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Virulence Functions of Proteobacterial Antimicrobial Compound Efflux (PACE) Family of Transport Proteins

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Declaration

This thesis does not contain any material which has been accepted for any other degree in any University or institution and to the best of my knowledge, contains no material written by another person, except where due references have been made within the text. The contributions of other's efforts and involvement has been acknowledged.

Alaska Pokhrel

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Abstract

Acinetobacter baumannii is a significant hospital-acquired pathogen due to its traits such as multidrug resistance. Multidrug efflux systems are one of the major causes of resistance to many antimicrobials in pathogenic bacteria. The multidrug efflux pumps in bacterial pathogens also play an important role in a range of cellular activities other than drug resistance, such as virulence, biofilm formation and the export of secondary metabolites.

Recent data using mouse and insect larval models suggest that the novel multidrug efflux pump AceI may play an important role in animal colonization and virulence. This project investigated the potential virulence functions of the *aceI* gene by performing a range of assays including growth in human serum, attachment to biotic and abiotic surfaces and iron starvation. It was found that *aceI* is not required for the overall fitness of the AB5075-UW for the growth in laboratory media or for the survival of this strain under iron-limiting conditions. However, an *aceI* knockout strain was not viable for growth in 100% human serum. Therefore, *aceI* might play a role in providing resistance to a component of human serum. Furthermore, biofilm formation and quorum sensing studies also suggested potential involvement of *aceI* in the pathophysiology of AB5075-UW.

1 Introduction

1.1 Antimicrobial resistance

Antimicrobial agents and biocides have long been used in various forms for the treatment of infectious diseases and to control the spread of pathogenic bacteria. However, the pathogenic bacteria encode a wide variety of mechanisms that can make them resistant to many otherwise effective drugs, thus creating problems in the medicinal sector. Studies suggest that most of these pathogens have become multidrug resistant (MDR), meaning that they have developed resistance to almost all of the antibiotics that were previously effective in treating clinical infections [1, 2]. Enteric bacterial strains such as *Escherichia coli* and *Salmonella* were among the first detected multiple drug resistant bacteria in the 1960s [3]. Such MDR organisms pose serious clinical problems and render therapies riskier and costlier, thus causing increasing morbidity and mortality particularly in developing countries [1, 3]. Gram-negative bacteria are inherently more resistant or tolerant to antibiotics and biocides due to their cell wall structure and the presence of multiple types of efflux pumps. Among the Gram-negative bacteria, hospital pathogens *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are resistant to almost all available antibiotics challenging the effective treatment of immunocompromised patients [4, 5].

Drug resistance can be intrinsic, adaptive or acquired characteristics of the microorganisms [6-8]. Intrinsic resistance of a bacteria is the characteristic level of susceptibility to a given compound, due to the inherent properties of a bacterial cell [8]. Adaptive resistance refers to the physiological alterations that are induced by environmental changes or by the characteristic expression of efflux pump in response to the drug substrates [8, 9]. Acquired resistance may arise from the mutation of genes involved in normal physiological processes or from the acquisition of foreign resistance genes or from a combination of these two mechanisms [10].

1.2 Multidrug efflux pumps

Multidrug efflux is a ubiquitous mechanism of drug resistance in bacterial pathogens that is mediated by integral membrane transport proteins. These transport proteins are able to recognize a huge range of antimicrobial substrates and transport these substrates across the membrane [11-13]. Efflux pumps are present in all bacterial species and the genes that encode efflux pumps can be located on the chromosome and also in mobile genetic elements, such as a plasmid, which can be acquired by bacterial pathogens through horizontal gene transfer [7, 13-15]. Efflux pumps can be specific to one substrate or one class of drugs (single transporters), or are able to transport a range of structurally dissimilar compounds including many antibiotics and other antimicrobial agents and thus those pumps are referred to as the multidrug resistant pumps [13]. MDR pumps are an important class of resistance determinants in pathogenic bacteria. Many studies have revealed that the bacterial efflux pumps not only confer resistance to antimicrobial agents but are also involved in extrusion of natural substrates produced by the host organisms, including antimicrobial peptides, bile salts and other host defense molecules [13, 14]. Additionally, it is becoming increasingly apparent that multidrug efflux pumps in bacterial pathogens play broader roles in the survival and fitness of a range of pathogenic organisms, due to their involvement in many cellular processes beyond drug resistance, such as virulence, biofilm formation, the removal of metabolic waste products and the export of important secondary metabolites, such as siderophores [9, 13, 15]. Therefore, multidrug efflux pumps are well recognized for their potential as drug targets to interfere with drug resistance and potentially virulence.

Five distinct families of transport proteins have been previously described, which include well characterized bacterial efflux systems: the major facilitator superfamily (MFS), the resistance/nodulation/division (RND) superfamily, the small multidrug resistance (SMR) family, ATP binding cassette (ABC) superfamily and the multidrug and toxic compound extrusion (MATE) family [13, 16] (Figure 1.1). Most of these families of multidrug transporters are distributed throughout all the kingdoms of life indicating their biological importance. The ABC transporters utilize ATP hydrolysis to drive the transport of substrates against their concentration gradient, whereas transporters in the other families utilize the proton motive force (PMF), or the sodium motive force to drive transport [7, 13, 17].

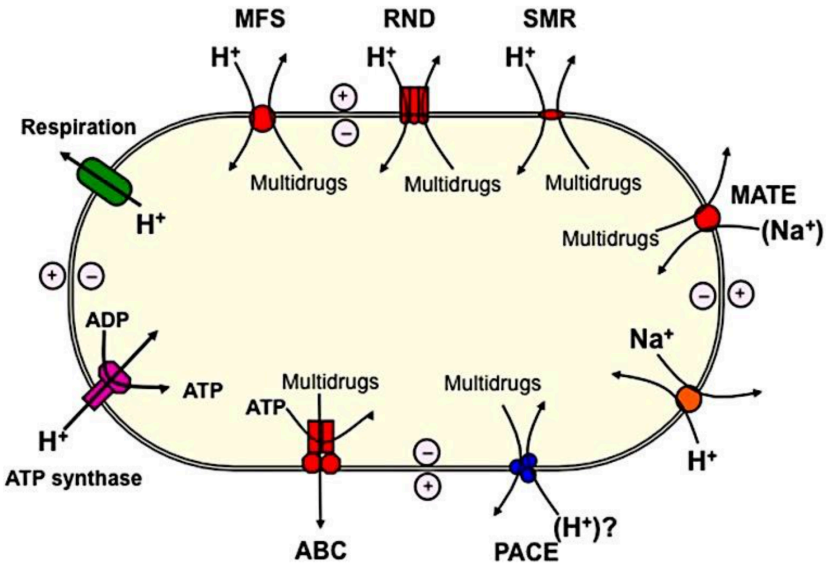


Figure 1.1: Schematic representation of topology and basis for the energization of multidrug efflux pumps operating in Gram-negative bacteria [9].

1.2.1 ATP binding cassette (ABC) superfamily

The ABC transporters comprise the largest known superfamily of integral membrane transporter proteins. These proteins are found ubiquitously in all the classes of living organisms and function in the uptake and extrusion of a range of substrates, including vitamins, metals, amino acids, metabolites, peptides, xenobiotics and chemotherapeutic drugs [18-21]. The functional unit of an ABC transporter consists of two transmembrane domains (TMD), and two cytoplasmic nucleotide binding domains (NBDs), which have ATPase activity [7, 22]. The extrusion of the substrate in this superfamily occurs via an altering access mechanism, where ATP binding and hydrolysis by the NBDs triggers conformational changes within or between transmembrane domains enabling the transportation of the bound substrates across the membrane [22-24]. The best characterized multidrug transporter from this superfamily is human P-glycoprotein which confers resistance to anti-cancer drugs [25]. A recent study by Li *et al.*, described an ABC efflux pump encoded by A1S_1535 from *A. baumannii,* which is the first drug transporter to be characterized from this superfamily in *A. baumannii* [26].

1.2.2 The major facilitator superfamily (MFS)

The MFS is a large and ubiquitous group of secondary active transporters, which function as uniporters, symporters or antiporters. MFS transporters are found across all kingdoms of life [27, 28]. MFS transporters can be divided into at least 74 families and they typically possess 12 transmembrane helices. MFS transporters share a high degree of structural similarity despite

their typically limited sequence homology, distinct substrate specificities and different vectorial mechanisms [7, 17, 29]. They function in the uptake and efflux of a huge range of substrates including metabolites, ions, carbohydrates, nucleosides, peptides and drugs [28-30]. Among all known membrane transport proteins in prokaryotes, a quarter of them belong to the MFS [28]. Members of MFS transporter proteins, namely the *Staphylococcus aureus* multidrug efflux transporter, QacA and the tetracycline transporters TetA(B) from *E. coli* and TetA(K) from *S. aureus* were amongst the first bacterial drug transporters to be described [31]. Crystal structures and other recent biochemical assays have suggested that MFS transporters also operate via an alternating-access mechanism, which involves exposing a single substrate binding site of MFS antiporters to the cytoplasmic and periplasmic sides of the membrane sequentially [28, 32]. There are several characterized MFS efflux pumps operating in *A. baumannii*, including CraA efflux pumps [33], AmvA [34], AedC and AedF [35] and Tet, tetracycline efflux pumps [36]. The Tet efflux pumps in *A. baumannii*, TetA and TetB, confer resistance to tetracycline and minocycline [36]. CraA has been found to be involved in chloramphenicol resistance of *A. baumannii* isolates [33] and the AmvA efflux pump has been identified in almost all of the *A. baumannii* isolates conferring resistance to ethidium bromide, benzalkonium chloride, SDS and other antimicrobials [34].

1.2.3 The multidrug and toxic compound extrusion (MATE) transporter family

The transporters that are classified within the MATE family are also known to be ubiquitous in all the classes of living organisms. MATE transporters mediate the transport via drug H+ or drug Na+ antiport mechanisms and thus are coupled with the proton (or sodium) motive force [37, 38]. The MATE transporter family is now considered the part of a new superfamily called the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase superfamily due to their phylogenetic relationship with the LPS flippase RfbX transporters [30, 38]. The first transporter identified from this family was the Na/cationic agent antiporter NorM from *Vibrio parahaemolyticus* [39], which has 12 transmembrane segments. These transporters confer resistance to aminoglycosides, cationic dyes and fluoroquinolones by mediating transport from the cytosol into the periplasmic space [30, 39-41]. The genes that encode MATE pumps have been cloned from many pathogens, however their contributions to resistance have not been studied in detail. H+ and Na+ play different roles in the transport of substrates by NorM proteins from *Vibrio*, where Na+ stimulates ethidium binding, which then drives proton (H+) release, suggesting these coupling ions might be involved in conformational switches that are

crucial for the transport cycle progression [17, 42]. The only MATE family efflux pump that has been characterized in *A. baumannii* is AbeM, which confers resistance to aminoglycosides, chloramphenicol, ethidium bromide and several other dyes [43].

1.2.4 The small multidrug resistance (SMR) transporter family

The SMR family of multidrug transporters is a member of the much larger drug/metabolite transporter (DMT) superfamily [44]. SMR multidrug transporters are known to be the smallest ion-coupled transporters consisting of four transmembrane domains which function as homodimers [45, 46]. This family of transporters are found in bacteria, for example, *E. coli* EmrE and *S. aureus* QacC multidrug transporters, and in archaea, for example, Hsmr transporter from *Halobacterium salinarium* [47, 48]. EmrE is a proton coupled multidrug transporter from *E. coli* and is one of the extensively studied efflux pumps from the SMR family [49, 50]. EmrE can actively transport a range of monovalent and trivalent amphiphilic cations such as tetraphenylphosphonium (TPP+), acriflavine, ethidium and methyl viologen and many other substrates in exchange with protons [51-53]. The functional transport unit of EmrE utilizes an alternating access mechanism, which facilitates the substrate binding to the negatively charged membrane embedded glutamate residue in the first transmembrane helix. When a substrate occupies the binding pocket, it induces a conformational change in the binding chamber opening it to the opposite side and the subsequent proton binding to the negatively charged glutamyl residue displaces the substrate, reorienting the binding pocket to the inward-facing position [52, 53]. This glutamic acid residue is fully conserved within all the members of the SMR family and is the only essential charged residue in EmrE [50, 51]. A chromosomally encoded SMR efflux pump called AbeS has been found in *A. baumannii* and it has been shown to confer resistance to fluoroquinolones, chloramphenicol, novobiocin and other dyes [54].

1.2.5 The resistance/nodulation/ cell division (RND) superfamily

The RND transporters are widespread in all classes of living organisms and recognize a very broad range of antimicrobial substrates such as tetracycline, chloramphenicol, fluoroquinolones and β-lactams. They are also known to transport detergents, cationic dyes [55] free fatty acids [56] and homoserine lactones, an autoinducer involved in quorum sensing (QS) [57] and disinfectants [17, 58]. AcrAB-TolC, the acridine resistance complex of *E. coli*, is a prototypic and well-characterized member of the RND family [7, 17, 59]. This tripartite

system assembly comprises of secondary efflux pump AcrB embedded in the inner membrane proteins (IMPs); an outer membrane protein (OMP) channel TolC and AcrA, a periplasmic membrane fusion protein (MFP), which connects these membrane proteins [17, 59, 60]. This tripartite complex facilitates the extrusion of substrates from the periplasm or within the inner membrane to the external medium [17, 61, 62]. AcrB is a homotrimer and has a 12-helix transmembrane domain along with the periplasmic section comprising of funnel domains [17, 63, 64]. The mechanism of drug transport involves proton translocation process and the conformational changes of AcrB, suggesting a functionally rotating mechanism of transport cycle that includes substrate access, binding and extrusion [17, 65]. Crystal structure studies have revealed a predominantly hydrophobic pocket within the periplasmic domain, following co-crystallization of AcrB with a substrate [30, 63, 66].

The RND transporters are clinically significant in MDR and are widely spread in Gram-negative bacteria including *Pseudomonas* and *Acinetobacter* species. The *P. aeruginosa* RND family efflux pumps include MexAB-OprM, MexCD-OprJ, MexXY-OprM and MexEF-OprN. All of these transport complexes are able to export a wide range of antibiotics dyes, detergents and other organic solvents [13, 67, 68]. The acylated homoserine lactones (AHLs) that are involved in QS are also substrates of the MexAB–OprM system of *P. aeruginosa* [13, 69, 70]. The *Acinetobacter* RND drug efflux systems include AdeABC, AdeFGH and AdeIJK. AdeABC was the first RND efflux pump described in *A. baumannii*, where AdeB was the secondary efflux pump protein; AdeA, periplasmic MFP and AdeC, an outer membrane protein [71]. Expression of each of these pumps is tightly regulated. The AdeABC efflux pump is regulated by two-component regulatory system called AdeRS, which controls *adeABC* gene expression [72]. Overexpression of the *adeABC* operon, following mutations in *adeRS* plays an important role in efflux mediated resistance in nosocomial pathogens [72, 73]. AdeFGH is regulated by the LysR-type transcriptional regulator AdeL and mutation within the *adeL* gene has been found to be associated with the overexpression of *adeFGH* gene [73] and AdeIJK efflux pump is regulated by a TetR family transcriptional regulator called AdeN [72, 73].

1.2.6 Proteobacterial antimicrobial compound efflux (PACE) family

A recent study by Hassan *et al.,* has described the novel AceI (*Acinetobacter* chlorohexidine efflux) protein from *Acinetobacter baumannii*, which conferred resistance to the widely used biocide chlorhexidine, via an active efflux mechanism [11, 12]. This gene was overexpressed by more than 10-fold in response to a sub-inhibitory chlorhexidine shock in *A. baumannii* ATCC17978 strain [12]. Heterologous expression in *E. coli* of *aceI* or homologs from *Acinetobacter baylyi* (ADP1), *Pseudomonas protegens* (Pf-5) and *A. baumannii* conferred resistance to chlorhexidine, and increased susceptibility to chlorhexidine was observed with an *aceI* knockout strain [11, 12]. Proteins that are homologous to AceI are encoded in the genome of many bacterial species and are particularly common within proteobacterial lineages. A subsequent study showed that homologs of AceI were able to confer resistance to a range of other biocides in addition to chlorhexidine [11]. These studies led to the finding that the group of AceI and its homologs represent a new class of multidrug efflux systems which is designated the "Proteobacterial antimicrobial compound efflux (PACE)" family of multidrug transporters. The PACE family is the sixth family of multidrug efflux systems and the first to be described in 15 years. Members of the PACE family confer efflux mediated resistance to structurally distinct biocides and are highly conserved in the genomes of several major opportunistic pathogens including *Acinetobacter, Klebsiella, Pseudomonas, Salmonella* and *Burkholderia* species. Importantly, a recent study suggested that genes encoding PACE family pumps maybe important for animal colonization and/or virulence [74].

The PACE family transporters that have been studied to date are each encoded adjacent to a divergently transcribed LysR family regulator [9]. The LysR family regulator adjacent to *aceI* in *Acinetobacter* has been designated *aceR* [9]. As previously mentioned, substrates of AceI such as chlorhexidine, are able to induce expression of AceI in wild-type *Acinetobacter* strain. However, in the *aceR* deletion strain of *Acinetobacter*, *aceI* expression was not induced by this substrate. Furthermore, similar to an *aceI* deletion strain, an *Acinetobacter aceR* mutant was susceptible to chlorhexidine. Recent biophysical assays showed that AceR can bind to AceI substrates and electrophoretic gel mobility shift assays and DNase I foot printing also demonstrated that the purified AceR protein was able to bind to the *aceI/aceR* intergenic region, suggesting *aceR* is a positive regulator of *aceI* gene expression (Liu *et al*., unpublished).

