A1S_1978_BamHI-R. The fragment was cloned into pCR-Xl-2-Topo plasmid using Topo XL-2 PCR cloning kit (Invitrogen) and transformed into DH5α competent cells. The pCR-XL-2-Topo:*A1S_1977-78* was extracted, digested with SacI-HF (NEB) and BamHI-HF (NEB), gel purified, ligated into pUC18T-mini-

Tn7T-LAC-Gm using T4 Ligase and transformed in DH5α competent cells to obtain the strain pPLS403 (pUC18T-mini-Tn7T-LAC-Gm:*A1S_1977-78*).

(ii) ***Introduction of A1S_1977-78 in AB199 by electroporation***

Chromosomal complementation of *A1S_1977-78* in the AB199 genome was performed following a previously described protocol (Ducas-Mowchun, De Silva, Patidar, et al., 2019). Briefly, the plasmids: pUC18T-mini-Tn7T-LAC-Gm:*A1S_1977-78*, pTNS2 and pFLP2 were extracted from overnight cultures of their respective bacterial cultures respectively, using BioBasic Plasmid Miniprep Kit (BioBasic Inc. Markham, ON, Canada) following manufacturer's protocol. Overnight culture of AB199, grown in LB broth at 37^{o}C and shaking at 250rpm, was centrifuged in a 1.5mL microcentrifuge tube at 12,000 X g for 2 minutes. The supernatant was carefully removed using a micropipette and the pellet was resuspended in ice cold MilliQ water. The cells were washed 3 times by centrifugation, resuspended in 200µL of ice cold MilliQ water. 100µL of resuspended cells were transferred into sterile electroporation cuvettes and kept on ice. 100-200ng of pUC18T-mini-Tn7T-LAC-Gm:*A1S_1977-78* and pTNS2 were added to the cuvettes and electroporated into freshly competent AB199 cells by charging at 2.1 KV using an Electroporator 2510 (Eppendorf, Mississauga, ON, Canada). Following this, 1mL of pre-warmed LB was added immediately into the cuvettes (only the ones with time constant values between 5 – 6ms), mixed, and the entire content was transferred into fresh centrifuge tubes, and incubated at 37^{o}C in a shaker incubator for 1 hour and 30 minutes. Only cuvettes which had time constant values between 5-6ms were used for recovery. The cell cultures were selected in LB agar supplemented with 15µg/mL Gentamicin. The colonies obtained were patched and confirmation of the insertion was done by Endpoint PCR using primers – *glmS-F* and *tn7R.* Further, the presence of *A1S_1977-78* was confirmed by end-point PCR using primers - A1S_1977_SacI-F and A1S_1978_BamHI-R. To obtain an unmarked complement strain, the removal of gentamicin cassette was attempted by electroporating pFLP2 into the marked complement strain. An empty vector AB452 was prepared alongside the *A1S_1977-78* complement strain.

3.2.4. Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) for the experimental strains were determined by E-test method using E-strips (Biomérieux, Montréal, Canada) following manufacturer's guidelines. The antibiotics tested include chloramphenicol, gentamicin, and ciprofloxacin. Briefly, a single isolated colony was picked and inoculated in 3mL of LB broth and incubated for 16-18 hours at 37^{o}C and shaking at 250rpm. These cultures were standardized by dilution in 0.85%(w/v) saline solution to 0.5 MacFarland using a DensiChek^{TM} Plus (Biomérieux, Montréal, Canada). Using a sterile cotton tipped applicator, the inoculums prepared were used to make a lawn on Muller Hilton Agar (MHB) plates. The plates were allowed to briefly dry for 4-5 minutes and E-strips of the desired antibiotics were carefully placed at the center of each plate. The plate was then incubated at 37^{o}C for 16-18 hours. The MIC of each antibiotic was measured by determining the zone of inhibition using scale provided on the E-strips. The assays were conducted in at least two bio replicates and three technical replicates per antibiotic for each strain.

3.2.5. Surface associated motility assay

Surface associated motility assay was conducted by following a previously described method with some modifications (Harding et al., 2013). Briefly, single isolated colonies of the experimental strains were inoculated in sterile LB broth and incubated at 37^{o}C and shaking at 250rpm for 16-18 hours. Using fresh LB media these cultures were standardized by adjusting the A_{600} value to 1.0 using a Spectrophotometer (Thermo Fisher Scientific). 3µL of the standardized inoculum was inoculated at the center of motility media plates by stabbing halfway through the media and careful dispensing with the micropipette. The plates were allowed to dry for 15-20 minutes and incubated at 37^{o}C in the dark for 16 hours. The Motility media plates were prepared by mixing 5g/L of tryptone, 2.5g/L of sodium chloride and 3g/L of Agarose in 1L of double distilled water and autoclaving for 15 minutes. Statistical analysis was done using Welch's t-test and graph was plotted using Graphpad prism 7.0. The experiment was conducted in three independent bioreplicates, and three technical replicates for each strain.

3.2.6. RNA extraction, preparation of cDNA, and RT-qPCR assay

(i) *RNA extraction*

A single colony of the experimental strains were inoculated in 3mL of LB broth and incubated at 37ºC with shaking at 250rpm for 16-18 hours. These were sub-cultured in fresh LB media at 1:100 ratio and grown to mid-log phase (A_{600} value of 0.5-0.8). 1.5mL of these cultures were aliquoted into sterile microcentrifuge tubes and centrifuged at 12,000 X g for 2 minutes. Using a micropipette, the supernatant was carefully discarded without dislodging the pellet. The centrifuge tubes containing the bacterial cell pellets were incubated for 16-18 hours at -72ºC.

