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Physiological functions of multidrug efflux pumps in the nosocomial pathogen *Acinetobacter baumannii.*

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Disclaimer

I certify that the work presented in this thesis has not been submitted for a higher degree to any other university or institution other than Macquarie University, Sydney, Australia. This thesis is my own original work and I have acknowledged all sources used and have cited these in the reference section.

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Abstract

The success of *A. baumannii* as a pathogen may be attributed to its broad resistance capabilities, and its ability to persist in the clinical setting and infect new hosts. Multidrug efflux pumps may play important roles in each of these factors. The role of multidrug efflux pumps in conferring resistance to antibiotics is of clinical relevance and has been well studied. However it is becoming increasing apparent that these efflux pumps may have physiological roles such as colonisation of the bacterium in hosts; maintaining cellular homeostasis by mediating extrusion of endogenous polyamines and playing a role in cell-to-cell signalling which ultimately controls biofilm formation.

To determine if multidrug efflux pumps have physiological roles in biofilm formation and resistance to host produced factors a mutant library from strain AB5075-UW was screened. Disruption of several chromosomally encoded efflux pump genes resulted in either significantly higher or lower biofilm formation. Efflux pumps which showed significant differences in biofilm were screened for polyamine transport. It was found that MATE efflux pump mutant ABUW_0109-152::T26, which displayed a loss of biofilm formation, was sensitive to the polyamine spermine. Therefore, spermine may be a native substrate of this efflux pump and play a role in biofilm regulation.

1 Introduction

1.1 *A. baumannii* as a global pathogen.

Members within the genus *Acinetobacter* are defined as Gram-negative, strictly aerobic and non-fastidious coccobacilli with a DNA G+C content of 39 – 47% (1, 2). Species belonging to genus *Acinetobacter* are omnipresent in nature and are frequently isolated from soil and water samples, yet the ubiquity of certain species in nature such as *Acinetobacter baumannii* is still largely unclear (3). Some species of *Acinetobacter* have been found to be part of normal skin flora of humans. Furthermore, epidemiological studies have found colonisation of skin and mucosal membranes with *Acinetobacter* species such as *A. lwoffii, A. johnsonii* and *A. junii* in non-hospitalised persons (1, 2). Contrarily, *A. baumannii*, the most significant nosocomial and community-acquired pathogen within this genus, are rarely found to be a part of normal human flora (1, 2).

The emergence of *A. baumannii* as a significant pathogen may be attributed to its association with nosocomial and community- acquired infections, as well as emergence of strains that are resistant to multiple if not all commercially available antibiotics (1). In clinical settings, *A. baumannii* has largely been affiliated with ventilator-associated pneumonia with majority cases reported in the ICU. It has also been isolated from nosocomial infections such as septicemia, endocarditis, meningitis and urinary tract infections (1, 2). Others factors contributing to risks of *A. baumannii* colonisation and infection include surgery, catheterisation, tracheostomy, mechanical ventilation and enteral feedings (1, 2). Aside from nosocomial infections, *A. baumannii* has also been affiliated with community-acquired infections in countries such as Australia, Asia and the Middle East (1, 2, 4). Incidents of community acquired *A. baumannii* infections reported in Australia have different epidemiology than those observed in a clinical setting. These differences include, aggressive community acquired pneumonia; pharyngeal carriage of *A. baumannii* strains and a high correlation between alcoholism and infections have been reported (1, 2, 4). In contrast, in Asia and the Middle East community acquired *A. baumannii* infections have primarily been associated with battlefield injuries incurred by soldiers and natural disasters such as the Southeast Asian tsunami of 2004(1, 2).

The infectious disease society of America has classified *A. baumannii* as one of the ESKAPE pathogens (5, 6). ESKAPE pathogens are a set of six highly drug resistant pathogens identified as a serious risk to public health. Pathogens, which form part of this classification include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp (7). Collectively, ESKAPE pathogens are adept at escaping antimicrobial activity of antibiotics and collectively represent a new paradigm in pathogenesis, transmission and resistance (7).

1.2 Multiple roles of efflux pumps

The success of *A. baumannii* as a pathogen may be attributed to its broad resistance capabilities (1), its ability to persist on medical devices in hospitals despite being subjected to adverse conditions such as desiccation (8), and its ability to cause infections (9). Efflux pumps encoded by *A. baumannii* may have played major roles in the resistance, persistence and pathogenicity of this pathogen. The role of multidrug efflux pumps in conferring resistance to antibiotics has been well characterised and is of clinical relevance. However, it is becoming increasing apparent that these multidrug efflux pumps may have roles other than decreasing susceptibility to antibiotics (14), such as colonisation and survival of the bacterium in hosts (17-20). Although most multidrug efflux systems recognise a broad range of chemically and structurally distinct antibiotics (10), some efflux pumps have a high specificity for single substrate. Substrates of these pumps may include, fatty acids (11), bile salts (12), polyamines (13), biofilm matrix components (14) and signalling molecules associated with quorum-sensing (15, 16). For example, the ability of Proteobacteria including *Campylobacter jejuni* (19), *Escherichia coli* (20) and *Vibrio cholera* (18) to colonise the gastrointestinal tract of hosts has been associated with extrusion of host-derived substrates such as bile and fatty acids (17). Therefore it can be suggested that extrusion of host-derived natural substrates by efflux pumps promotes survival of bacteria in its ecological niche and therefore may impart bacterial pathogenicity (17).