1.3 Physiological roles of MDR efflux pumps

In addition to antimicrobial resistance, the MDR pumps in Gram-negative bacteria are involved in many other physiological processes including bacterial resistance to host derived molecules, cell adherence, invasion, virulence/colonization in animals/plants and biofilm formation [13, 75].

1.3.1 Roles in bacterial pathogenicity and virulence

In order to infect a host, pathogenic bacteria must resist host defenses, acquire nutrients in the host environment and in some cases, produce specific toxins. Some multidrug efflux pumps are essential in these activities and could be considered as pathogenicity factors. For example, some multidrug efflux pumps from Gram-negative bacteria transport not only antimicrobials and other substrates including host encoded factors such as, antimicrobial peptides (AMPs), bile salts, free fatty acids (FFAs) and polyamines [13, 76]. Host encoded factors are the integral components of the host defense mechanisms against the wide range of invading microorganisms.

AMPs are an intrinsic and important part of the innate immune system in human and other vertebrates. These are relatively small size host defense peptides with a net positive charge and are involved in a broad range of antimicrobial activity. Studies have shown that AMPs inhibit or kill bacteria using multiple mechanisms, which includes binding to cell wall materials usually lipopolysaccharides and peptidoglycan; by using electrostatic reactions to penetrate the phospholipid bilayer acids or by directly attacking intracellular targets without damaging the host tissue [77, 78].

FFAs are another type of host defense molecule, which is normally found on the surface of the skin. FFAs are produced on the skin by the lipolytic cleavage of lipids secreted by the sebaceous glands and the biological activities of these molecules have roles in defense of skin and mucosal surfaces [79, 80]. The antimicrobial protective mechanisms mediated by FFAs include maintenance of acidic pH to make surface conditions unfavorable for bacteria to adhere and grow [81] and disrupted expression of bacterial virulence factors such as cell to cell signaling [82, 83].

Polyamines are aliphatic polycationic molecules, that are found in almost all cells of living organisms and are involved in a broad range of physiological processes including cell

proliferation, membrane stabilization, regulation of gene expression [76, 84] biofilm formation [85] and also in microbial carcinogenesis [86].

Bile salts are an excretory and digestive secretion that promote lipid absorption in the small intestine, which are able to mediate antimicrobial effects in distal small intestine. These molecules regulate the expression of host defense genes, thus promoting intrinsic defense against luminal bacteria [87, 88].

Bacteria have evolved a number of different strategies to respond to these host derived molecules that they encounter at various sites in the body. One of these mechanisms is active transport of these natural toxic substrates. In addition to the natural substrates, the bacterial efflux pumps might also be involved in secretion of other virulence factors such as adhesins and toxins that are important for colonization and infection [13, 89]. Studies have shown that *E. coli, Salmonella typhimurium* and *Campylobacter jejuni* mutants lacking components of AcrAB-TolC or CmeABC pumps are hyper susceptible to fatty acids and bile salts that are present in natural environment of enteric pathogens. Whereas, overexpression of these pumps render them resistant to long chain fatty acids and high concentration of bile salts [90-93]. Furthermore, Bina *et al*., reported the importance of TolC for the resistance to bile in *Vibrio cholera* [91]. A study by Lin and colleagues also showed that the CmeABC pumps mediate resistance to bile salts in chicken intestinal tracts thus enabling *C. jejuni* to colonize in chickens [90]. Similarly, *S. aureus* has been studied in a mouse skin infection model in the presence of fatty acids and polyamines, which were found to be the natural substrates of chromosomally encoded the Tet38 efflux transporter [94]. The MtrCDE system has been shown to be important for the survival of gonococci in the genito-urinary tract of female mice and also in mice that secrete gonadal hormones [95]. This MtrCDE efflux pump has also been shown to resist antimicrobial peptides such as human LL-37 and porcine protegrin-1 [96, 97], suggesting RND efflux pumps also include hormones in their substrate profile.

Similarly, MDR efflux pumps from *P. aeruginosa* were found to control various genes required for the virulence and resistance to antimicrobial peptides [98, 99]. The *P. aeruginosa* produce highly acylated lipid A species and aminoarabidose modified lipid A species, thus preventing antimicrobial peptides binding and disruption of the bacterial surfaces [100]. A study on *P. aeruginosa* by Hirakata *et al*., reported that the mutants lacking components of MexAB-OprM efflux pump were not able to invade the epithelial (Madin Darby canine kidney (MDCK)) cells but after the complementation of the strain that invasion could be restored [99].

Furthermore, *P. aeruginosa* isolates that overexpress Mex efflux pumps were identified in an experimental model of *P. aeruginosa* induced acute pneumonia in rats [101]. These findings implicate Mex efflux pumps from *P. aeruginosa* are involved in virulence. Likewise, reduced bacterial fitness and host virulence was observed in *Enterobacter cloacae* with disrupted AcrAB-TolC [102].

1.3.2 Roles in cell to cell communication (Quorum Sensing)

Quorum sensing (QS) is a regulatory mechanism, which allows bacterial cells to communicate with each other by producing signaling molecules called autoinducers such as acylated homoserine lactones (AHL). QS mainly relies on the cell population density and upon reaching a certain concentration, the cell to cell communication is established [103]. The autoinducers specifically bind to the transcriptional regulators altering gene expression and other physiological activities. QS is required in bacterial populations to coordinate social activities, such as that require a large population to act in unison to mediate a meaningful outcome. For example, the production of virulence factors, motility, conjugation, antibiotic production, sporulation, bioluminescence and biofilm formation are all QS controlled activities [103, 104]. The prototypical QS system is from the bioluminescent marine bacterium *Vibrio fischeri* [105]. The light emission by this bacterium was studied to be heavily correlated with the cell population density and controlled by QS. The luciferase enzymes involved in light production in *V. fischeri* are encoded by *luxCDABE*, a part of the *luxICDABE* operon [106, 107]. In this organism, the QS system is comprised of two regulatory proteins called LuxI and LuxR. LuxI is an autoinducer synthesizing enzyme and is involved in the production of AHL, which binds to LuxR and the LuxR-AHL complex, then binds to the *luxICDABE* promoter activating the transcription [106, 108].

QS in the opportunistic pathogen *P. aeruginosa* is also controlled by two pairs of LuxI/LuxR homologs named LasI/LasR and Rh1I/Rh1R. Both of these autoinducer synthases LasI and Rh1I are involved in the production of autoinducers called *N*- (3-oxododecanoyl)-homoserine lactone (3-oxo-C12-HSL) and *N*-(butryl)-homoserine lactone (C4-HSL) respectively. The QS mechanism in *P. aeruginosa* also includes two quinolone signaling molecules called PQS and HHQ [109-111]. These QS circuits function to control the regulation and expression of *P. aeruginosa* virulence factors. QS also plays a vital role in regulating biofilm related genes in *P. aeruginosa* enabling it to persist in a desiccated state for a long period of time [112]. QS has been linked to the generation of extracellular DNA in *P. aeruginosa* biofilms [113] and also in

the production of a biosurfactant rhamnolipid which is important for biofilm formation in *P. aeruginosa* [114]. QS signaling in *P. aeruginosa* also controls the production of siderophores, which are required for iron acquisition [115].

The QS autoinducers synthesized *in vivo* need to be exported to the extracellular matrix for the QS regulatory functions. The ability of these autoinducers to diffuse across the membrane differ according to their size. Many studies have suggested a link between the expression of RND efflux pumps and the QS regulatory mechanism in *P. aeruginosa* [100, 110, 116]. Pearson *et al*., reported the increased secretion of 3-oxo-C12-HSL, a LasI derivative during the over expression of MexAB-OprM pump, and deletion of the *mexAB-oprM* resulted in decreased secretion of this autoinducer suggesting this autoinducer molecule is secreted by this MexAB-OprM efflux pump [57]. Mutations in another RND multidrug efflux system encoded by *mexEF-oprN* resulted in decreased intracellular concentrations of 3-oxo-C12-HSL suggesting this could also be a substrate for the MexEF-OprN pump [57, 110]. Further investigations on this pump have shown the substrate profile of this pump also include non-native N-acylated homoserine lactones and their related derivatives [117]. However, short chain autoinducers of *P. aeruginosa* like C4-HSL and 3-oxo-C6-HSL of *Photobacterium fischeri* could freely diffuse across the bacterial cell membrane, suggesting efflux pumps are involved in transport of large autoinducers from the bacterial cell [57, 110].

Similarly, RND pump MexEF-OprN had been found to be involved in active extrusion of intracellular signaling molecule PQS [15]. These signaling molecules are reported to play a role in the formation of extracellular biofilm matrix in conjugation with autoinducers [113]. Another RND efflux pump MexGHI-OpmD was also reported to export a toxic metabolite called anthranilate and the precursor of PQS autoinducer [118]. The involvement of MDR efflux pumps in QS has also been studied in other bacterial species. BCAL1675 and BCAL2821 pumps from *B. cenocepacia* have also been found to mediate extrusion of N-octanoyl homoserine lactone, an autoinducer of this pathogen [119]. Furthermore, Martinez *et al*., reported that the efflux pumps enable the elimination of QS response in bacteria by increasing the efflux of autoinducer molecules and its precursors, thus allowing bacteria to quickly respond to the changes in its ecological niche [120].

1.3.3 Roles in biofilm formation

Microorganisms typically do not live as dispersed single cells but instead they accumulate to form aggregates or clusters such as biofilms. The microbial communities in biofilms live in a self-produced matrix composed of extracellular polymeric substances (EPS) such as, extracellular DNA, lipids, proteins, fibers and polysaccharides that form an immediate environment for those microorganisms [121]. The EPS serves as a protective barrier from the host immune system; provide structural stability of the biofilm, and enables cell adherence to solid surfaces. Most prolonged and persistent bacterial infections are associated with their ability to form biofilms [122]. Biofilms may also have accelerated the emergence of MDR bacteria, because concentrations of most antibiotics are typically lower inside the biofilm environment [123]. Biofilm formation and development consists of series of coordinated steps which includes initial attachment of the bacteria to the biotic/abiotic surface and formation of micro colonies followed by the development of biofilm structures where the bacterial cells are enclosed in EPS thus allowing intense microbial interactions, followed by biofilm detachment and dispersal into the surrounding environment [121, 124]. Bacterial clusters can also form at the air-liquid interface and these aggregates are generally referred to as a "pellicle". A pellicle is a floating structure observed on the liquid surface and thus requires a higher level of organization, as it does not possess the solid surface for it to initially establish an attachment. This air-liquid interface is known to be favorable for the aerobic bacteria that can obtain nutrient from the liquid media and oxygen from the air [125]. The general factors that influence biofilm formation include nutrient availability, hydrodynamic concentrations, bacterial motility and intracellular communication (QS), as well as biofilm associated proteins and exopolysaccharides. Biofilms are considerably more resistant to host defense mechanisms, antimicrobial treatments, UV lights and desiccation enabling the microbes to persist in harsh environments such as clinical settings [126-128].

Several studies have reported that the defects in efflux pump genes impairs biofilm formation suggesting the role of efflux pumps in biofilm formation. A study by Kvist *et al*., also showed that the use of efflux pump inhibitors (EPIs) in *E. coli* and *Klebsiella* strains reduced the amount of biofilm formation and with subsequent treatment with more EPIs abolished the formation of biofilm completely [129]. Another study reported compromised ability of *Salmonella* in biofilm formation due to the chemical inhibition or genetic inactivation of efflux pumps [130]. Similar results were observed in other species including *P. aeruginosa* and *S. aureus* with the

use of EPIs, further supporting the involvement of efflux pumps in biofilm formation and regulation [131].

Several factors have been shown to influence the adhesiveness and biofilm formation in *A. baumannii*. The CsuA/BABCDE pilus chaperone-usher system has been demonstrated to be essential for the initial adhesion, surface colonization and subsequently in biofilm formation of *A. baumannii* [132]. In addition, the outer membrane protein OmpA has been reported to be involved in the development of robust biofilms on abiotic surfaces and bacterial attachment to human alveolar epithelial cells [133]. Similarly, the outer membrane protein homolog of staphylococcal biofilm-associated protein (Bap) and other Bap like proteins such as BLP1 and BLP2 were also found to be involved in adherence to the epithelial cells and biofilm formation in *A. baumannii*. Inactivation of these biofilm associated genes resulted in reduced ability of *A. baumannii* to form thick biofilms and water channels [134, 135]. The *A. baumannii pgaABCD* gene cluster encodes the biosynthesis of the polysaccharide poly-β-(1-6)-N-acetyl glucosamine (PNAG), which is also critical for biofilm formation [136]. A recent study reported that the overexpression of *Acinetobacter* drug efflux (Ade) RND systems such as AdeABC and AdeIJK impairs biofilm and pellicle formation in *A. baumannii* [137]. Mutants defective in the Ade efflux pump had decreased fitness and persistence in mouse pneumonia model [128]. Similarly, the deletion of the *A. baumannii* two component system AdeRS, which regulates the expression of RND MDR efflux pump gene *adeABC,* has led to reduced multidrug efflux, virulence and biofilm formation on mucosal tissue in an *ex vivo* model, suggesting inhibition of multidrug efflux pumps might help prevent bacterial colonization in patients associated with *A. baumannii* infections [73, 128].

1.3.4 Roles in nutrient acquisition

Humans and other vertebrates maintain extracellular free metals at low levels through several mechanisms including the expression of metal-binding proteins and intracellular localization, limiting the metal availability to invading pathogens. Thus, for these pathogens to adapt to host niches, they must possess metabolic flexibility and employ specialized systems for nutrient acquisition. Metal limitation is thus considered a host defense mechanism against the invading microbes [138, 139]. One of the challenges that the host niches present for the bacterial pathogens to overcome is iron acquisition. Iron is a micronutrient that is necessary for almost all living organisms. In the human host, most of the iron is sequestered by high affinity iron binding proteins, such as lactoferrin, transferrin and hemoglobin in order to control microbial

infections and also to avoid cytotoxic effects [139].

Many studies have demonstrated the response of various bacteria in iron-limiting conditions and reported that the bacterial pathogens secrete iron chelating compound called siderophores, which help them acquire iron from the host iron binding proteins [140, 141]. Siderophores are low molecular weight iron chelators, which are able to bind Fe^{3+} from transferrin and lactoferrin with high affinity. The bound siderophore-iron complexes are too large to diffuse through the membrane and thus require an energy dependent transport by outer membrane TonB-dependent receptors for transport into bacterial cells. These TonB proteins, encoded by many Gram-negative bacteria are required for different purposes in addition to metal transport [142-144]. Another mechanism that bacteria employ to respond to low iron availability in the host is the use of iron dependent repressor ferric uptake regulator (Fur) to regulate gene expression, which works by binding the conserved FUR box DNA sequence upstream of target genes. Fur controls expression of siderophores and the other iron acquisition systems, as well as expression of some iron dependent enzymes and iron storage systems [138, 143].

Similar mechanisms have been observed in *A. baumannii* in iron-limiting conditions. *A. baumannii* is known to encode the synthesis of several iron chelating siderophores, and the most studied *A. baumannii* siderophore is acinetobactin, which was found in numerous clinical isolates. Acinetobactin is composed of equimolar amounts of 2,3-dihydrobenzoic acid (DHBA), N-hydroxy histamine and threonine [138, 145]. A study investigated *A. baumannii* under iron limitation and reported the involvement of efflux pump genes in recognition and transport of siderophores. MFS efflux pump named A1S_1649 was found to facilitate the extrusion of siderophores and PepSY-associated transmembrane helix family protein were reported to be involved in recognizing and reducing the ferric siderophores [140, 146]. Another efflux pump A1S_2562, which is a member of MATE superfamily was also reported to have a putative role in siderophore efflux [140]. Among the sequenced strains of *A. baumannii*, at least five gene clusters required in siderophore synthesis and transport have so far been discovered and the genes that are involved in synthesis, uptake and extrusion of the siderophores are usually clustered within the bacterial genome [144, 147]. The study by Eijkelkamp *et al.,* [140], also examined the transcriptional changes in *A. baumannii* ATCC17978 after exposure to the iron chelator 2,2-dipyridyl (DIP). Most of the genes in three siderophore biosynthesis gene clusters were highly up-regulated under iron-limiting conditions demonstrating the importance of iron uptake in this bacterial pathogen [140]. Another study

reported a decrease in expression in all siderophore clusters as the FUR box was moved away from those genes, suggesting the potential requirement of FUR regulation in production of siderophores [140, 144]. All of these findings point to the importance of iron in *A. baumannii* virulence.

1.4 Species of interest: *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram negative bacillus, that has been designated as a "red alert" human pathogen in clinical settings due to its high level of multidrug resistance [4, 148]. It is an opportunistic pathogen which is associated with a wide range of nosocomial infections including ventilator associated pneumonia, meningitis, bacteremia, urinary tract infections and other wound infections [149, 150]. These infections are facilitated by traits such as, prolonged survival in a hospital environment enabling nosocomial transmission and its ability to rapidly acquire multidrug resistance mechanisms [9, 75]. In addition to nosocomial infections, *A. baumannii* is also associated with community acquired infections such as, community acquired pneumonia, battlefield injuries in soldiers and infections associated with natural disasters in many countries [149]. *A. baumannii* has been known to use major resistance mechanisms such as drug or enzymatic inactivation, target site modifications, reduced cellular uptake or increased extrusion from the cells as a result of efflux mechanisms [75, 137, 151]. The multidrug efflux pumps in *Acinetobacter* may play a significant role in survival and fitness of the organisms, due to their involvement in a range of cellular activities other than drug resistance; such as virulence, biofilm formation and the export of secondary metabolites [13, 152].