RNA extraction was performed using RNeasy mini kit (Life Technologies) following manufacturer instructions. Briefly, the bacterial cell pellets frozen at -72ºC was resuspended in 100µL of 1mg/mL lysozyme. Following this 600µL of lysis buffer and 3.5µL of β-mercaptoethanol. 700µL of 95% ethanol was added to the mixture and the tubes were mixed thoroughly using vortex. The solution was then transferred to spin cartridges provided in the kit and centrifuged at 12,000 X g for 15 seconds. Once all the sample was centrifuged, the spin cartridges were washed using the wash buffers provided in the kit and dried by centrifugation at 12,000 X g for 15 seconds. RNA was extracted from the spin cartridges using 50µL of RNase free water.

(ii) *RNA purification by DNase I treatment and cDNA synthesis*

Following RNA extraction of the experimental strains, the concentration of the RNA samples was measured using Nanodrop Spectrophotometer (Thermo Scientific). The RNA samples were treated with 1µL (2.73U/µL) of DNase I enzyme (Qiagen) to obtain purified RNA samples. The reaction mixture comprised of 1µg of RNA, 2µL of RDD buffer, 1µL of DNase I (2.73U/µL) enzyme and sterile MilliQ water up to a final volume of 20µL. Reaction was performed in a BIORAD thermocycler with the following rection temperatures, Incubation temperature of 37ºC for 30 minutes, enzyme inactivation temperature of 62ºC for 5 minutes, and holding temperature of 4ºC. These samples were used as template and reverse transcribed using SuperScript VILO

cDNA synthesis kit (Invotrogen) following manufacturer instructions. A 20µL reaction mixture comprising of 4µL of DNase treated RNA, 4µL of 5X RT buffer, 2µL of 10X Reverse transcriptase enzyme and 10µL of MilliQ water, was prepared. The reaction mixture was run in a BIORAD thermocycler using the following temperature settings: annealing temperature of 25^{o}C for 10 minutes, Incubation temperature of 42^{o}C for 1 hour and 30 minutes, enzyme inactivation temperature of 85^{o}C for 5 minutes and holding temperature of 4^{o}C.

(iii) *RT-qPCR*

Following the synthesis of cDNA from the experimental strains, the tubes containing cDNA were briefly centrifuged and the samples were diluted to 1:5 ratio using sterile MilliQ water. A master mix of each gene of interest was prepared using SYBR Select Master mix (ThermoFisher Scientific) following manufacturer guidelines. The Master mix prepared for each gene of interest contained 100nM of forward and reverse primers. All primers were standardized by 10-fold dilution of genomic DNA of ATCC 17978 to confirm 95-100% efficiency. 2.3µL of cDNA template was added to 5.3µL of master mix to get a final reaction volume of 8µL per well. RT-qPCR reactions were performed in a StepOne Plus machine (Applied Biosystems) using the following reaction temperatures: initial denaturation of 95˚C for 2 minutes, 40 cycles of 95˚C for 15 seconds, 60˚C for 1 minute. To measure product specificity, a melt curve was obtained using the following conditions: 95˚C for 10 seconds, 60˚C for 5 seconds, and 95˚C for 10 seconds. Relative gene expression was calculated using Pfaffl's method and 16S rRNA was used as internal gene for normalization of the expression levels of each gene of interest. Graphs were plotted using Graphpad Prism 7.0. Statistical analysis was performed using Microsoft Excel and 2-way ANOVA (Turkey's multiple comparison test) in Graphpad Prism 7.0. The experiment was conducted with three independent bioreplicates. Each bioreplicates comprised of three technical replicates per strain.

3.3. Results

3.3.1. Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) of AB199 was determined for three different classes of antibiotics by E-test method using E-strips (Biomérieux, Montréal, Canada) (Table3.3). The deletion mutant AB199 was found to be 2-fold more sensitive to ciprofloxacin compared to the wild-type strain ATCC 17978. No difference in MIC was observed between ATCC 17978 and AB199 for chloramphenicol and gentamicin (Table 3.3).

3.3.2. Motility Assay

Surface-associated motility assay was performed in at least 4 independent bio-replicates and three independent technical replicates. No significant change in motility was observed in knockout mutant AB199 compared to the wild type ATCC 17978 (Figure 3.2).

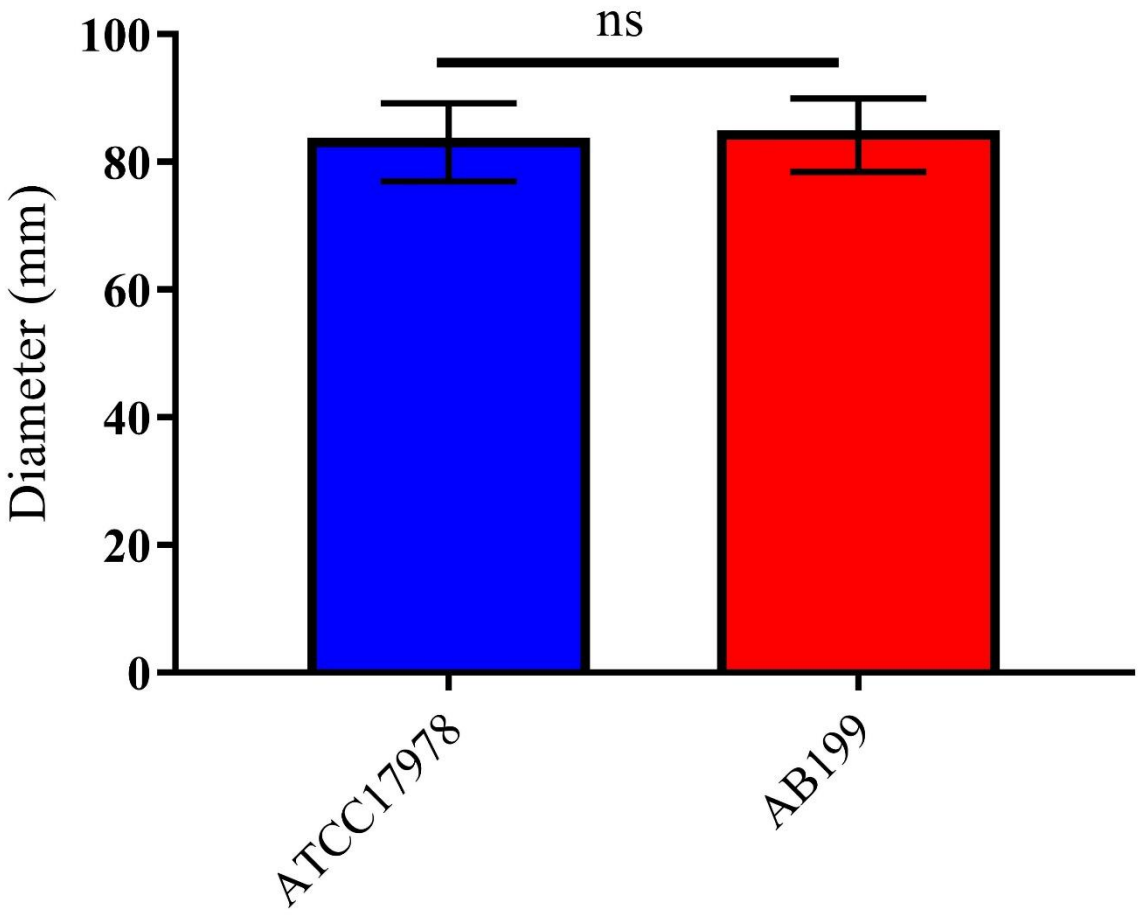
3.3.3. Gene expression assay using RT-qPCR