The AB5075-UW strain belongs to the first international clonal complex group (ICCI), one of the two major clonal lineages of *A. baumannii* [75]. This strain was isolated from a soldier with osteomyelitis. The comparatively recent isolation and MDR phenotype makes it a more representative strain of current clinical outbreaks compared with older strains classically used in scientific studies [153]. AB5075-UW is a good model strain for the study of *A. baumannii*, due to its genetic tractability and its high virulence in animal models [75]. These traits of AB5075-UW makes it a good candidate for virulence and pathogenic studies of this species as well as other novel therapeutic studies [75].

1.5 Objective of the study

A recent study by Gebhardt *et al*., used transposon insertion sequencing (TnSeq) to identify potential virulence determinants of the contemporary virulent MDR isolate of *A. baumannii*, AB5075-UW. This study revealed the importance of 300 genes for the survival and/or growth of *A. baumannii* inside *Galleria mellonella* larvae. The set of genes also included the *aceI* gene and its positive regulator *aceR* from recently described PACE family [74]. Considering those findings, we examined the role of AceI in virulence using a mouse model in collaboration with Prof Anton Peleg (Monash University). This experiment also demonstrated an almost complete loss of virulence in an *aceI*-inactivated mutant compared to the parental strain (Figure 1.2). The bacterial load of the wild type AB5075-UW and an *aceI*-inactivated mutant in different organs of mice was examined after colonization. A reduced load of bacteria in organs infected with an *aceI*-inactivated mutant compared to the parental strain was observed (Figure 1.3). These results suggest that AceI plays a significant role related to virulence and colonization.

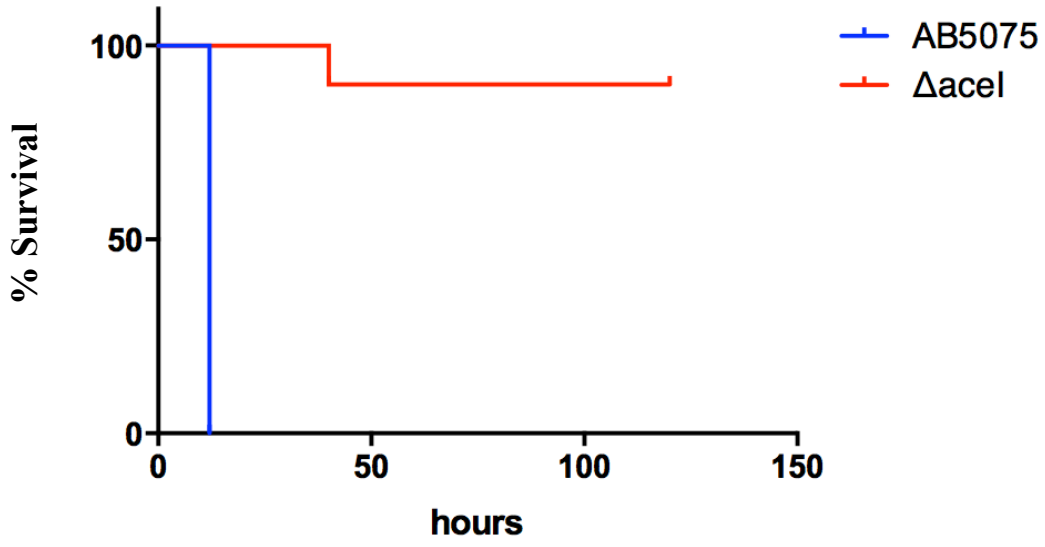


Figure 1.2: Survival curves of mice (10/group) infected with *A. baumannii* AB5075-UW (blue) and *aceI* inactivated mutant (red) (Monash University).

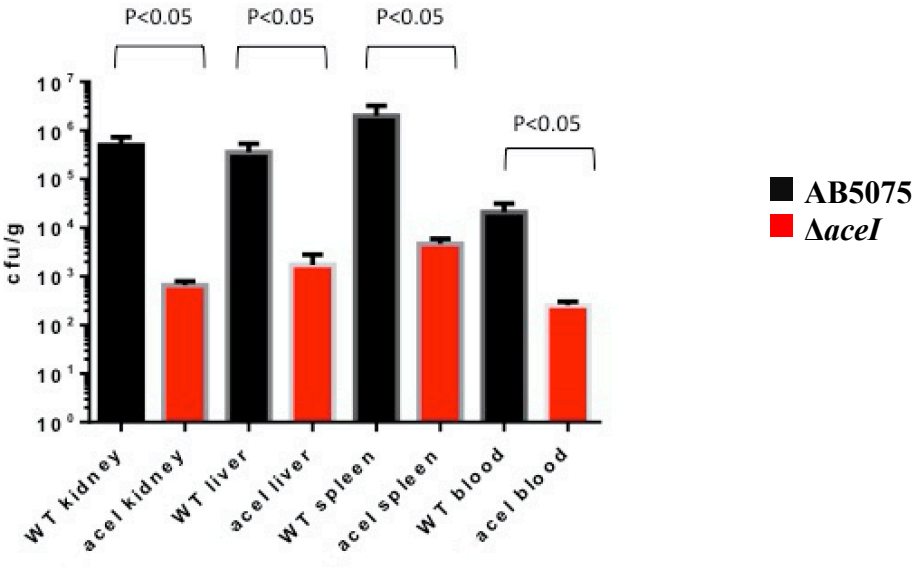


Figure 1.3: Bacterial load of *A. baumannii* AB5075-UW (black) and *aceI* inactivated mutant (red) in mouse organs/tissues (Monash University).

Since clinical isolates of *A. baumannii* are increasingly resistant to antimicrobials, this raises a disturbing potential link between virulence and multidrug resistance. As previously mentioned, multidrug efflux pumps are highly conserved in the genomes of many bacterial pathogens [14]. Many studies have demonstrated the role of MDR efflux pumps in other physiological functions beyond bacterial cell detoxifications, which included bacterial survival and persistence within the host organism either by recognizing a broad range of substrates including host defense molecules or by facilitating bacterial cell communication (QS), thus enabling prolonged persistence of the bacterial pathogens within the ecological niche [13, 74]. However, it is still not very clear that the main role of multidrug efflux pumps is to protect the bacterial pathogens against antimicrobial therapy. Furthermore, the ability of clinical isolates of *A. baumannii* to tolerate stress conditions and form biofilms enabling themselves to persist in clinical settings illustrates the survival mechanism of these bacterial pathogens [4]. These recognized roles of multidrug efflux pumps are likely to contribute in virulence and pathogenesis.

In addition, the preliminary data (Figure 1.2 and 1.3) suggested a strong selection against the loss of *aceI* gene for the growth and colonization in the animal infection models. Therefore, the aim of my MRes Y2 project was to investigate the roles of AceI in colonization and

virulence. An *aceI* knockout mutant strain of AB5075-UW was compared to the wild type AB5075-UW strain using a range of assays. Minimum inhibitory concentration (MIC) assays were used to examine resistance to substrates and stress tolerance under iron-limiting condition; growth in laboratory media/human serum to investigate the fitness defect of the parental AB5075-UW strain and *aceI*-inactivated mutant. This project also investigated the role of AB5075-UW and the *aceI*-inactivated mutant in biofilm formation and cell adherence in order to analyze the ability of *A. baumannii* to adhere to biotic and abiotic surfaces.

2 Material and Methods

2.1 Materials

2.1.1 Bacterial strains and storage

Bacterial strains used in this study are listed in Table 2.1. *Acinetobacter baumannii* AB5075-UW was isolated from a wound infection of a soldier [153], it has been fully sequenced (NCBI accession: PRJNA243297) and subjected to transposon mutagenesis to generate a mutant library [153].

Bacterial AHL biosensor strains, *Agrobacterium tumefaciens* A136 [154] and *Chromobacterium violaceum* CV026 [155] were obtained from the University of New South Wales. The α-Select gold competent *E. coli* DH5α cells were obtained from Bioline. All bacterial strains were preserved at -80°C in 25-30% glycerol for the long-term storage.

2.1.2 Bacterial culture media and growth conditions

Growth media were sterilized by autoclaving at 121° C for 20-30 minutes. Unless otherwise stated, bacterial strains were routinely cultured on Luria Bertani (LB) broth (Becton Dickinson and company) or Muller Hinton (MH) broth (Becton Dickinson and company). For selection of single colonies in plate culture, bacterial strains were grown at 37°C on LB agar plates. Where required, appropriate antibiotics were used at a required concentration for the selection in both broth and plate culture. Measurements of bacterial growth were made by determining the optical density of the growing broth cultures at 600nm (OD_{600nm}) using a BioPhotometer (Eppendorf).

Table 2.1: Bacterial strains, plasmid and human cell line used in this work and their relevant characteristics.

Strain or plasmid	Relevant characteristic(s)	Source/reference
A. baumannii		
AB5075-UW	Wound isolate	[153]
ABUW_1673-156::T26	*aceI* knockout mutant	This work
P. aeruginosa		
PAO1	Laboratory reference strain (Burn wound isolate)	[156]
C. violaceum		
CV026	AHL biosensor strain	[155]
A. tumefaciens		
A136	AHL biosensor strain	[154]
E. coli		
DH5α gold competent cells	Cloning host	Bioline
Plasmids		
pWH1266	Shuttle vector *(E. coli-A. baumannii)*; *Acinetobacter lwoffii* plasmid cloned into the pBR322 PvuII site; Ampicillin resistance (Amp^r), Tetracycline resistance (Tc^r)	[157]
Cell line		
A549 (ATCC® CCL-185™)	Human type II pneumocyte cell lines	[158]

2.1.3 Oligonucleotide primers

Oligonucleotides were obtained from Integrated DNA Technologies. The pair of primers used for polymerase chain reaction (PCR) and DNA sequencing are listed in the Table 2.2.

Table 2.2: Primers used in this work.

Primer name	Sequence 5'-3'
aceI_compf	GCACTGCAGTACTCACAGCAGAGGGGACT
aceI_compr	GCACTGCAGCCATCTGGGTGCTTATTTCA

2.2 Methods

2.2.1 Complementation of the *aceI* inactivated mutant

2.2.1.1 Plasmid/Genomic DNA isolation

Well-isolated single colonies from each bacterial transformation plate were used to inoculate 5-10 ml of sterile LB broth with appropriate antibiotics. The inoculated cultures were incubated overnight at 37°C on a shaker at 200rpm and bacterial cells were harvested by centrifugation. Plasmid DNA was isolated using the Promega Wizard SV plus Miniprep DNA purification kit, according to the manufacturer's instructions. Genomic DNA was extracted using QIAGEN DNeasy blood and tissue kit following manufacturer's instructions. DNA concentrations were determined using a Nanodrop ND1000 spectrophotometer.

2.2.1.2 Restriction endonuclease digestion

DNA restriction endonucleases were obtained from New England Biolabs (NEB) and were used with supplied buffers, according to the manufacturer's instructions.

2.2.1.3 DNA ligation

Purified restriction endonuclease digested DNA fragments were diluted to a vector:insert ratio of approximately 1:3 in a 15-20 µl volume containing 1 unit of T4 DNA ligase (Promega) and 1X T4 DNA ligation buffer, supplied by the manufacturer. The ligation mixture was incubated overnight in an ice bath at starting temperature of <4°C to a final temperature of approximately

17°C. The ligation mixture was then transformed into α-Select gold competent *E. coli* DH5α cells.

2.2.1.4 Transformation of competent *E. coli* cells

Aliquots of competent *E. coli* DH5α cells (20- 50µl) were thawed on ice prior to use. A 0.5-1µl aliquot of purified pWH1266 plasmid DNA (approximately 100ng) or 5 µl of ligation mixture containing approximately 100-200 ng (vector and insert combined) was added to an aliquot of competent *E. coli* cells and incubated on ice for 45 minutes. The cells were then heat shocked by heating to 42°C for 30 seconds and allowed to recover on ice for 2 minutes. Ten volumes of LB broth was added to each transformation mix and cells were incubated at 37°C for approximately 1 hour. The transformed cells were plated onto solid LB agar plates containing appropriate antibiotic selection (100 µg/ml ampicillin or 10 µg/ml tetracycline) and incubated overnight at 37°C. Single isolated colonies were chosen for further analysis.

2.2.1.5 Colony screening PCR

Aliquots of colony screen mix for the colony screening PCR were prepared in 0.5ml PCR tubes. Each 12µl mix contained 10mM of dNTPs, 1 unit of GoTaq® polymerase (Promega), 1X of PCR buffer (supplied by the manufacturer), 25mM of $MgCl_2$ and 5µM of each primer (Table 2.2). Well-isolated colonies from the bacterial transformation plates were transferred into the LB plates containing 10 µg/ml tetracycline and a part of the same colony was also transferred into the PCR colony screen reaction mixture as a template for colony PCR plasmid insert screening. The PCR reaction mixtures containing the bacterial colony material were transferred to an Eppendorf thermal cycler for DNA amplification. DNA was amplified using the GoTaq® DNA polymerase and the primers listed in Table 2.2. PCR reactions were cycled using an Eppendorf thermal PCR cycler. Cycling conditions included a 30 second denaturation step at 95°C, a 20 second annealing step at 55°C and 5-minute extension step at 72°C. The PCR products were separated by agarose gel electrophoresis and visualized under UV trans illuminator (Gel Logic).

2.2.1.6 DNA sequencing

Plasmid DNA was isolated using the Promega Wizard SV plus Miniprep DNA purification kit, according to the manufacturer's instructions. DNA sequencing samples were prepared by mixing 600-1500ng of purified plasmid DNA with 10pmol of an appropriate sequencing primer

in 12µl volume. DNA sequencing reactions were performed at the Australian Genome Research Facility (AGRF), Sydney.

2.2.2 Minimum inhibitory concentration (MIC) analysis and growth curve assays

The minimum inhibitory concentrations of an iron chelator 2,2'-dipyridyl (DIP) (Sigma Aldrich), human serum (Sigma Aldrich) and bile salt mix (approximately 50% sodium cholate and 50% sodium deoxycholate) (Sigma Aldrich) were determined using a broth dilution method. The wild type AB5075-UW and its *aceI* inactivated mutant were diluted to 1:50 dilution from overnight cultures and grown to OD_{600nm} of 0.6 in 10ml cation-adjusted MH-broth. Stock solution of compounds at the desired starting concentration (100% for human serum, 10mM for DIP, 100mg/ml for bile salt mix) were diluted in cation adjusted MH-broth. The cultured cells were then inoculated into broth micro dilution plates with two-fold serial dilutions. The 96 well microtiter plates were incubated at 37°C, 200rpm for 24 hours and following incubation, optical density was measured at 600nm using the PHERAstar FS-BMG Labtech plate reader. The MIC was determined as the lowest concentration of any compound required to fully inhibit bacterial growth as previously described [159] .

For the growth curve analysis, the serially diluted subculture (with/without any compound) in 96 well microtiter plates were incubated at 37°C with shaking at 400rpm for 24 hours in PHERAstar FS-BMG Labtech plate reader with optical density being measured every 6 minutes at 600nm.

For the bacterial growth analysis in human serum, a 1:50 dilution from overnight cultures were grown to OD_{600nm} of 0.6 in 10ml MH-broth, which were pelleted and resuspended in the sterile 0.01M phosphate buffered saline (PBS). Following resuspension of the pellets, optical density (OD_{600nm}) was measured and adjusted to OD_{600nm} of 0.6 for both parental AB5075-UW and an *aceI* inactivated mutant, which were then subjected to 2-fold serial dilution in PBS. The serially diluted cells were used to inoculate human serum and 1µl of tetrazolium redox dye was added to the inoculated wells. The plate was incubated at 37°C for 48 hours in an Omnilog Phenotype Microarray system (Biolog Inc.) with the bacterial growth being measured every 15 minutes as a colorimetric change in the tetrazolium redox dye. This method of growth determination was required for serum due to its high opacity and interference with OD_{600nm} measurements.

2.2.3 Biofilm formation assay and scanning electron microscopy (SEM)

Overnight cultures of the parental AB5075-UW parental and the isogenic *aceI* inactivated mutant were diluted 1:100 in fresh MH-broth and the diluted cultures were dispensed into 96 well microtiter plates. The inoculated 96 well-plates were incubated aerobically at 37°C for different time points (24h, 48h, 72h and 96 h). Following incubation, the plates were gently shaken to sufficiently mix the unbound cells and the optical density of planktonic cells was measured at 600nm using the PHERAstar FS-BMG Labtech plate reader. The supernatant from each well was removed and each well was gently washed twice with 0.01M sterile PBS. The wells containing biofilms were stained with 0.1% crystal violet (CV) for 15-20 min. After staining, each well was washed with 0.01M sterile PBS twice and air dried for 30 minutes. The biofilm in each well was then destained and solubilized by adding 80% ethanol. The solubilized biofilm cells were diluted 1:10 in fresh 80% ethanol and the optical density was measured at 570nm using the PHERAstar FS-BMG Labtech plate reader. A negative control was setup with the wells containing sterile MH-broth only to measure the background. The OD_{570}/OD_{600} ratio was then used to normalize the amount of biofilm formed to the planktonic cells growth of each sample.

For the scanning electron microscopy (SEM), static cultures were grown in sterile 6 well-plates with round Nunc Thermanox plastic coverslips (Thermo Fisher Scientific) submerged in 6ml of 1:100 diluted culture. The plates were incubated for different time points (24h, 48h, 72h and 96h) at 37°C with slight shaking (50-100 rpm). After the respective time points, the supernatant was removed from the wells and the coverslips were washed gently with 0.01M sterile PBS. The coverslips were then transferred to the fresh plate containing fixative (3% glutaraldehyde in PBS) and allowed to sit at room temperature for 2 hours followed by 4°C for 24 hours. Following fixation, glutaraldehyde was removed and coverslips were washed in 0.01M PBS twice and stored in the same until all the other samples were ready for dehydration. Dehydration of the samples on coverslips was performed with sequential ethanol washing steps (30%, 50%, 70%, 80%, 90% and 100%) for 10 minutes each in the sterile conditions. The samples were then subjected to critical point drying and all the coverslips were left to air-dry overnight, which were mounted the next day and sputter gold-coated using an EMITECH K550X Sputter Coater (Quorum Technologies, UK). Samples were analyzed using a JSM-6480LA (JOEL, Japan) scanning electron microscope.

2.2.4 Cross streaking assay using biosensor strains for the detection of acyl homoserine lactones (AHLs)

A set of LB agar plates with no selection and another set of plates covered with 65µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were prepared for cross streaking assays. These assays consisted of streaking the no selection LB agar plates with the AHL biosensor *C. violaceum* CV026, which detects AHLs with *N*-acyl side chains of four to eight carbons and the presence of those AHL molecules restore violacein production turning *C. violaceum* CV026 colonies purple [155]. Similarly, LB agar plates covered with 65µg/ml X-gal were streaked with the AHL biosensor *A. tumefaciens* A136, which recognizes N-3-(oxooctanoyl)-L-homoserine lactones (OOHL) and the presence of those AHLs activates a *traG*::*lacZ* fusion turning the *A. tumefaciens* A136 colonies blue [154]. The *A. baumannii* AB5075-UW and *aceI* knockout mutant strains were streaked in parallel to the biosensor strains. The cross streaked plates were incubated at 28°C for 48 hours. *P. aeruginosa* PAO1 strain and the wild type *C. violaceum* were included in the experiment as positive controls for CV026 strain. The isolates were considered positive for AHLs production when CV026 colonies turned purple and A136 colonies turned blue following incubation.

2.2.5 Pellicle formation assay at the air-liquid interface

Overnight cultures of *A. baumannii* AB5075-UW and the *aceI* inactivated mutant were diluted 1:100 in 8 ml of fresh MH-broth in Falcon round-bottom polypropylene tubes. The subcultures were grown for 72 hours to stationary phase at the room temperature (RT). Negative control containing nutrient broth (MH) only was also included in the experiment. Pellicle formation was observed visually.

2.2.6 Bacterial adherence to A549 human alveolar epithelial cells

Adherence of the parental AB5075-UW and *aceI* inactivated mutant to A549 cells (human type II pneumocyte cell lines) [158] was determined as previously described [160]. Briefly, cell lines were grown in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Gibco, Life Technologies) and 2mM glutamine (Thermo Fisher Scientific) at 37°C and 5% CO_2. The cells were frequently examined under the microscope to confirm >95% confluency prior to infection. *A. baumannii* strains were diluted to 1:50 dilution from overnight cultures and grown to OD_{600nm} of 0.6 in MH-broth. The bacterial cells were washed and resuspended in PBS and were serially diluted to obtain an OD_{600nm} of 0.001, which was

then used to inoculate the A549 cell monolayers in the 12 well-plates. The plates were incubated at 37°C in presence of 5% CO_2 for 4 hours to allow bacteria to adhere to the A549 cells.

For adherence analysis by SEM, the cell monolayers growing on round Nunc Thermanox plastic coverslips (Thermo Fisher Scientific) were gently washed with 0.01M PBS three times and then transferred into the fixative (3% glutaraldehyde in PBS) and allowed it sit at RT for 2 hours followed by 4°C for 24 hours. The coverslips were further prepared for SEM as described in section 2.2.3.

Adherence analysis using viable count assays was also performed as previously described [161]. The cell monolayers grown in 12 well-plates were gently washed with 0.01M PBS three times. The A549 cells were detached from the wells with an addition of 0.25% trypsin with EDTA (Gibco, Life Technologies) followed by 0.025% of Triton X-100 (Sigma Aldrich) to lyse the epithelial cells. The lysed cells were then serially diluted 10-fold and plated on LB agar in order to determine the number of adherent bacteria per well.

3 Results

3.1 Growth of AB5075-UW and *aceI* inactivated mutant in laboratory media

To examine whether *aceI* contributes to the overall fitness of the AB5075-UW strain *in vitro*, conventional laboratory media (MH-broth) was inoculated with the parental AB5075-UW and an *aceI* inactivated mutant. The growth of strains was observed using optical density measurements over a timecourse of 15 hours. The growth assay revealed that there is no detectable growth fitness defect in the *aceI* inactivated mutant, suggesting that *aceI* is not required for the overall fitness under standard laboratory conditions of the AB5075-UW strain (Figure 3.1).

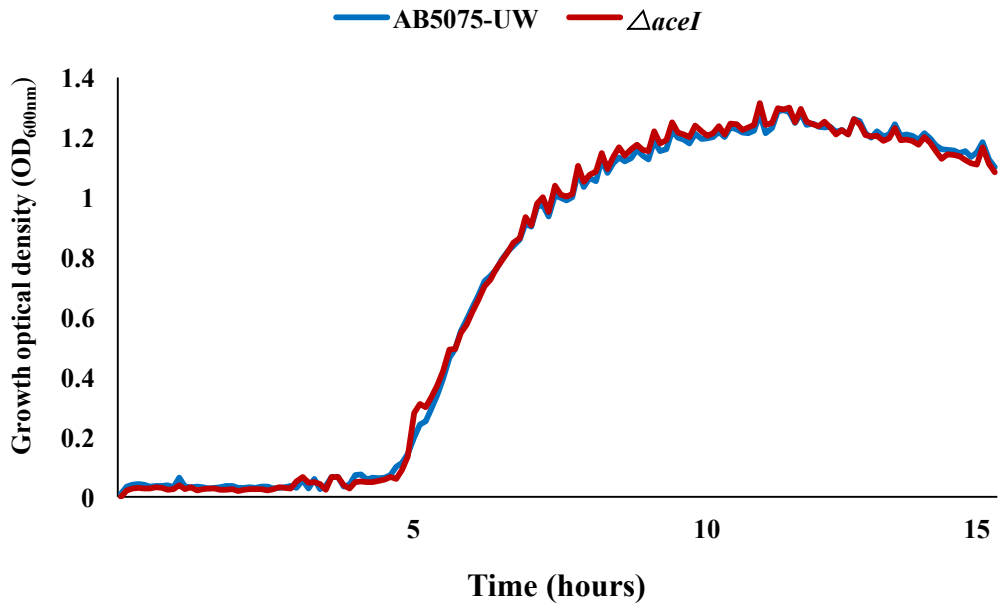


Figure 3.1: Growth curves of *A. baumannii* AB5075-UW (blue) and *aceI* inactivated mutant (red) in MH-broth. Growth optical density was measured at OD_{600nm} every 6 minutes for 15 hours.

3.2 Growth of AB5075-UW and *aceI* inactivated mutant in 100% human serum

A. baumannii has increasingly been identified to cause bloodstream infections which have a mortality rate, above 40% [162]. To investigate the fitness of the virulent *A. baumannii* AB5075-UW strain and *aceI* inactivated mutant in *in vitro* conditions that mimic these bloodstream infections, the cells were cultured in 100% human serum. Following 48 hours of

incubation in human serum, it was found that, the parental strain had an extended lag phase of approximately 20 hours, however a strong growth rate was observed after this time point. Whereas, the *aceI* knockout strain was unable to grow at this concentration of human serum (Figure 3.2). This suggests that *aceI* might play a role in providing resistance to a component of human serum, thus allowing growth of parental AB5075-UW strain.

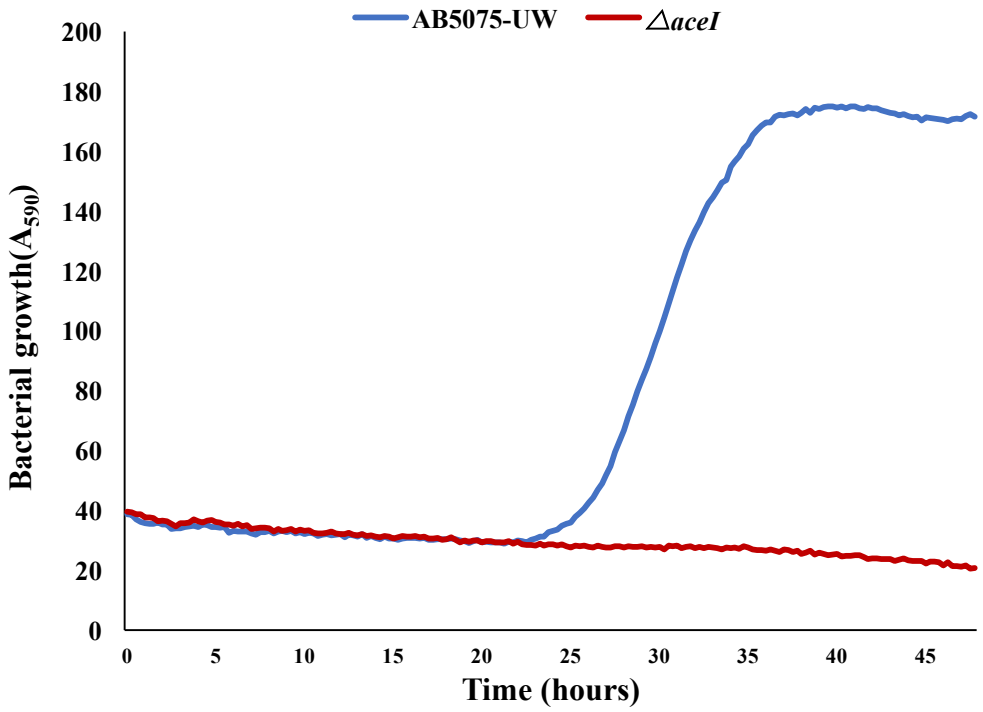


Figure 3.2: Growth curves of *A. baumannii* AB5075-UW (blue) and *aceI* inactivated mutant (red) in 100% human serum. Bacterial growth was measured every 15 minutes as a colorimetric change (purple formazan absorbance at 590nm) for 48 hours. Data represent the average from three independent experiments.

The data suggested that the bactericidal factors in human serum could directly kill or inhibit the growth of the *aceI* knockout strain. Therefore, MIC analysis was performed to determine the minimum concentration of human serum required to inhibit the growth of the *aceI* knockout strain. This analysis revealed that the human serum at concentrations higher than 25% had an inhibitory effect on the *aceI* knockout mutant while 12.5% had a moderate inhibition but non-lethal effect and the lower concentrations of serum were unlikely to affect its growth (Figure 3.3A). The parental AB5075-UW strain was also included as a positive control in the experiment, which showed shorter lag phases as the dilution of human serum increased (Figure

3.3B). The parental AB5075-UW took almost half the time to enter log phase after 1:1 dilution of human serum, when compared to the stock concentration. Similarly, as the serum was diluted further below 50%, shorter lag phases were observed (Figure 3.3B).

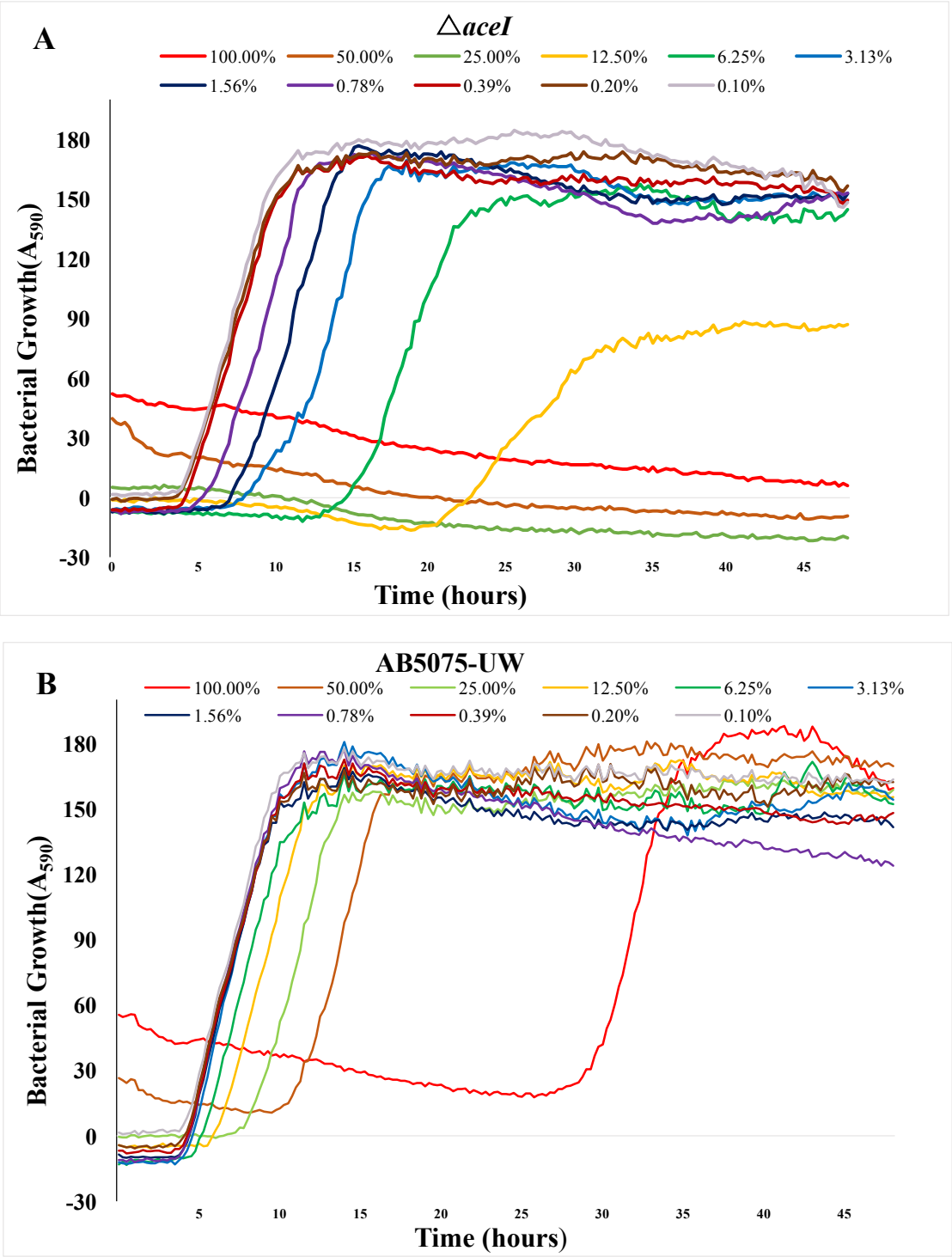
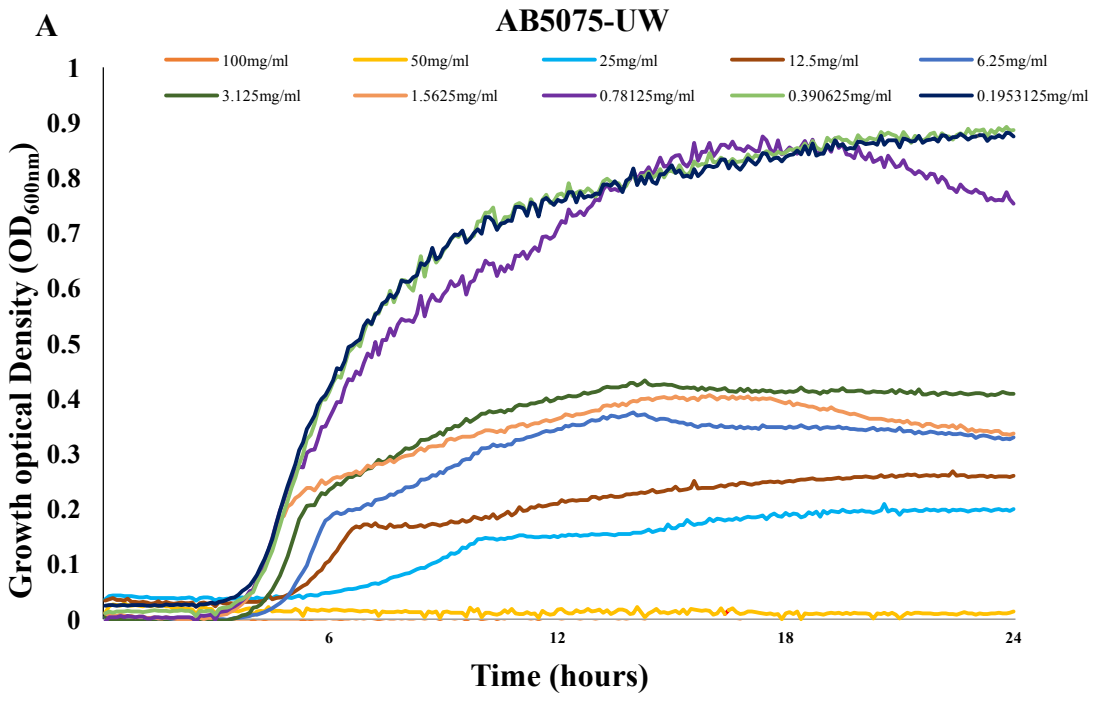


Figure 3.3: Growth curves of *aceI* inactivated mutant (A) and *A. baumannii* AB5075-UW (B) at varying concentrations of human serum. Bacterial growth was measured every 15 minutes as a colorimetric change (purple formazan absorbance at 590nm) for 48 hours.