Comparative expression analysis of the genes *adeN, adeJ, adeI* and *adeK* was performed between ATCC 17978 and deletion mutant AB199. Results from three independent bio-replicates did not show any significant difference in expression of either *adeN* or *adeIJK* (Figure3.3). Since both experimental strains were grown in nutrient rich LB media, we repeated the experiment with strains grown on VBMM minimal media. Despite the nutritional stress, no change in expression was observed in *adeN or adeIJK* (Figure 3.4). Overall, this indicates that disruption of A1S_1977 and A1S_1978 from the genome of ATCC 17978 has no impact on expression of either AdeN or AdeIJK efflux pump.

Table 3.3. Minimum Inhibitory Concentration assay forATCC 17978 and AB199 (ATCC 17978:Δ*A1S_1977-78*): The MIC values of all antibiotics were determined using E-strip test in two independent bio-replicates. All values were determined in µg/mL.

Antibiotics	ATCC 17978	AB199
Gentamicin	0.5	0.5
Ciprofloxacin	0.125	0.064
Chloramphenicol	96/128	96/128

(A)


ns
100
80
60
40
20
0
Diameter (mm)
ATCC17978
AB199

(B)

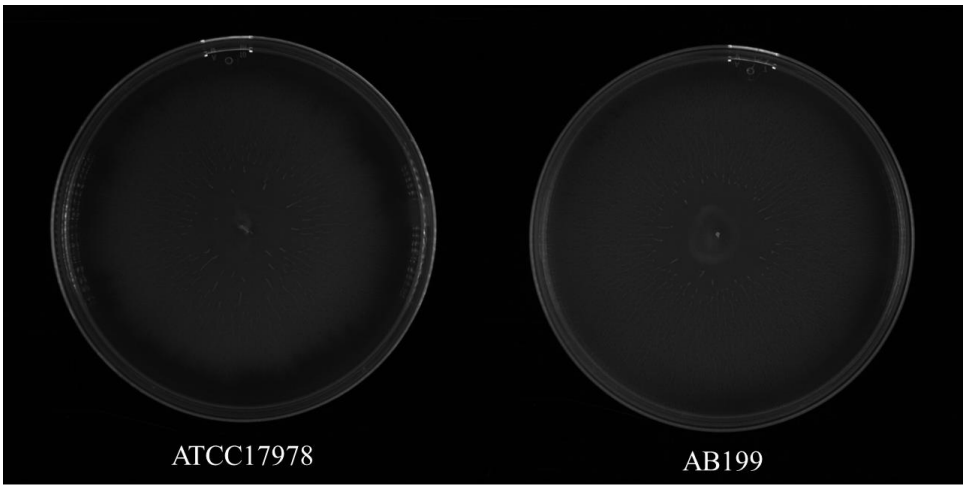

ATCC17978
AB199

Figure 3.2. Surface-associated motility assay: A) No significant difference in motility was observed between wild type strain ATCC 17978 and deletion mutant AB199 (AB199:mini-Tn7T-LAC-Gm:*A1S_1977-78*). B) Representative images of motility assay plates of wild type ATCC 17978 and deletion mutant AB199.