3.3 MIC analysis with bile salts mix

Blood serum carries various small molecules in the body and contributes to many important biological functions. A human serum metabolomics study described blood serum to be composed of fatty acids, phospholipids, steroid derivatives, glycerides and other small nutrients [163] and a very small amount of conjugated bile acids [164]. Many studies have demonstrated the involvement of efflux pumps in providing resistance to bile salts, polyamines and other natural substrates. To examine if bile salts are a natural substrate of AceI, the growth of AB5075-UW and *aceI* inactivated mutant was observed in the presence of different concentrations of unconjugated bile salts mix (sodium cholate and sodium deoxycholate (1:1)). The resulting growth curves showed that the bile salt had a lethal effect on both the parental and mutant strains at the stock concentration of 100 mg/ml and a partial inhibitory effect at concentrations ranging from 50mg/ml to 0.16mg/ml. However, both the strains showed a non-inhibitory effect of bile salt mix at concentrations lower than 0.16mg/ml (Figure 3.4A, B), suggesting that bile salts may not be a substrate of the AceI pump.



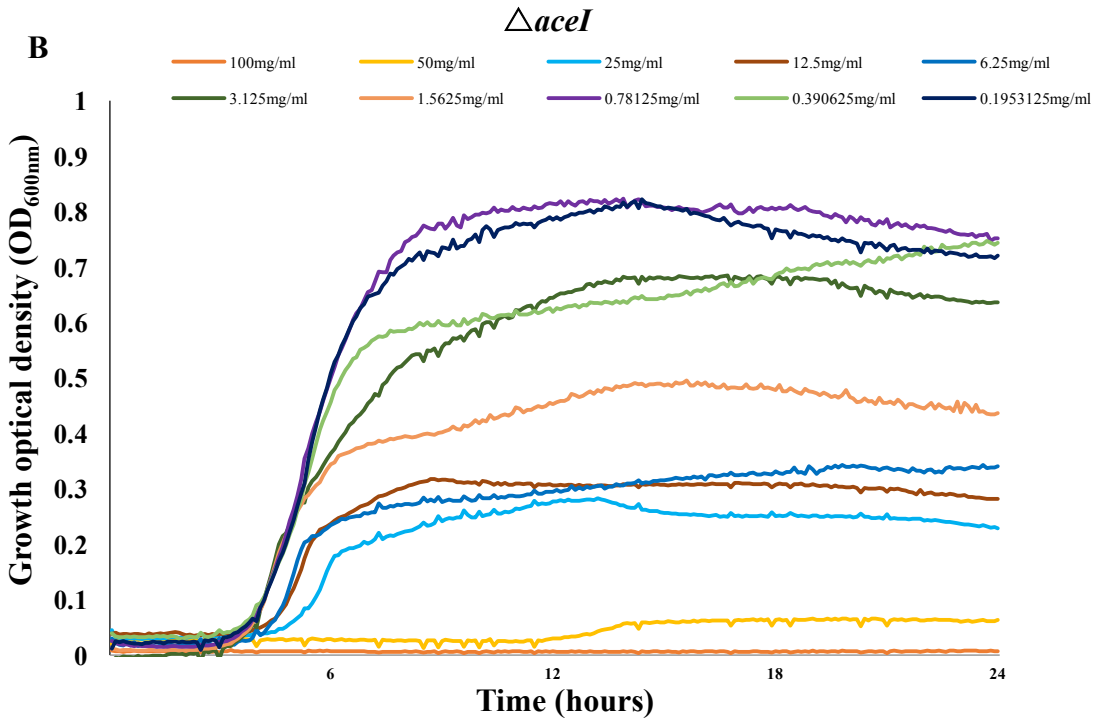


Figure 3.4: Growth curves of *A. baumannii* AB5075-UW (A) and *aceI* inactivated mutant (B) at varying concentrations of bile salt mix (sodium cholate and sodium deoxycholate (1:1)). Growth optical density was measured at OD_{600nm} every 6 minutes for 24 hours.

3.4 Effect of *aceI* gene inactivation in biofilm formation

Efflux pumps encoded by opportunistic pathogens such as *P. aeruginosa* have been identified to play important roles in biofilm formation and regulation. Very recently a study reported that the deletion of some RND efflux pumps in *A. baumannii* led to reduced biofilm formation [128]. Thus, to examine, whether the *aceI* gene plays a role in biofilm formation and maintenance in AB5075-UW, the biofilm formation abilities of the parental AB5075-UW strain and *aceI* inactivated mutant were investigated. A quantitative biofilm formation assay performed in 96 well microtiter plates with crystal violet staining, showed a significant difference in biofilm formation by the parental strain and the *aceI* inactivated mutant. The quantitation of biofilm formation by parental AB5075-UW and the *aceI* knockout strain at different time points (24h, 48h, 72h, 96h) is shown in Figure 3.5. The inactivation of *aceI* gene resulted in a significantly reduced biofilm formation at all time points ($P< 0.05$), except for 96 hours, when compared with the biofilm formed by the parental strain under the same experimental conditions.

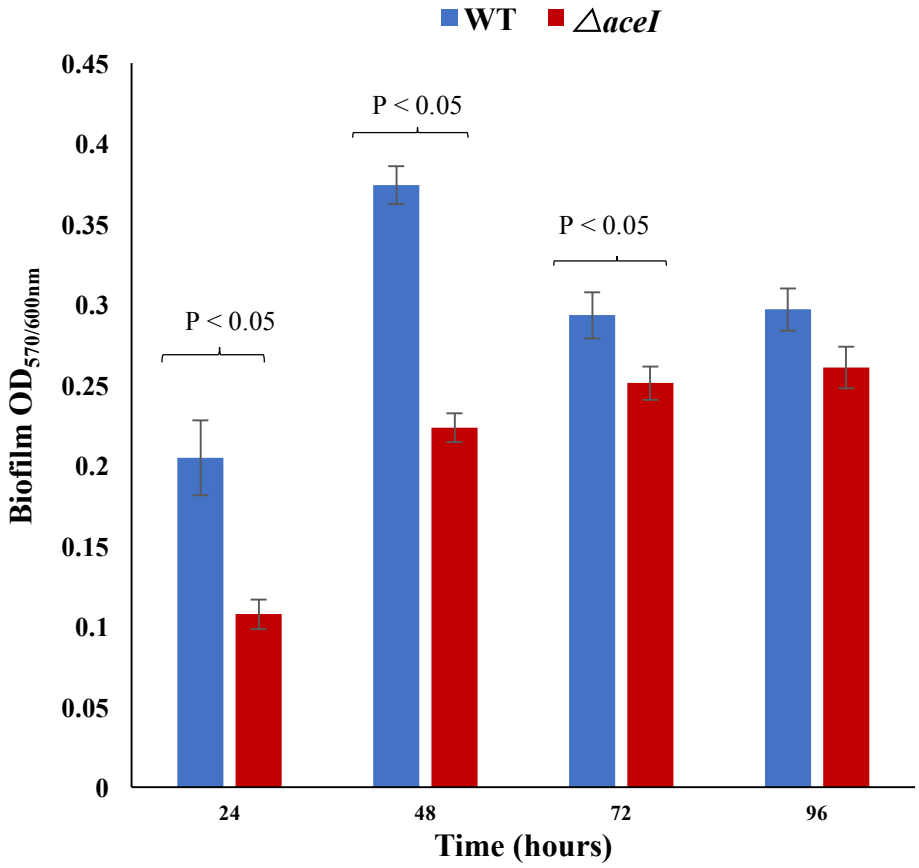
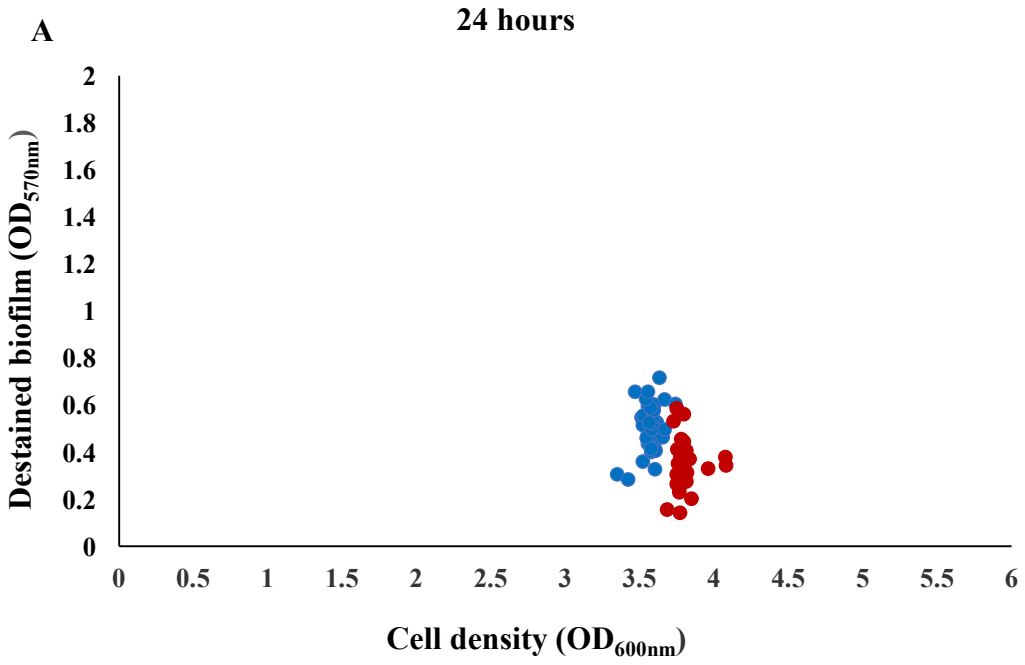
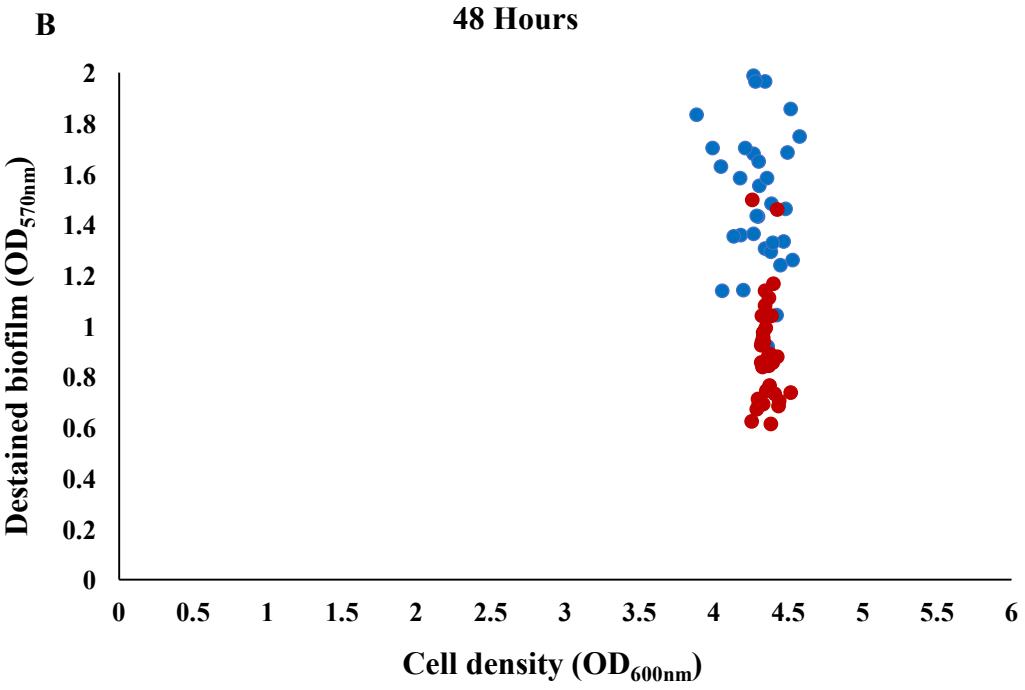


Figure 3.5: Quantitation of biofilm formation by *A. baumannii* AB5075-UW (blue) and *aceI* inactivated mutant (red) at 24h, 48h, 72h and 96 h. Bars represent the means from three independent experiments; error bars indicate the standard error of the means. Biofilm formation by *aceI* inactivated mutant at 24h, 48h and 72h had statistically significant difference in comparison to the parental strain ($P<0.05$).

Bacterial cell density has been found to be an essential factor in biofilm formation and maturation [165]. Thus, biofilm formation by the parental and mutant strains were normalized against their planktonic growth to best characterize their biofilm forming capacity. Based on the quantitative and normalized data, biofilm formation by the parental strain was at its lowest at 24 hours, highest at 48 hours, showed a slight decline at 72 hours and remained fairly similar at 96 hours (Figures 3.5,3.6). It could also be observed that the cell population density was directly related to the increased biofilm forming capacity of the strains. Furthermore, the rapid biofilm formation was observed by the parental strain at 48 hours (Figure 3.5, 3.6B), which may have led to earlier nutrient limitation and thus potential biofilm dispersal could be seen after this time point (Figure 3.6C, D). Whereas, a constant increase in cell population density and biofilm formation capacity was observed with the *aceI* inactivated mutant with a potential

dispersal behavior at 72hours (Figure 3.6C) and 96 hours (Figure 3.6D), similar to the parental AB5075-UW strain. Overall, *aceI* gene inactivation seem to have significantly affected the biofilm formation ability of *A. baumannii* AB5075-UW.





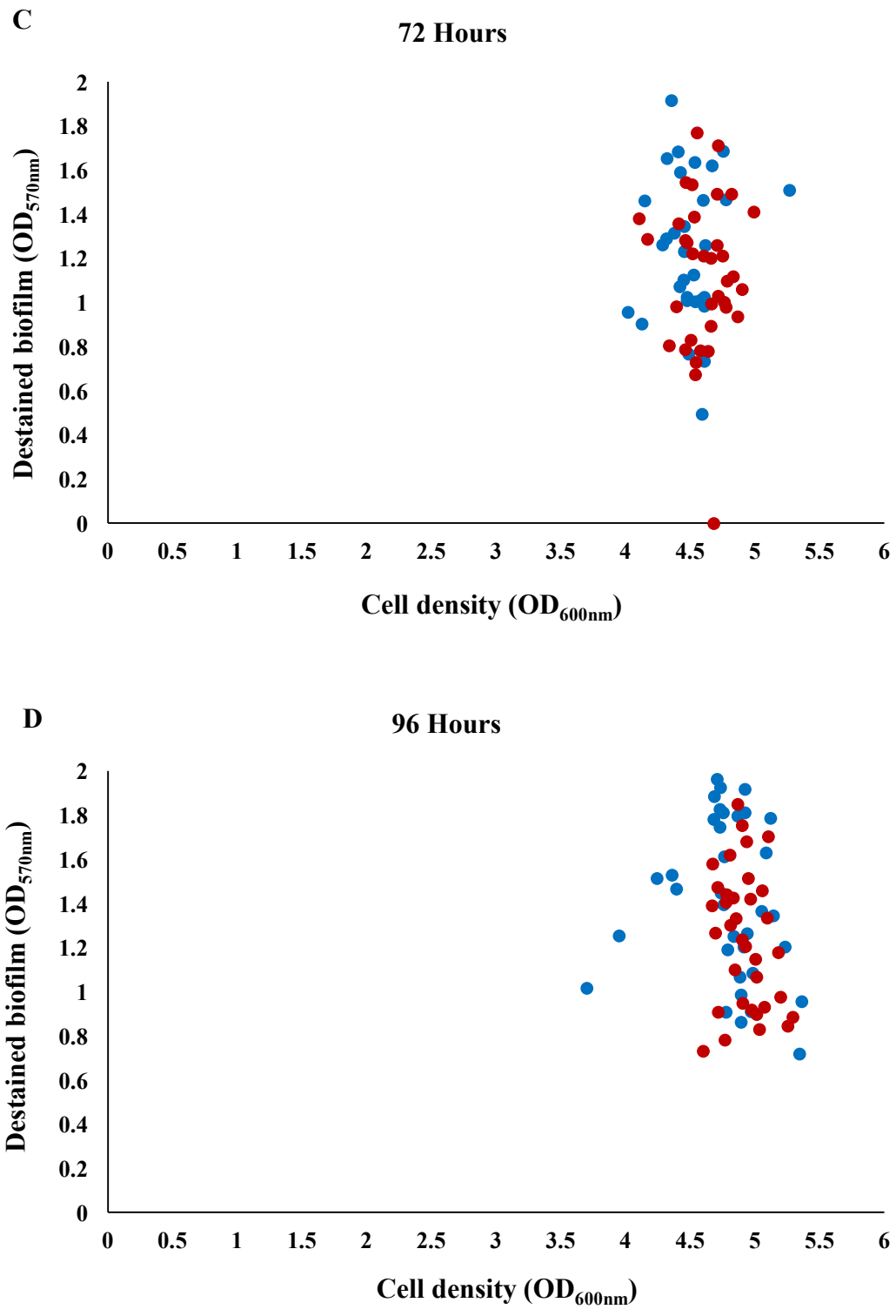


Figure 3.6: Scatter plot of cell density (OD_{600nm}) and destained biofilm (OD_{570nm}) for *A. baumannii* AB5075-UW (blue dots) and *aceI* inactivated mutant (red dots) at 24h (A), 48h (B), 72h (C), 96h (D). Each dot represents readings from three independent experiments.

The biofilm formation capacity of the parental and *aceI* knockout strains were further confirmed using SEM to analyze the role of *aceI* on biofilm architecture. Both the parental and mutant strains showed a remarkable capacity to attach to the plastic coverslips, on which the strains were tested for their biofilm formation capacity. In addition, images obtained from SEM are in agreeance with the quantitative biofilm formation data (Figures 3.5-3.7). As observed in the scatter plots of the parental strain (Figure 3.6), bacterial aggregation at 24h, higher cell density and biofilm formation at 48h and potential dispersal behaviour at 72h and 96h was observed with SEM (Figure 3.7). Furthermore, the parental AB5075-UW and *aceI* inactivated mutant were observed to be similar in terms of their morphology and both the strains possessed bacterial appendages on the cell surface (Figure 4.1). These cell surface structures have been found to play an important role in cell-cell interactions and their attachment to biotic and abiotic surfaces [165]. The cells were observed to be linked with each other via a dense extracellular polymeric substance, which is known to provide structural stability for the biofilms (Figure 4.1).

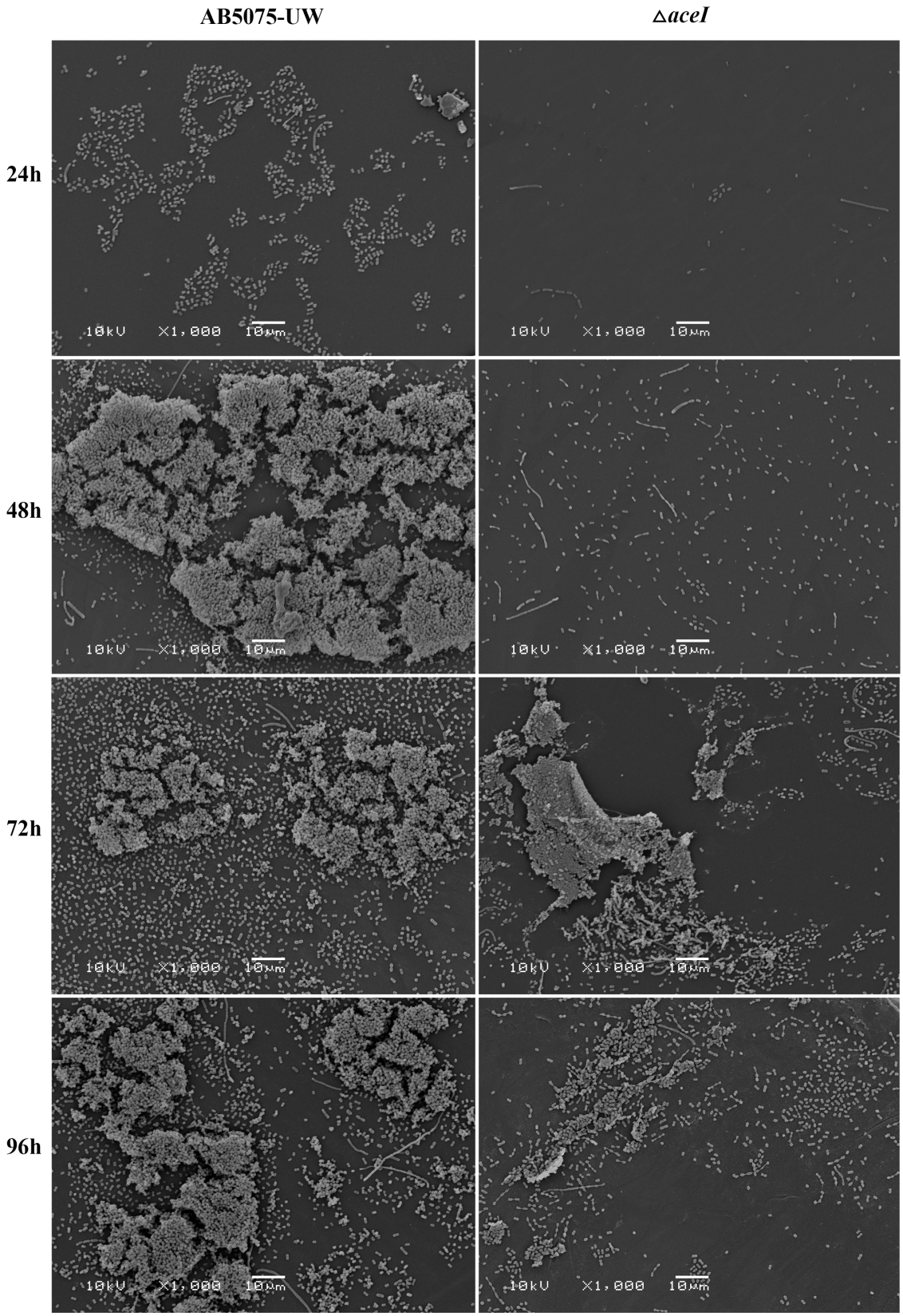


Figure 3.7: Scanning electron microscopy images of *A. baumannii* AB5075-UW and *aceI* inactivated mutant at 24h, 48h, 72h and 96h. Cells were cultured on 13 mm plastic coverslips in MH-broth with shaking at 100 rpm at 37 °C. Scale bar:10 µm, Magnification: X1000.

3.5 Pellicle formation by AB5075-UW and *aceI* inactivated mutant

The capacity of the parental AB5075-UW strain and *aceI* inactivated mutant to form pellicle in an air-liquid interface was also observed in polypropylene tubes. A very thin pellicle with some hanging structures were observed at the end of the 72 hours incubation, in both the tubes containing the parental AB5075-UW and *aceI* inactivated mutant (Figure 3.8). Whereas, the tube containing nutrient broth only, did not have any pellicle looking structures attached at the air-liquid interface (Negative control). The pellicle forming capacity of the strains were confirmed with three independent experiments.

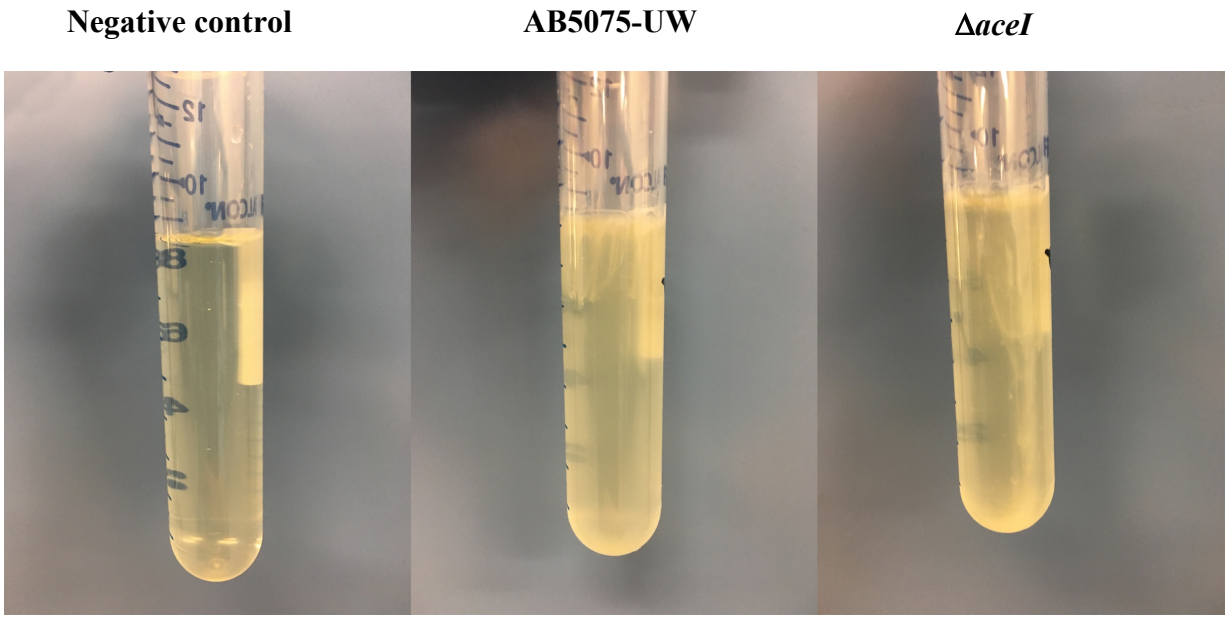


Figure 3.8: Pellicle formation by *A. baumannii* AB5075-UW and *aceI* inactivated mutant at the end of 72 hours of incubation. Negative control represents MH-broth only (left), with no bacterial aggregation on the air-liquid surface; parental AB5075-UW (middle) and *aceI* inactivated mutant (right) formed thin pellicles with some cell hanging structures on top of the liquid media.

3.6 Bacterial adherence to A549 human alveolar epithelial cells

The capacity of the parental AB5075-UW strain and *aceI* inactivated mutant to interact with the biotic surface was also examined by infecting A549 human alveolar epithelial cells with the parental AB5075-UW and *aceI* inactivated mutant. A549 human alveolar cells were infected for 4 hours at 37°C in presence of 5% CO_2. The bacterial cells attachment to A549 alveolar cells was then analyzed by both viable count assay as well as by SEM observations.

The colony forming units of *aceI* inactivated mutant that adhered to A549 cells were found to be greater than the parental AB5075-UW in viable count assays (Figure 3.9). This observation was further confirmed by infecting the A549 cells grown in plastic coverslips with the known number of bacterial cells and observed under SEM (Figure 3.10). The SEM observations also suggested an increased adherence phenotype of *aceI* inactivated mutant to the eukaryotic cell monolayers, compared to the parental AB5075-UW strain. The uninfected A549 cells representing a healthy monolayer and the infected A549 cells are shown in Figure 3.10.

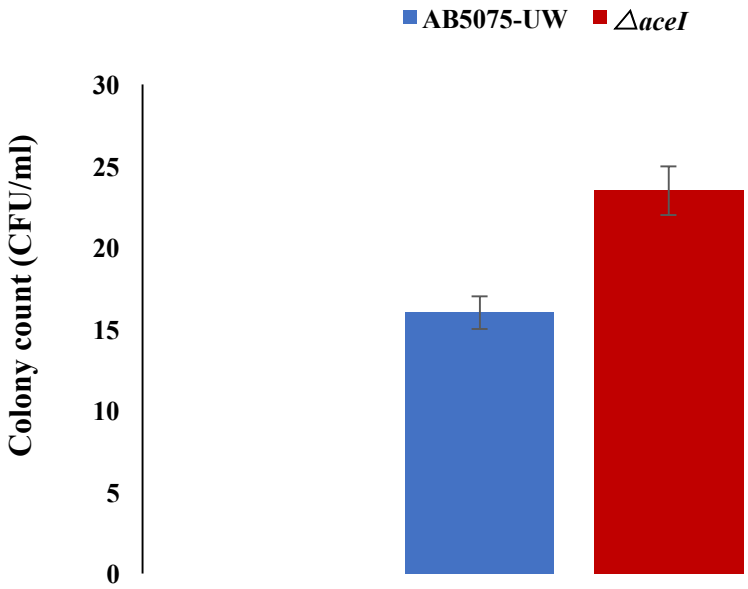


Figure 3.9: Adherence to A549 epithelial cells. Data shows the mean of five technical replicates (CFU/ml). Error bars show the standard error of the mean. The parental AB5075-UW strain (blue) showed the significantly lower binding to A549 cells ($P<0.05$) compared to the *aceI* inactivated mutant (red).

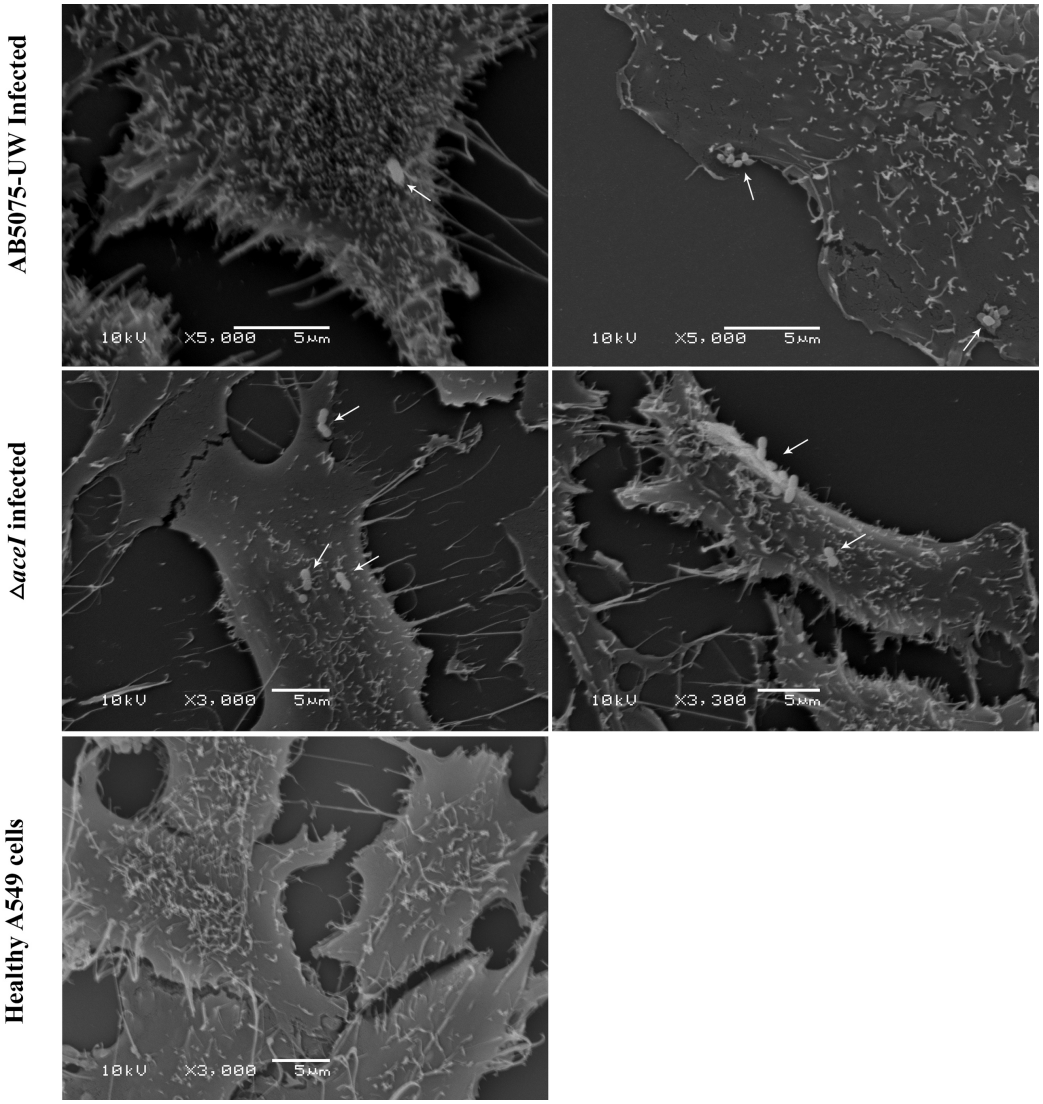
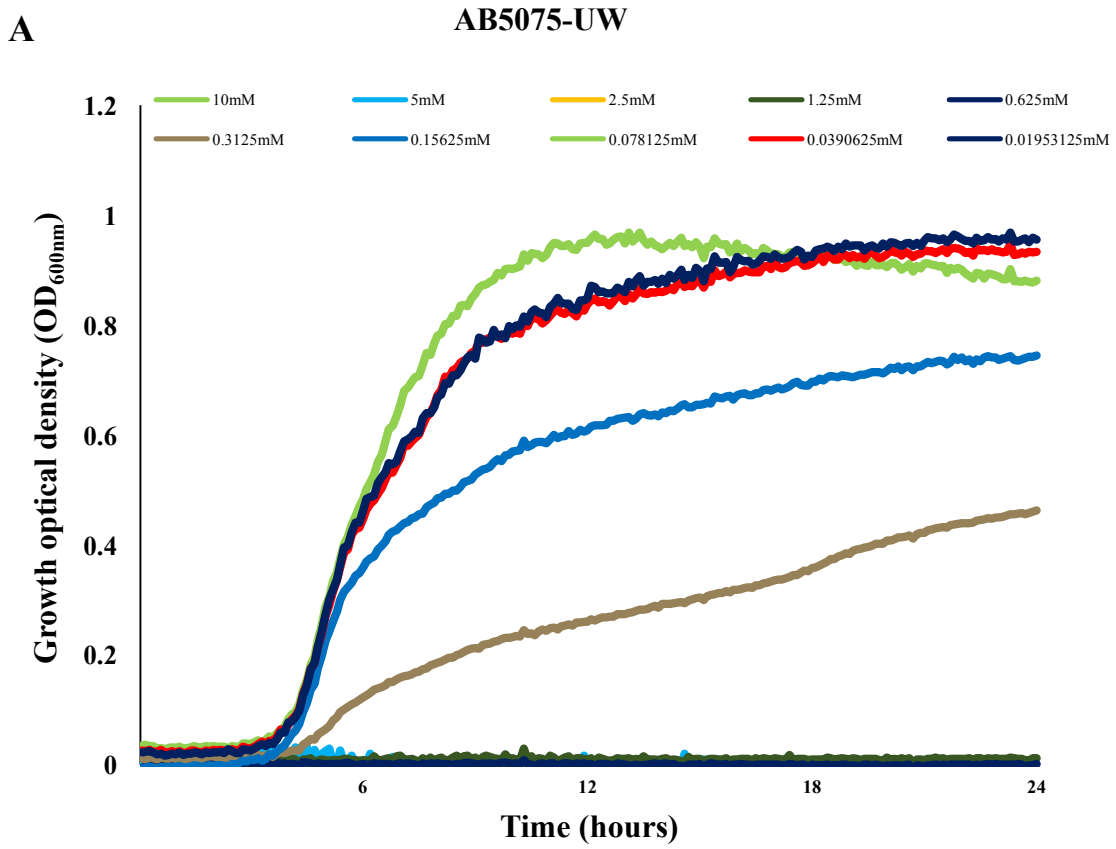


Figure 3.10: SEM images of cell adherence assay. Images show the adherence of *A. baumannii* AB5075-UW cells (top) and *aceI* inactivated mutant cells (middle) to the A549 human alveolar cells and uninfected A549 cells (bottom) following 4h incubation at 37°C in presence of 5% CO_2. White arrows show the presence of bacteria on A549 cell surface. Scale bar: 5µm

3.7 Effect of *aceI* gene inactivation in iron acquisition

Iron is an essential micronutrient that bacteria require for the survival and persistence within the host and the reduction of this micronutrient in the bacterial growth medium is likely to impact cell survival. Efflux pumps can play important roles in iron acquisition mediated by iron chelating compounds called siderophores, presumably by mediating secretion of the siderophores out of the bacterial cells [140, 145]. In this study, iron available in MH-broth was reduced by supplementing the MH medium with a synthetic iron chelator 2,2'-dipyridyl (DIP)

and the growth of the parental AB5075-UW strain and isogenic *aceI* mutant were examined under a range of DIP concentrations for 24 hours. Following 24h incubation, none of the strains showed any indication of growth at high DIP concentrations ranging from 0.6mM to 10mM. The growth of both the strains were still impacted at 0.3mM and 0.16mM (Figure 3.11A, B), however concentrations below 0.16mM were unlikely to affect their growth. Based on this result, *aceI* might not be involved in iron acquisition in AB5075-UW.