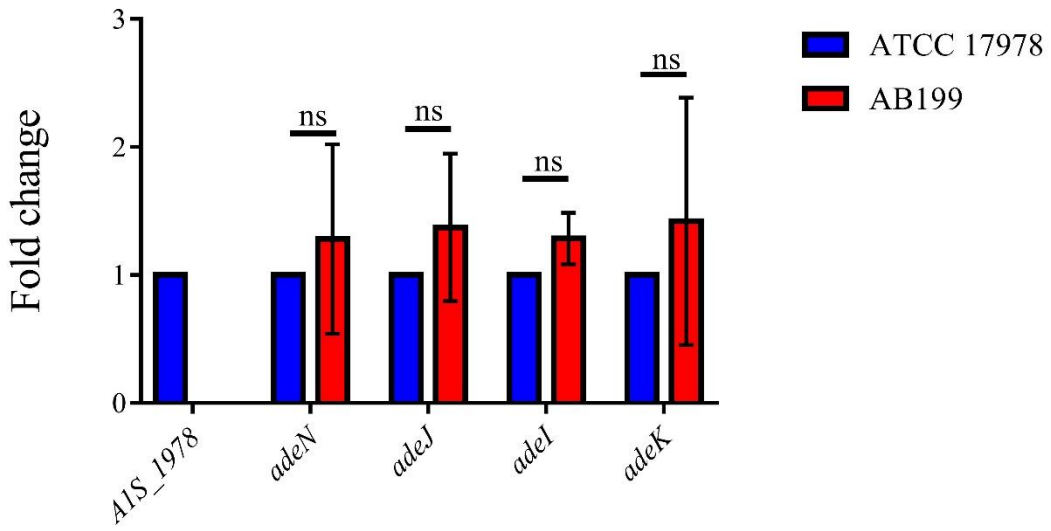


Figure 3.3. Expression of AdeN and AdeIJK efflux pump in AB199 grown in nutrient rich media (LB): Comparative gene expression analysis for the AdeN regulator protein (*adeN*) and AdeIJK efflux pump (*adeJ, adeI* and *adeK*) was done in wild type strain ATCC 17978 and deletion mutant AB199. No change in expression was observed for *adeN* and *adeIJK* in AB199 compared to ATCC 17978. *A1S_1978* was used as reference control to rule out any possible contamination during the experiment. *A1S_1978* was not detected in deletion mutant AB199 as expected. Statistical analysis was performed using 2-Way ANOVA Turkey's multiple comparison test in Graphpad Prism 7.0. P values for all genes were greater than 0.9.

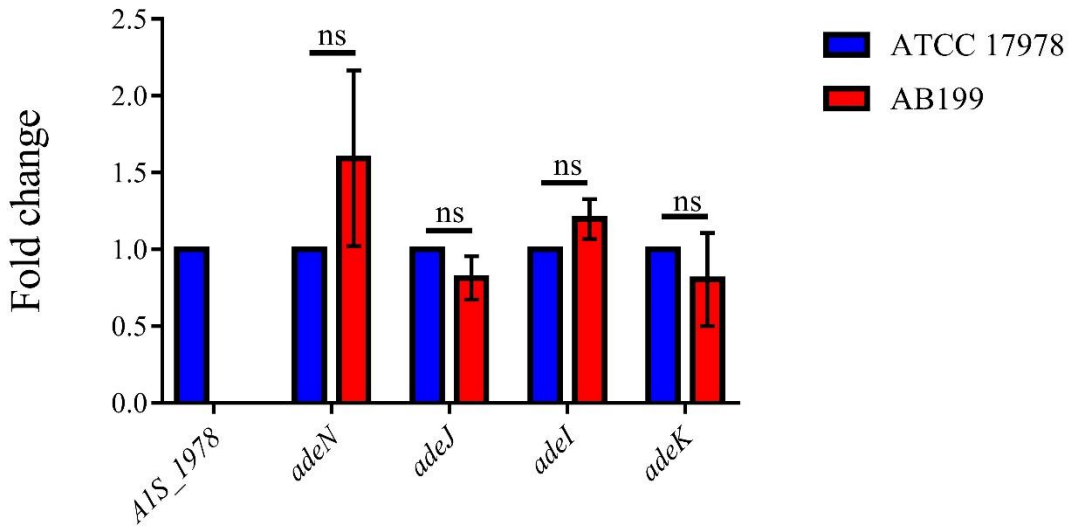


Figure 3.4. Expression of AdeN and AdeIJK efflux pump in AB199 grown in minimal media (VBMM): Comparative gene expression analysis for the AdeN regulator protein (*adeN*) and AdeIJK efflux pump (*adeJ, adeI* and *adeK*) was done in wild type strain ATCC 17978 and deletion mutant AB199. No change in expression was observed for *adeN* and *adeIJK* in AB199 compared to ATCC 17978. *A1S_1978* was not detected in deletion mutant AB199 as expected. Statistical analysis was performed using 2-Way ANOVA Turkey's multiple comparison test in Graphpad Prism 7.0. P values for all genes were greater than 0.1.

3.4. Discussion

Signal transduction is an essential mechanism which enable bacterial cells to adapt to their environmental conditions (De Silva & Kumar, 2019). Two Component Systems are the most abundant signal transduction systems in bacteria and have been associated with regulation of various adaptive phenotypes in response to environmental stress (Alm et al., 2006). Numerous studies have also demonstrated their involvement in regulating antibiotic susceptibility and virulence phenotypes in different bacterial species (Bhagirath et al., 2019). In this study, we investigated the functional characteristics of the putative TCS A1S_1977-78 and its potential link with AdeN and AdeIJK efflux pump expression. In a previous study, comparative genotypic analyses between clinical isolates *A. baumannii* AB030, AB031 and lab strain ATCC 17978 revealed point mutations in the *A1S_1977-78* operon in AB030, which resulted in a significant decrease in expression of *A1S_1978* in this strain (Alsaadi, 2014). Comparative genomic analysis between ATCC 17978, AB030 and AB031 using multi-sequence alignment on NCBI revealed a percentage identity score > 97% between these strains (Alsaadi, 2014). *A. baumannii* AB030 is an XDR strain which exhibits resistance to all clinically used antibiotics except for colistin (Loewen et al., 2014). Since *A1S_1977-78* is in close proximity to *adeN*, and downregulation of *adeN* is associated with increased expression of the AdeIJK efflux pumps, we came to a hypothesis that the downregulation of this TCS may have had an impact on the antibiotic susceptibility profile of this strain. Further, AB031, another clinical isolate, exhibited higher expression of *A1S_1978* compared to AB030 and ATCC 17978, and was found to have higher biofilm formation compared to AB030 (Alsaadi, 2014). Since AdeN expression in *A. baumannii* has been directly linked with regulation of virulence associated phenotypes including biofilm formation and motility (Saranathan et al., 2017), this further strengthened our hypothesis. Studies have also shown that this operon is conserved in *A. baumannii* (Alsaadi, 2014). We, therefore, decided to investigate the potential link between A1S_1977-78, AdeN and AdeIJK.