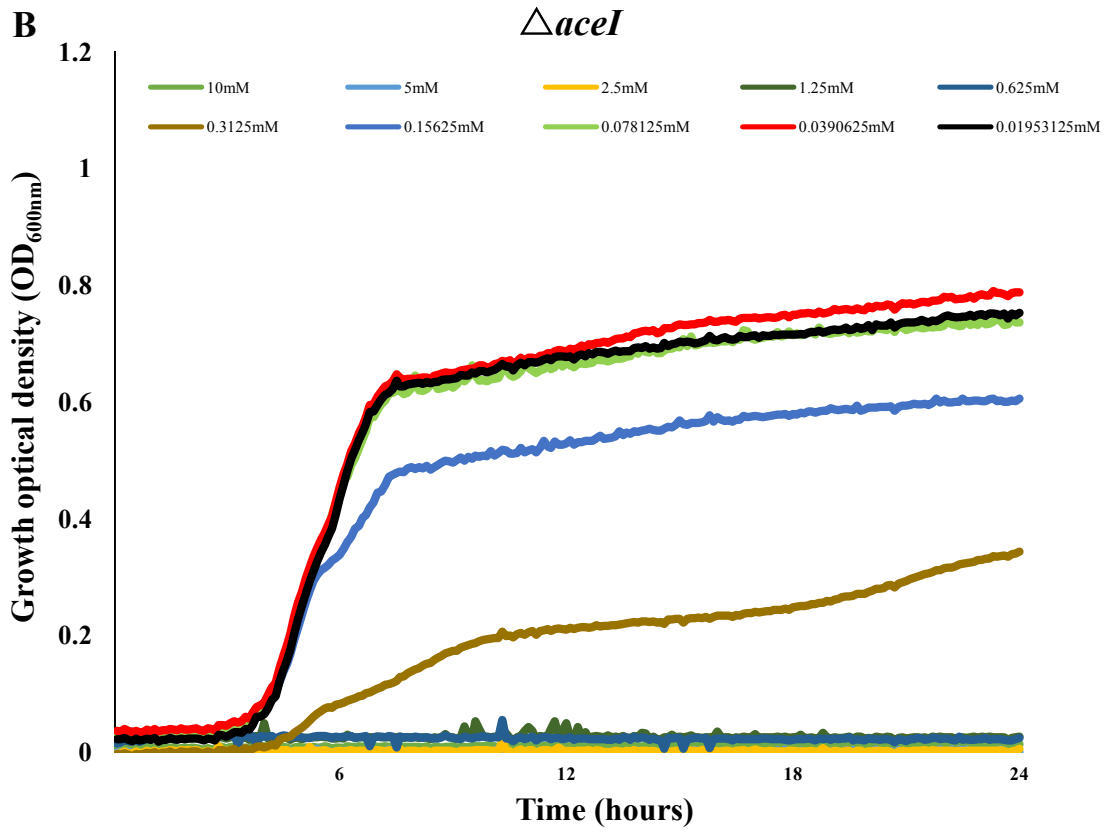


Figure 3.11: Growth curves of *A. baumannii* AB5075-UW (A) and *aceI* inactivated mutant (B) at varying concentrations of 2,2'-dipyridyl (DIP). Growth optical density was measured at OD_{600nm} every 6 minutes for 24 hours. Data represents the average from three independent experiments.

3.8 Effect of *aceI* gene inactivation in AHL production

QS is an important regulatory mechanism that allows bacterial communication with the production of diffusible signalling molecules called N-acyl homoserine lactones (AHLs). Efflux pumps have been found to actively extrude these signalling molecules thus allowing bacteria to quickly respond to the changes in its surrounding environment. When a sufficiently high concentration of signalling molecules are available in the medium, resulting in a high internal concentration, they bind to the transcriptional activators to induce the expression of target genes [15, 110]. The use of two AHL biosensor strains, *A. tumefaciens* A136 and *C. violaceum* CV026 in cross streaking assays allowed the screening of AHLs production by the parental AB5075-UW strain and the *aceI* inactivated mutant. The parental AB5075-UW strain was found to produce AHL molecules detectable by *A. tumefaciens* A136, whereas the *aceI* inactivated mutant did not induce any blue pigment production (Figure 3.12A, B). Both the

strains were AHL negative with the detection of *C. violaceum* CV026 (Figure 3.12C, D). The positive control *P. aeruginosa* PAO1 strain (Figure 3.12E) promoted production of a small amount of purple violacein pigment in the CV026 biosensor strain. The wild type *C. violaceum* was also included in the assay as a positive control for CV026, which can produce purple violacein pigments on its own (Figure 3.12F). The A136 detectable AHL molecules produced by the parental AB5075-UW strain might be either longer carbon chains AHLs ranging from 6-12 or 3-oxo derivatives of AHL molecules [154].

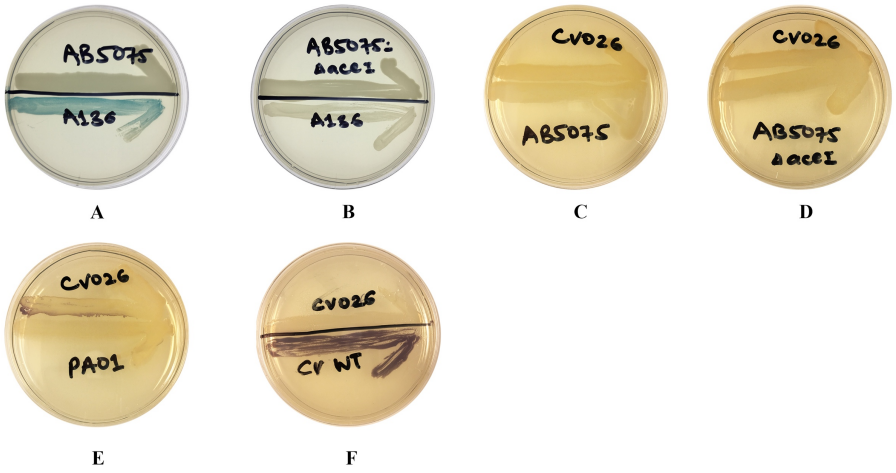


Figure 3.12: Cross streaking assays. Indication of AHLs production by *A. baumannii* AB5075-UW, *aceI* knockout mutant and *Pseudomonas* PAO1 strains in presence of A136 and CV026 biosensor strains. Plate A: AB5075-UW/A136, Plate B: Δ*aceI*/A136, Plate C: AB5075-UW/CV026, Plate D: Δ*aceI*/CV026, Plate E: PAO1/CV026, Plate F: Wild type CV/CV026

3.9 Construction of complemented strain for *aceI* inactivated mutant

An *A. baumannii* mutant with an inactivated *aceI* gene showed reduced virulence in a mouse infection model. Therefore, to confirm whether this observed virulence defect was due to the deletion of *aceI,* but not due to secondary mutations elsewhere in the genome, it is necessary to construct a complemented *aceI* strain. The complementation experiment was initiated during this study, where the *aceI* sequence was cloned in an *E. coli-Acinetobacter* shuttle vector, pWH1266 [157]. The cloned pWH1266 vector carrying *aceI* was transformed into *E. coli* DH5α gold competent cells. Colony screening PCR was performed to amplify the insert gene and gel electrophoresis was performed to visualize the presence of the insert. Visualization of PCR products suggested the successful amplification of *aceI* gene. The presence of a band of

approximately 500-600bp suggests the successful integration of DNA encoding AceI. Thus, to confirm the successful integration of *aceI* gene into pWH1266 in *E. coli* DH5α was performed using Sanger sequencing. However, the sequencing data obtained from AGRF did not confirm the presence of an *aceI* insert in the transformants screened. Thus, future optimization and repetition of this experiment will be required, but due to time constraints this was not feasible within the scope of this project.

4 Discussion

The success of *A. baumannii* as a global pathogen is likely to be related to various factors. The clinical importance of this pathogen can be attributed to its ability to adhere to biotic and abiotic surfaces such as medical devices [166]; to persist in a variety of environmental conditions including the severely dry surfaces [167]; to be able to survive in an iron-limiting conditions such as that within the human host [147]; and most importantly its MDR phenotype [149]. It is very important to investigate and understand these attributes, which could be exploited as potential targets in developing effective strategies for treatment and prevention of *A. baumannii* related infections in the future.

In a recent study, the membrane protein AceI from *Acinetobacter baumannii* was found to be important for the growth of AB5075-UW in insect larvae [74] as well as in the virulence and colonization in mouse infection model (Figures 1.2-1.3). Therefore, this project focused on investigation of possible physiological and molecular roles of *aceI* gene in the virulence of *A. baumannii* AB5075-UW.

4.1 *A. baumannii* growth in biologically relevant growth media

In this study, *A. baumannii* was cultured in 100% human serum as it mimics *in vivo* bloodstream related infections, which comprises 40% of hospital acquired infections caused by *A. baumannii* [162]. Specifically, the growth of *A. baumannii* AB5075-UW and an *aceI* inactivated mutant strain was observed in human serum. The parental AB5075-UW could grow in 100% human serum, however the growth of the *aceI* inactivated mutant was significantly inhibited (Figure 3.2), suggesting *aceI* might play an essential role in proliferation of *A. baumannii* in the bloodstream. The parental AB5075-UW strain displayed an extended lag phase in human serum possibly to adapt to the growth media, probably by expressing the factors essential to overcome the bactericidal effect of human serum. The growth defect observed in the *aceI* inactivated isogenic derivative might be because *aceI* is required to provide resistance to a component of human serum, thus allowing AB5075-UW to survive and propagate in the serum. To confirm the role of *aceI* during growth of *A. baumannii* AB5075-UW in human serum, the gene expression of *aceI* in response to human serum could be measured using qRT-PCR. The up-regulation of *aceI* would suggest the importance of *aceI* gene for survival of AB5075-UW in the bloodstream.

Human serum carries various small molecules in the body and contributes to many important biological functions. This biofluid is known to play an important role in transporting nutrients, metabolic wastes and hormones and in addition it also provides defense against pathogens and toxins. Serum is also essential in regulation of pH and iron composition in interstitial fluids and in stabilization of body temperature [163]. Blood serves as a "liquid highway" in the body as it bathes all tissues and organs and transports the molecules secreted or excreted by the tissues, such as enterohepatic circulation of bile acids [163, 168]. The human serum metabolomics study reported blood serum composition is mostly dominated by fatty acids, phospholipids, steroid derivatives, glycerides, and other small nutrients such as amino acids, iron, glucose, lactate and several waste and catabolic byproducts [163, 169].

Many Gram-negative bacteria, especially enteric pathogens are found to be involved in bile-mediated virulence. Inactivation of the AcrB pump from *S. typhimurium* and *E. coli* were found to affect the colonization of these organisms in the intestinal tract [170, 171]. Similarly, conjugated bile acids, which are also found in a very small amount in human serum were found to contribute to *Vibrio* pathogenicity by enhancing the production of virulence factor called thermostable direct hemolysin [170]. Likewise, CmeABC efflux pump from *C. jejuni* was also found to be essential in mediating resistance to bile salts thus allowing successful colonization in chickens [90]. Therefore, to examine if bile salts are a natural substrate of *aceI,* the growth of AB5075-UW and the *aceI* inactivated mutant was observed in the presence of different concentrations of unconjugated bile salts mix (sodium cholate and sodium deoxycholate (1:1)), which revealed no change in sensitivity to bile salts in *aceI* inactivated mutant derivative, suggesting bile salts may not be a substrate for AceI. This hypothesis can be further tested by performing the antimicrobial susceptibility tests in presence of the glycine or taurine conjugates of bile acids, which are major bile salts found in human bile [170].

In addition to the other constituents, serum is also known to contain a very small amount of polyamines, typically in picomolar concentrations [172]. Several studies have described the physiological functions of polyamines in bacteria including their involvement in microbial carcinogenesis [86], biofilm formation [85] and providing defense against acid and oxidative stresses [173]. Polyamines have been found to be the natural substrates of several efflux pumps such as the SMR efflux pump MdtJI in *E. coli* [174], RND efflux pump BpeAB-OprB from *Burkholderia pseudomallei* [175] and the MFS efflux pump Blt from *Bacillus subtilis* [176]. In addition, polyamines were recently identified as substrates of PACE family multidrug

transporters (Liu *et al*., unpublished). Therefore, the involvement of polyamines in AB5075-UW pathogenesis could be investigated in future.

4.2 Biofilm formation and adherence contribute to pathogenicity

A. baumannii possess a remarkable capacity to persist and propagate in hospital environments, largely due to its ability to form biofilms on wide range of surfaces including abiotic substrata commonly found in clinical settings [126, 133]. *A. baumannii* is also known to adhere to medical devices including catheters and respiratory equipment as well as biotic surfaces, such as human alveolar cells, which might be a target during respiratory infections [177]. The ability of *A. baumannii* to persist in medical settings despite being subjected to range of adverse conditions including desiccation, nutrient limitations and use of disinfectants might explain its success as a hospital pathogen [178].

Biofilm formation processes in *A. baumannii* are known to be affected by complex regulatory mechanisms, efflux pump expression, cell population density (via QS), cell surface charge and other environmental conditions [178]. A recent study by Rumbo-Feal *et al.*, revealed 1,621 genes that are essential for biofilm formation in ATCC17978 strain as compared to the planktonic cells [179]. The CsuA/BABCDE pilus chaperone-usher assembly system which is regulated by a two component regulatory system was found to be essential for initial attachment to abiotic surfaces [132]. Similarly, the outer membrane protein OmpA and its homolog of the staphylococcal biofilm associated proteins (Bap) have been described to be involved in development and stabilization of *A. baumannii* biofilms [134]. In addition, other bacterial surface structures like fimbriae have been found to play an important role in bacterial adhesion to the biotic and abiotic surfaces [180].

In this study, the biofilm and pellicle forming abilities of the parental AB5075-UW and *aceI* inactivated mutant were tested to examine the effect of *aceI* gene inactivation in bacterial attachment to abiotic surfaces and biofilm formation (Figures 3.5-3.8). The quantitative biofilm formation and SEM images revealed that the *aceI* gene inactivation led to reduced biofilm formation.

The involvement of MDR pumps in biofilm formation has been reported in other bacterial pathogens including *Klebsiella* [129], *Pseudomonas* and *Salmonella* species [131]. However, the structure and composition of the biofilms are diverse and can vary widely among the strains of the same species. Furthermore, the construction and composition of biofilm matrices and

the timing of their synthesis can differ in response to the small changes in environmental conditions [165]. The biofilm formation assays in microtiter dishes allows large number of samples to be tested rapidly. This method also allows testing of different environmental and growth conditions, leading to identification of genes that are involved in biofilm formation and maintenance. However, one limitation of this method is that it does not allow continuous flow of nutrients, as in a flow cell environment, which allows denser biofilm formation. Study of biofilm formation using flow cells provides more detailed information on biofilm architecture, which consists of mushroom and pillar like structures separated by water channels, that serves as a circulatory system allowing exchange of nutrients and waste products [165, 181]. Thus, flow cells could be used in future for further, more detailed investigations on the role of AceI in biofilm structure and composition.

As previously mentioned, biofilm formation is a multistep process, the first step being initiation of biofilm formation, which is characterized by the cell-cell interactions and cell-surface interactions [182]. In this study, biofilm formation by the parental AB5075-UW strain was first observed by SEM at 24h, as cell aggregation can be clearly observed at this stage (Figure 3.7). The next stage involves multiplication of bacteria on the surface and once enough cells have aggregated, mature biofilms are formed by synthesizing EPS [181]. The EPS serves as a protective barrier holding the bacterial cells together and firmly attaching the bacterial mass to the underlying surface, which could be observed after 48h of biofilm formation by the parental strain (Figure 3.7). Next stage of biofilm formation consists of dispersal of cells, which allows release of bacteria into the environment to colonize into the new surface or sites [181]. This stage of biofilm formation could be observed by quantitative analysis and SEM images at 72h and 96 h of biofilm formation by the parental strain (Figures 3.6,3.7). The final stage of biofilm formation involves detachment of cells from the biofilm community and their subsequent dispersal into the surrounding environment. The detachment and dispersal stage of biofilm formation is likely to play an important role in disease transmission within clinical settings or bacterial spread within the host, thus allowing bacterial persistence [181, 182]. The reduced biofilm formation phenotype observed in the *aceI* inactivated mutant during this study could be due to *aceI* playing a role in bacterial attachment to plastic surfaces as well as for the development of mature biofilms.