Generally, the sensory HK domain and RR domain of a TCS have been found to exhibit high specificity (Casino et al., 2009), however, there have been cases where certain sensory kinases have been found to interact with multiple RRs and vice versa (López-redondo et al., 2010)(Laub & Goulian, 2007). Taking this into consideration, in our study, both the HK and RR domains were deleted from the ATCC 17978 genome to obtain the strain AB199 (ATCC

17978:Δ*A1S_1977-78*). To investigate the impact of this deletion on the expression of AdeN and AdeIJK efflux pump, we performed RT-qPCR assays on *adeN, adeJ, adeI* and *adeK.* Interestingly, no change in expression was observed in either of the genes in AB199 compared to wild-type strain ATCC 17978. Since the experimental strains were grown in LB, which is a nutrient rich media, we assumed that the observed results may have been due abundance of nutrients. We, therefore repeated this experiment using strains grown in VBMM minimal media. This, however, made no difference, as no change was observed in either of the genes tested between ATCC 17978 and AB199. Contradictory to our hypothesis, these results indicate that disruption of *A1S_1977-78* did not have any impact on AdeN and AdeIJK expression. However, since A1S_1977-78 is a novel TCS and the environmental signals to which it responds to is yet to be determined, further investigation using different environmental stress may provide a better insight into the relationship between A1S_1977-78, AdeN and AdeIJK.

To further investigate the impact of *A1S_1977-78* disruption on the phenotypic profile of AB199 in comparison to the wild type of strain ATCC 17978, we conducted an MIC assay on these strains using antibiotics belonging to three different classes. The antibiotics used for this experiment included chloramphenicol, ciprofloxacin, and gentamicin. Results from this experiment revealed a small but reproducible increase in susceptibility of AB199 to ciprofloxacin compared to the wild type ATCC 17978. However, no significant change in MIC was observed for gentamicin and chloramphenicol. Since chloramphenicol is a known substrate of AdeIJK, the lack of change in MIC for this antibiotic together with our RT-qPCR data indicate that A1S_1977-78 may be involved in regulation of ciprofloxacin resistance mechanisms independent of AdeIJK pump expression. However, further investigation needs to be carried out using other antibiotics and molecules that are known substrates of AdeIJK. While gentamicin is not a substrate of AdeIJK (Damier-Piolle et al., 2008), it was included in our experiment to investigate the possible impact of A1S_1977-78 disruption on resistance mechanisms other than AdeIJK pump expression. Our results showed that deletion of *A1S_1977-78* has no impact on mechanisms regulating gentamicin resistance. To expand upon this observation, further MIC experiments need to be carried out using other aminoglycosides. To further investigate the fluroquinolone resistance mechanism(s) that is impacted by the disruption of *A1S_1977-78*, expression analysis of the AB199 genome in comparison to wild type, needs to be performed. RNA-sequencing of AB199 will reveal the variations in expression of all the

genes impacted by gene deletion. Further, proteomic analysis can further strengthen the data obtained from RNA-seq by revealing the changes in the protein expression profile because of this deletion. Apart from AdeIJK pump activity, fluroquinolone resistance in *A. baumannii* is greatly impacted by various other mechanisms. DNA gyrase and Topoisomerase IV are the primary and secondary targets of Fluoroquinolones respectively, and mutations in *gyrA* (encoding DNA gyrase) and *parC* (encoding Topoisomerase IV) is a major mechanism involved in conferring resistance to such antibiotics (Seward & Towner, 1998). AdeFGH efflux pump expression is another mechanism associated with fluroquinolone resistance in *A. baumannii* (Coyne et al., 2010). Other contributors of fluroquinolone resistance include, AbaQ (MFS transporter)(Perez-Varela et al., 2018), AbeM (MATE transporter) (Su et al., 2005), AbeS (SMR transporter) (Srinivasan et al., 2009)and AdeABC (F. G. Adams et al., 2018). Gene expression analysis of these potential contributors using RT-qPCR assay may provide further insights into the mechanisms that are regulated by A1S_1977-78.

Since AdeN is associated with regulation of virulence associated phenotypes in *A. baumannii* (Saranathan et al., 2017), we investigated the role of A1S_1977-78 in regulation of AdeN expression, and its impact on surface associated motility assay in *A. baumannii*. Our results showed that disruption of A1S_1977-78 had no impact on motility in AB199 compared to wild type. This further suggest that A1S_1977-78 does not regulate AdeN expression. Since AdeN has also been associated with biofilm formation in *A. baumannii*, biofilm assays experiments ATCC 17978 and AB199 may provide better clarity into this.

To further support the results from our experiments for this study, we had attempted to prepare an unmarked complement strain using the single copy complementation vector pUC18T-mini-Tn7T-LAC-Gm (Kumar et al., 2010). We have successfully prepared a marked complement strain AB451 (AB199:mini-Tn7T-LAC-Gm:*A1S_1977-78*) and an empty vector AB452 (AB199:mini-Tn7T-LAC-Gm). Future experiments required involve removal of the gentamicin cassette from AB451 and AB452 to obtain an unmarked complement and empty vector respectively. Repeating all the experiments using these strains will give us further insights into the results observed.

Chapter 4: Conclusion and future directions

The role of Two Component Systems in regulating antibiotic susceptibility and virulence in response to environmental stimuli has been extensively studied in different bacterial species. *Acinetobacter baumannii* is a highly resilient bacterial pathogen and is known for its ability to resist a broad spectrum of antibiotics as well as efficiently adapt to environmental changes. In this project, our overall objective was to investigate the significance of TCS in regulating antibiotic susceptibility and virulence phenotypes in response to environmental stress in *A. baumannii.*

In the first part of our project, we studied the functional characteristics of AvnR, a novel response regulator, in a hypervirulent clinical isolate, *A. baumannii* AB030. Preliminary studies on this RR in lab strain ATCC 17978 had shown that AvnR is associated with regulation of various virulence associated phenotypes including motility, biofilm formation, virulence in *G. mellonella* and mice infection models, adhesion to A549 epithelial cells, and nitrogen metabolism. Previous studies on *A. baumannii* AB030 revealed a natural disruption of the gene encoding AvnR in AB030 by a 3902bp transposon insertion, resulting in loss of functionality. In our study, we attempted to restore these functions by complementation of *avnR* sourced from ATCC 17978 in AB030 by using single copy complementation method. To investigate the effect of *avnR* expression on virulence associated phenotypes in the AvnR complement strain, AB408 (AB030:mini-Tn7T:*avnR*), we investigated the changes in biofilm formation and motility in this strain. Results from our study showed that expression of *avnR* in AB030 did not alter expression of these phenotypes. Further, using P3MB BIOLOG^{TM} microarray plates, we investigated the changes in nitrogen metabolism in AB408 because of *avnR* expression. We observed increased growth of AB408 in L-Serine and L-Arginine. Metabolism pathways for these amino acids have been associated with biofilm formation, motility, adherence, and invasion of epithelial cells across a wide variety of gram-negative and gram-positive pathogens including *E. coli, P. aeruginosa, Salmonella enterica,* and *B. abortis.* Since AvnR has been previously shown to be involved in expression of these phenotypes in ATCC 17978 and our observations for L-serine and L-arginine are consistent with previously observed data, this indicates that AvnR may be involved in expression of these pathways. RNA sequencing of AB408 is required to gain a broader insight into the genes affected by the expression of AvnR in this strain. RT-qPCR assays on the genes involved in these pathways can be used to confirm the results obtained from RNA sequencing experiment. Further, proteomic analysis can be used to investigate if differential

expression of the involved genes has any effect on protein expression in this strain. Since the genome of AB030 contains several accessory elements which may be involved in expression of the phenotypes tested in this study, AvnR can be further studied in a different strain of *A. baumannii.* The clinical strain, *A. baumannii* AB031 may be ideal for this investigation. An RT-qPCR assays from a previous study had shown that this strain has higher expression of AvnR compared to lab strain ATCC 17978. Further, this strain has been found to produce more biofilm compared to ATCC 17978 and AB030. Therefore, the effect of disruption of *avnR* in this strain on virulence associated phenotypes may further provide an insight into the role of AvnR in regulating these phenotypes.

In the second part of this project, we have investigated the functional characteristics of the putative TCS, A1S_1977-78. Previous studies involving comparative RT-qPCR analysis between lab strain ATCC 17978, XDR strain, AB030, and MDR strain, AB031, had revealed differential expression of this TCS due to mutations in its operon. AB030 was found to have a significantly lower expression for *A1S_1978* compared to ATCC 17978 and AB031. AB031, on the other hand, showed higher expression for *A1S_1978* compared to both ATCC 17978 and AB030. The operon for A1S_1977-78 has been found to be situated 276bp upstream of AdeN, a Tet-R type regulator protein associated with regulation of AdeIJK pump expression, and virulence associated phenotypes including biofilm formation and motility. We, therefore investigated if A1S_1977-78 was involved in regulating the expression of AdeN and consequently involved in antibiotic resistance and virulence in *A. baumannii.* Results from MIC experiments on A1S_1977-78 deletion mutant AB199 (ATCC 17978: Δ*A1S_1977-78*) revealed a 2-fold increase in sensitivity to ciprofloxacin and moxifloxacin. However, on conducting RT-qPCR assays, no significant change in expression was seen for either *adeN* or *AdeIJK.* This result was found to be consistent across experiments conducted with strains grown in nutrient rich and minimal media. Further, no significant changes in motility were observed in the deletion mutant AB199 compared to wild type ATCC 17978. These preliminary observations suggest that, in contradiction to our hypothesis, A1S_1977-78 may not be involved in regulation of AdeN or AdeIJK. However, further investigations are required to strengthen this finding. Since A1S_1977-78 is a putative TCS and the environmental signals sensed by this system is not known, expression analysis using RT-qPCR on the experimental strains grown under different environmental stress. Subsequently, MIC assays with substrate antibiotics for AdeIJK other than

the ones used in this study. To further investigate the impact of *A1S_1977-78* disruption on the gene expression profile of AB199, RNA sequencing of this strain is required. Subsequently, RT-qPCR experiments can be used to confirm the data obtained from RNA-seq experiments. To Further strengthen the data obtained from the RNA- seq experiments proteomic analysis is required to further to check the effect of the differential expression of genes on protein expression in AB199. Finally, experiments performed and proposed in this study needs to be repeated with an unmarked complement strain for A1S_1977-78 in ATCC 17978 to further strengthen our findings.

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Appendix

Appendix I. Nitrogen utilization assay using BIOLOG™ PM3B Microarray plates. The numbers in columns AB030 and AB408 are A_{595nm} values obtained after 24 hours of incubation of the respective strains in BIOLOG™ PM3B Microarray plates at 37°C. Percentage growth was calculated by normalizing the A_{595nm} values for AB408 with AB030. Obtained values have been color coded from red (low) to green (high) and is indicative of the variation in percentage growth in AB408 relative to AB030 in different nitrogen sources.