This study also examined the role of *aceI* in AB5075-UW biofilm formation properties at the air-liquid interface, also known as a “pellicle”. Most of the *Acinetobacter* biofilm formation

studies are focused on biofilm formed at solid surfaces, yet factors influencing biofilm formation at the air-liquid interface remain largely unknown. The air-liquid interface of biofilm formation is considered to be a favourable niche for aerobic bacteria like *A. baumannii* because the liquid media provides the cells with nutrients while they get enough oxygen from the air and this type of biofilm formation has mostly been found to be associated with the more pathogenic species [125]. Similar to biofilm formation on solid surfaces, the pellicles formed in liquid media are known to vary greatly in their morphology, pellicle organisation and the composition of the matrix among the different strains [125, 183]. A recent study by Chabane *et al*., reported the *A. baumannii* and *A. nosocomialis* species to have high pellicle forming ability compared to other *A. baumannii* species. The researchers also classified the strains into three groups based on their pellicle morphology, including egg-shaped, ball–shaped and irregular pellicles. The pellicle forming capacity was found to be associated with the high strain hydrophobicity combined with surface-interactions [125]. In the present study, a very thin pellicle with irregular hanging structures was observed with both the parental and *aceI* inactivated derivative after incubation at RT for 72 hours. Based on this observation, pellicle formed by the *aceI* inactivated strain does not seem to possess strong surface cohesion as detachment of the surface structure could be observed in the tube (Figure 3.8). Therefore, the hydrophobicity of the test strains can be tested in future, which might provide a possible explanation to the thin pellicle formation and detachment properties observed during this study.

It is hypothesized that pellicle formation in hospital settings might take place in tiny droplets which allows bacterial colonization and persistence observed in different clinical substrata, serving as a potential source of transmission and with the bacterial detachment and dispersal characteristics that these pathogens possess, they might spread and transfer themselves causing nosocomial transmission [125, 184]. Therefore, it is very important to examine the colonizing and persisting behaviour of these pathogens using a good biofilm and pellicle forming model, which might assist in potential identification of novel drug targets and also in development of hospital disinfectants and antiseptics that target these bacterial structures.

4.3 Bacterial adherence to the human alveolar cells

The ability of *A. baumannii* to adhere to eukaryotic cells has also been identified as one of the major steps in bacterial pathogenesis. Many studies have examined *A. baumannii* isolates for their ability to bind to human cell lines and revealed significant variations between the isolates in cell adherence [132, 185]. In this study, the adherence of the parental AB5075-UW to A549

human type 2 pneumocyte was tested, as this cell line represents a potential model for *A. baumannii* caused pneumonia. An *aceI* inactivated mutant strain was also included in the experiment to examine the effect of *aceI* gene inactivation in adherence to epithelial cells. This adherence study did not show any similarity to the results observed in abiotic surface attachment studies, instead the *aceI* knockout strain showed increased adherence affinity to A549 cells when compared to the parental AB5075-UW strain (Figures 3.9,3.10). This suggests that the mechanism(s) involved in adherence to the biotic and abiotic surfaces might be different, as suggested by other studies [161, 185]. However, several studies have described the importance of similar bacterial components in *A. baumannii*'s biofilm formation and epithelial cell adherence. This includes the outer membrane protein OmpA, which has been characterized as an important component for bacterial attachment to A549 epithelial cells [133]; biofilm associated protein, Bap has been found to be the surface structure that is involved in adherence of *A. baumannii* to human cell lines [186]. In addition, bacterial surface structures such as pili/fimbriae and the synthesis of exopolymeric matrices have also been found to contribute in bacterial adherence to biotic surfaces [132]. Therefore, the reduced adherence phenotype of AB5075-UW parental strain could be due to the involvement of *aceI* in transportation of components of exopolymeric substances or other outer membrane components, thus leading to lower adhesion and aggregation on the A549 cell surfaces. However, *aceI* was likely to be important for the initial attachment and mature biofilm formation on abiotic surfaces, thus further investigations might be necessary to confirm this hypothesis more specifically. Furthermore, attachment of AB5075-UW to other epithelial cells types could be tested in future to observe the importance of *aceI* in adherence to different cell types.

4.4 Bacterial surface structures and formation of exopolymeric substances

Bacteria produce various cell surface structures that assist them in attachment to the surfaces as well as in cell adhesion. SEM images showed that the cells were linked with each other through extracellular appendages that looked like pili (Figure 4.1A). Pili are 1-3 μm long protein fibers, like bacterial appendages and are made up of protein subunits called pilins. The extracellular bacterial structures like pili, flagella and fimbriae have been studied to be involved in cell adhesion, extracellular matrix stabilization in biofilms also in bacterial motility [121, 165]. In addition, a large amount of extracellular material was also observed holding the biofilm cells together (Figure 4.1B). This extracellular polymeric matrix is known to be

composed of polysaccharides, proteins, DNA and other amphiphilic molecules. These components of EPS serve the bacterial biofilm community by assisting in adhesion, initial aggregation of the bacterial cells, nutrient acquisition, exchange of genetic information, retention of ions and also serve as a protective barrier by conferring resistance to antimicrobial agents and the host defense molecules. EPS are known to differ between biofilms depending on the type of microbes, nutrient availability, temperature and other environmental conditions [121]. The biofilm formation on abiotic surfaces revealed that the AB5075-UW pathogenic strain is able to produce these bacterial components, similar to those of other pathogenic species and *aceI* gene inactivation was unlikely to affect the production of these surface appendages and EPS synthesis.

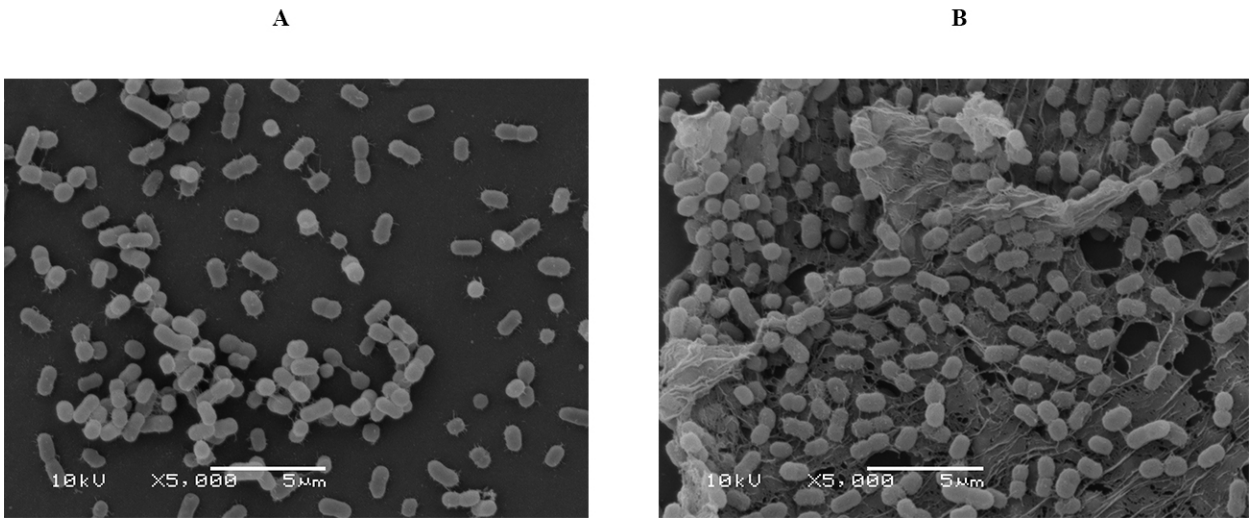


Figure 4.1: Synthesis of bacterial surface appendages (A) and exopolymeric matrices (B) by *A. baumannii* AB5075-UW. Scale bar: 5µm

4.5 Use of biosensor strains in QS signals detection

As mentioned earlier, QS is one of the most important regulatory mechanisms that bacteria use to communicate with each other by producing signalling molecules called AHLs. The AHLs are produced only by Gram-negative bacteria, however different bacterial species produce one or more forms of AHLs and they differ according to their length and specific substitutions of acyl side chains. QS in *A. baumannii* has been found to be involved in range of bacterial pathogenic activities including production of virulence factors and biofilm formation [104]. Studies have shown the involvement of many MDR pumps in extrusion of QS signals. Therefore, to examine whether *A. baumannii* AB5075-UW produces any QS signalling

molecules and if *aceI* gene inactivation affects the production of QS signals, these strains were cross streaked with the AHL biosensor strains. The biosensor strains used in this study included a mini-Tn*5* mutant of *C. violaceum* (CV026) and *A. tumefaciens* reporter strain (A136) (Table 2.1).

C. violaceum is a Gram-negative bacterium that produces the characteristic purple pigment called violacein and is commonly found in soil and water. CV026 is a violacein-negative white mutant, which is defective in the production of the factors, such as AHLs that can induce violacein production. However, violacein production can be induced by the unsubstituted AHL molecules with *N*-acyl side chains of four to eight carbon atoms. The presence of AHL molecules with carbon chain longer than ten, however does not induce expression of the violacein production [155].

A. tumefaciens is also a Gram-negative soil bacterium, which contains a plasmid with a *traR* promoter and *traG*::*lacZ* transcription fusion. This reporter strain is able to detect a broad range of AHLs including N-3-(oxooctanoyl)-L-homoserine lactones (OOHLs) and other AHLs with acyl side chains of eight to twelve carbon atoms in length by activating a traG::lacZ fusion and thus producing blue pigments [154, 187]. Therefore, these strains are considered as uncomplicated, model biosensor strains for the detection of AHLs.

In this assay, the parental AB5075-UW strain was found to induce blue pigment production with the A136 strain (Figure 3.12A). The production of blue colonies by AB5075-UW indicates that it either produces one type of AHL molecule at high concentrations or it produces a range of different A136 detectable AHL molecules. Furthermore, since purple pigment production by the parental AB5075-UW was not detected with the CV026 biosensor strain (Figure 3.12C), it can be hypothesised that AB5075-UW only produces longer carbon chains AHLs or the 3-oxo-derivatives of AHLs, which are only detected by A136 reporter strain. Contrarily, the *aceI* inactivated mutant showed an AHL negative phenotype with both the AHL biosensor strains (Figure 3.12B, D), suggesting that the *aceI* gene inactivation might have impacted the AHL production. The reason why AHLs were not detected might also be because the tested strains did not produce detectable quantities of AHLs. Purple pigments production could be observed with the PAO1 strain from *P. aeruginosa* (Figure 3.12E). This strain was included as a positive control along with the wild type *C. violaceum* (Figure 3.12F) which can produce purple violacein pigments on its own. The AHL negative phenotype observed with *aceI* inactivated mutant, suggest that *aceI* might be involved in transportation or secretion of

AHLs.

The use of these reporter strains facilitated very simple and rapid screening of multiple strains for the production of AHL molecules directly from the pure cultures. Thus, this method can be used as a first step of QS signal detection before moving onto in depth testing of any organisms. This direct detection method has been used by several studies to detect QS signal activity in biofilms [154, 155]. Some of the studies also have coupled this method with techniques such as thin layer chromatography and high performance liquid chromatography that allows extraction and quantitation of the signaling molecules [188, 189]. Therefore, similar techniques can be used in future to confirm these findings as well as to identify and quantify the AHL molecules produced by these isolates.

4.6 Chelator-mediated iron starvation

In order to prevent infection caused by pathogenic organisms, humans and other mammals limit the availability of essential metals, such as iron as a countermeasure against invading microbes. Therefore, in this study, available iron in MH medium was reduced by supplementing the medium with a synthetic iron chelator called 2,2'- dipyridyl (DIP) and growth of the parental AB5075-UW and *aceI* inactivated mutant was observed at varying DIP concentrations. The supplementation of DIP had been found to have no effect in pH of the MH medium [140]. The MICs of DIP was determined by performing serial dilution, starting with the highest concentration of 10mM of DIP. The MICs of DIP for both the strains were found to be 0.6mM, since it was the lowest concentration where the bacterial growth was still inhibited. Efflux pumps can participate in iron acquisition in bacteria, such as via the efflux of siderophores. However, the parental AB5075-UW and *aceI* inactivated mutant displayed similar levels of DIP-mediated inhibition, suggesting that *aceI* in AB5075-UW is not involved in iron acquisition. A genome-wide transcriptomics study of AB5075-UW in iron-limiting conditions might assist in identification of the potential iron acquisition mechanisms that this pathogenic strain utilizes to survive within the human host.

5 Future research

A range of assays performed during the course of this project have provided an important insight into the potential virulence mechanisms of the pathogenic AB5075-UW strain of *A. baumannii* as well as the effect of *aceI* gene inactivation in those mechanisms. However, there are further avenues of investigation required to fully understand the role of *aceI* in AB5075-UW virulence.

5.1 Construction of complemented *aceI* mutant strain

The construction of a complemented *aceI* mutant derivative had been attempted during the project but due to time constraints and other unforeseen circumstances, it could not be completed. The construction of the complemented strain is crucial in confirming the virulence attenuation phenotype observed due to the inactivation of *aceI* in the preliminary data as well as the phenotypic defects or changes observed during this project. The construction of complementation vectors should restore the wildtype phenotype and thus use of which will confirm that the observed phenotype was due to the primary mutation of *aceI* but not because of the undetected mutation or some downstream effect elsewhere in the genome.

5.2 Study of expression changes of *aceI* and potential substrate identification

Growth assays of the parental AB5075-UW and an *aceI* inactivated mutant revealed that the *aceI* mutant could not grow in 100% human serum, whereas the parental strain was able to grow well after an initial extended lag phase. Therefore, it is possible to hypothesize that *aceI* might be required to provide resistance to a component of human serum. Since efflux pumps are frequently expressed in response to their substrates, some additional evidence for this hypothesis could come from gene expression studies. In order to measure the expression of *aceI* in response to the human serum, mRNA can be extracted from the wild-type AB5075-UW cells grown in human serum and subjected to qRT-PCR to measure changes in gene expression relative to the housekeeping genes. This would confirm if *aceI* is upregulated during exposure to human serum and may thus suggest the importance of the *aceI* gene in the survival of *A. baumannii* AB5075-UW in human serum.

Furthermore, in order to identify the potential substrate(s) of AceI in human serum, the interaction of purified AceI protein with human serum could be investigated using techniques such as,equilibrium dialysis, ultrafiltration, ultracentrifugation, size-exclusion chromatography

and solid phase immobilization techniques in order to identify any component of human serum that binds to AceI [190].

5.3 Genome wide transcriptomic studies

In order to quantify differential gene expression in response to range of different conditions, such as human serum, iron limitation and biofilms, whole transcriptome sequencing could be performed. This technique will provide a broad overview of transcriptional changes under particular conditions. For example, the growth of AB5075-UW in serum mimic the biologically relevant growth condition of bacteraemia, which might lead to an identification of condition specific essential genes that are required for the survival and persistence of AB5075-UW in blood. In addition, a study of transcriptomic changes in biofilms as well as in iron-limiting conditions will further enhance our understanding and knowledge of the genes that are involved in these virulence functions at the transcriptomic level.

5.4 Confirming the involvement of *aceI* in epithelial cell adherence

In order to confirm the higher adherence phenotype observed with the *aceI* knockout strain in bacterial adherence assays with A549 human alveolar cells, further investigations would be required. Due to time constraints, this experiment could not be repeated and the effect of incubation time in bacterial adherence could not studied. Therefore, this result needs to be confirmed by including one or more biological replicates in the assays, as well as by increasing the incubation duration to observe the effect of incubation time in bacterial adherence. Furthermore, attachment of AB5075-UW to other epithelial cell types could be tested in future to observe the importance of AceI for adherence to different cell types. For instance, epithelial cell type relevant to intraperitoneal infection model could be used to confirm the virulence defect phenotype observed in the preliminary mouse model studies.

6 Conclusion

In summary, results presented in this work indicate the involvement of the *A. baumannii* AB5075-UW clinical strain in different aspect of pathogenesis. The findings from comparative analyses between an *aceI* inactivated mutant and the parental AB5075-UW strongly suggest the involvement of the *aceI* gene in many physiological processes relevant to infections including survival in serum, biofilm formation and QS. However, further investigations are required to fully understand the adherence and persistence mechanisms of AB5075-UW in human epithelial cell lines and also to compare the mechanisms employed by AB5075-UW in attachment to biotic and abiotic surfaces. Collectively, the observations from this study indicate the potential role of *aceI* gene in *A. baumannii* AB5075-UW pathobiology and thus may make a good target to impair pathogenesis and virulence of this pathogen.

Furthermore, the potential role(s) of *aceI* in pathophysiological mechanisms of AB5075-UW identified in this study, may contribute in development of *aceI* specific efflux pump inhibitors (EPIs), which could serve as the anti-virulence factors in fighting many infectious diseases associated with AB5075-UW. The EPIs could be used as an alternative to antibiotics or together with antibiotics, thus reducing the selective pressure for bacterial resistance.

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