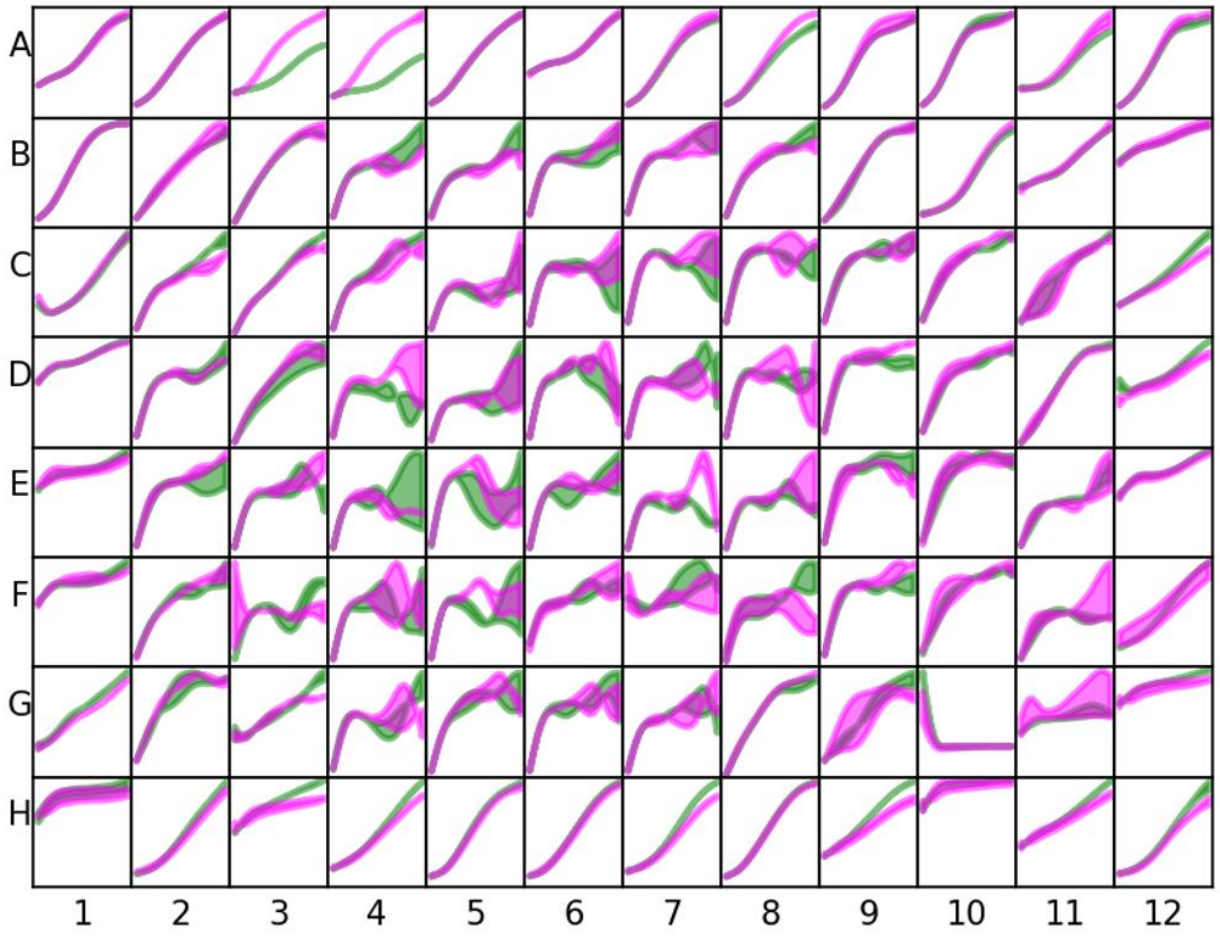
Nitrogen Source	AB030	AB408	% Growth of AB408 relative to AB030
Thymine	0.44235	0.20345	45.99299197
Alloxan	0.37665	0.20155	53.51121731
D,L-Lactamide	0.16885	0.0913	54.07166124
N-Amylamine	0.1757	0.09715	55.29311326
Formamide	0.2862	0.1802	62.96296296
Adenosine	0.37615	0.2426	64.49554699
Acetamide	0.28755	0.19285	67.06659711
L-Leucine	0.50955	0.36315	71.26876656
Allantoin	0.42455	0.3258	76.74007773
Uric Acid	0.626	0.4983	79.60063898
D-Galactosamine	0.26665	0.207	77.62985187
ε-Amino-N-caproic acid	0.2718	0.2216	81.53053716
Ala-Glu	0.09655	0.0797	82.54790264
L-Phenylalanine	0.48205	0.39795	82.553677
Gly-Gln	0.1663	0.1377	82.80216476
L-Ornithine	0.2155	0.1812	84.08352668
L-isoleucine	0.4523	0.38215	84.49038249
D-Asparagine	0.5379	0.45665	84.89496189
Met-Ala	0.37355	0.31775	85.06224066
D-Alanine	0.91965	0.7901	85.91311912
Ala-Thr	0.32895	0.2847	86.54810762
Ala-Gly	0.2793	0.2422	86.71679198
Agmatine	0.1456	0.1271	87.29395604
Cytidine	0.28115	0.2455	87.31993598
Gly-Met	0.1291	0.11285	87.41285825
Methylamine	0.43625	0.3818	87.51862464
L-Valine	0.54355	0.47605	87.58163922
Guanosine	0.4389	0.38685	88.14080656
N-Phthaloyl-L-Glutamic acid	0.36185	0.32895	90.90783474
Ala-Asp	0.07855	0.0718	91.40674729
Alpha-Amino-N-Valeric acid	0.07355	0.06735	91.5703603
Ala-Gln	0.39975	0.3673	91.88242652

N-Acetyl-D-Mannosamine	0.09515	0.0875	91.96006306
Xanthine	0.3162	0.29335	92.77356104
Inosine	0.27245	0.25305	92.87942742
Histamine	0.09905	0.09265	93.53861686
L-Homoserine	0.39475	0.37485	94.95883471
Ala-His	0.45745	0.4373	95.59514701
L-Citruline	0.32655	0.3124	95.66681978
Xanthosine	0.26105	0.2531	96.9546064
Putrescine	0.9382	0.91225	97.23406523
Ala-Leu	0.4566	0.4446	97.37187911
Cytosine	0.2525	0.2463	97.54455446
N-Acetyl-D-Galactosamine	0.09715	0.0948	97.58106022
N-Acetyl-L-Glutamic acid	0.1094	0.10725	98.03473492
L-pyroglutamic acid	0.7211	0.70865	98.27347109
Gly-Glu	0.067	0.06595	98.43283582
Gly-Asn	0.4581	0.4511	98.47194936
N-Butylamine	0.2323	0.22905	98.60094705
L-proline	0.8111	0.8037	99.08765874
N-Acetyl-D-Glucosamine	0.4107	0.4102	99.87825664
L-Glutamine	0.7772	0.77705	99.98069995
D,L-alpha-Amino-Caprylic acid	0.05295	0.05305	100.1888574
Parabanic acid	0.30955	0.3102	100.2099822
Ammonia	0.59145	0.59285	100.2367064
L-tyrosine	0.474	0.4752	100.2531646
L-Aspartic acid	0.5767	0.5791	100.4161609
Urea	0.547	0.54985	100.5210238
L-tryptophan	0.11855	0.11965	100.9278785
Biuret	0.19535	0.1973	100.9982083
Adenine	0.46035	0.46715	101.477137
Ethylamine	0.24785	0.25335	102.2190841
L-Aspargine	0.63155	0.6462	102.3196897
Uracil	0.27165	0.2781	102.3743788
D-Mannosamine	0.2984	0.3055	102.3793566
Glycine	0.61205	0.6275	102.5243036
Glucuronamide	0.36735	0.3805	103.5796924
L-threonine	0.2051	0.21295	103.8274013
L-Methionine	0.34705	0.36035	103.8323008
D-Valine	0.314	0.3267	104.044586
L-Glutamic acid	0.6096	0.6358	104.2979003
L-Alanine	0.5592	0.5833	104.3097282
Ethylenediamine	0.2944	0.30925	105.0441576
D-Aspartic acid	0.39665	0.41705	105.1430732

L-serine	0.54675	0.57955	105.9990855
L-Histidine	0.81295	0.8618	106.0089796
Guanine	0.67995	0.7235	106.4048827
Gamma-Amino-N-Butyric acid	0.7899	0.84495	106.9692366
L-Lysine	0.35745	0.38555	107.8612393
L-Arginine	0.53095	0.59015	111.1498258
L-Cysteine	0.25265	0.2881	114.0312686
Thymidine	0.25845	0.3132	121.1839814
Beta-Phenylethyl-amine	0.29895	0.36475	122.0103696
D-Lysine	0.22225	0.27195	122.3622047
Ethanolamine	0.2365	0.29475	124.6300211
D-Serine	0.23725	0.2961	124.805058
Delta-Amino-N-Valeric acid	0.07305	0.09255	126.6940452
D-Glutamic acid	0.218	0.3089	141.6972477
Nitrite	0.25205	0.3599	142.7891291
Uridine	0.23065	0.34135	147.9947973
Hydroxylamine	0.1777	0.2871	161.5644344
Nitrate	0.2319	0.3791	163.4756361
D,L-Alpha-Amino-N-Butyric acid	0.23955	0.39245	163.8280109
D-Glucosamine	0.20915	0.3465	165.6705714
Tyramine	0.2274	0.40615	178.6059807

Appendix II. Raw Data of growth kinetics assay using BIOLOG™ PM3B Microplates. (A) Growth curves of AB030 (Green) and AB408 (Magenta) in different nitrogen sources. (B) Template of a BIOLOG PM3B Microplate™ Nitrogen sources plate. Graphs were generated using DuctApe (Galardini et al., 2014).

(A) **BIOLOG PM3B Microplate**

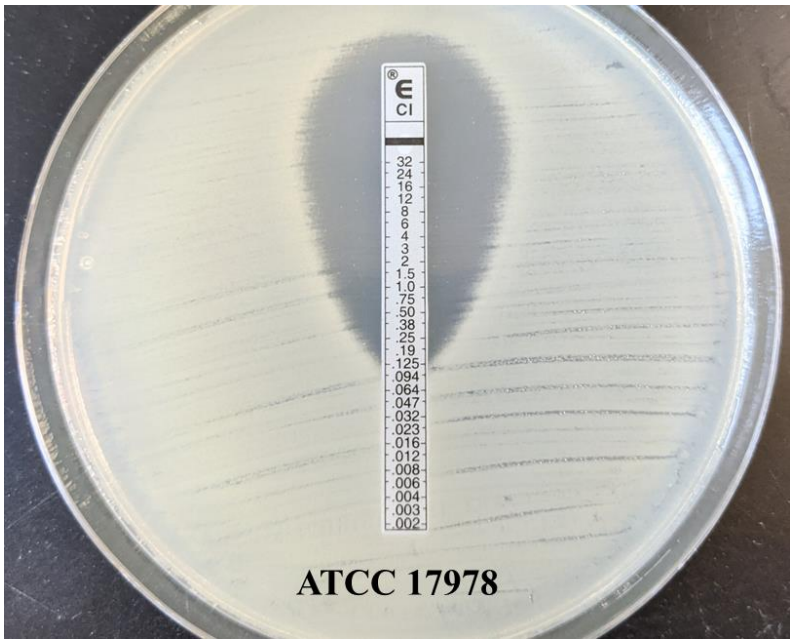


(B)

A1 Negative control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic acid	A11 L-Cysteine	A12 L-Glutamic acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D- Alanine	C4 D-Asparagine	C5 D-Aspartic acid	C6 D-Glutamic acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D1 N-Acetyl-L-Glutamic acid	D2 N-Phthaloyl-L-Glutamic acid	D3 L-Pyroglutamic acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 B-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric acid	G4 Alloxan	G5 Allantoin	G6 Parabanic acid	G7 D,L-α-Amino-N-Butyric acid	G8 γ-Amino-N-Butyric acid	G9 ε-Amino-N-Caproic acid	G10 D,L-α-Amino-Caprylic acid	G11 δ-Amino-N-Valeric acid	G12 α-Amino-N-Valeric acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

Appendix III: Representative images of MIC of (A) ATCC 17978 and (B) AB199 (ATCC 17978: Δ*A1S_1977*-78) for Ciprofloxacin.

(A)



(B)