In addition to promoting survival, multidrug efflux pumps have also been associated with extrusion of homoserine lactones associated with quorum sensing (15, 16, 21). Quorum sensing is a signalling system which regulates the expression of genes in response to the cell population (22). Bacteria regulates a range of physiological activities, including biofilm formation, using this signalling system (22). Recently, multidrug efflux pumps were also found to be involved in the extrusion of essential biofilm matrix components (14). The ability of many *A. baumannii* strains to form biofilms has been associated with their ability to persist on a range of surfaces in hospitals (8, 23).

Finally, while polyamines have pleiotropic effects in bacteria, high concentrations of polyamines can be toxic to bacteria as they can inhibit protein synthesis; therefore efflux pumps which have been implicated in biofilm formation, have also been shown to modulate intracellular polyamine levels to maintain homeostasis (24).

The goal of the work presented in this thesis was to investigate the physiological roles of drug efflux pumps in *A. baumannii* in functions other than drug resistance, focussing on biofilm formation, polyamine transport, and resistance to host produced factors such as fatty acids. Furthermore, the role of polyamines in biofilm formation by *A. baumannii* was also investigated.

1.3 Efflux pumps encoded in the core genome of *A. baumannii*

There are almost 50 putative drug efflux pumps encoded in the core genome of *A. baumannii*, (i.e. genes conserved in all strains) (25). However, less than 20% of these are known to have resistance functions, while the roles of others are unknown. These efflux pumps could have alternative physiological roles, but are poorly studied (25). Analysis of diverse *Acinetobacter* spp, including non-pathogenic strain *A. baylyi* ADP1, clinical strains *A. baumannii* ATCC 17978, *A. baumannii* AYE, and the commensal strain *A. baumannii* SDF showed that almost 75% of the putative multidrug efflux pumps encoded in *A. baylyi* ADP1 are common with at least one clinical strain of *A. baumannii* (25). Furthermore, twenty-one multidrug efflux pumps (almost half of the efflux pumps encoded) are conserved across all four *Acinetobacter* spp (25). Interestingly, non-pathogenic soil commensal *A. baylyi* ADP1 encodes almost as many multidrug efflux pumps as clinical strains of *Acinetobacter* (25). Given their broad conservation, efflux pumps encoded in the core genome of *A. baumannii* may have functions that are unrelated to drug resistance.

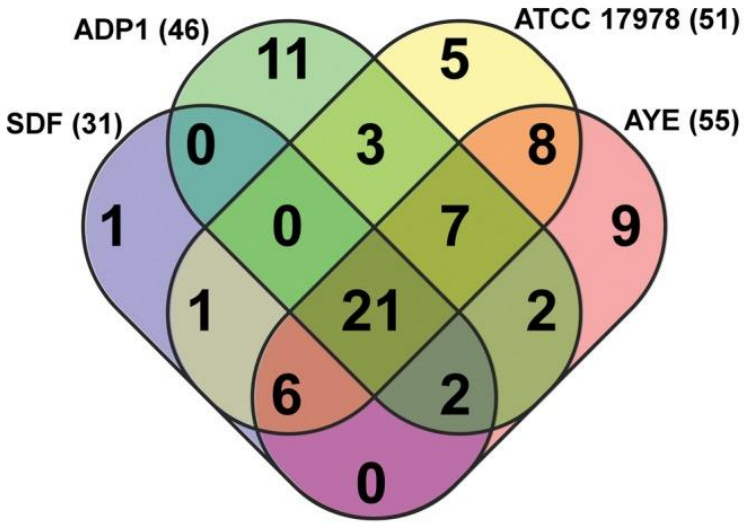

Figure 1.1 (Figure credit: Brzoska *et al.*, 2013) Venn diagram illustrating conservation of multidrug efflux pumps across *Acinetobacter* species (25). Each colour-coded ellipse represents a strain, and the total number of putative multidrug efflux pumps encoded within each strain is specified next to the strain name (25). Common putative multidrug efflux pumps are represented by intersection of ellipses, whereby 21 efflux pumps are conserved across all four strains (25).

Conserved genes that are encoded in the core genome, such as efflux systems may contribute to intrinsic resistance through active extrusion of antimicrobials (26). In contrast, acquired genes can emerge through acquisition and dissemination of mobile elements, such as insertion sequences, integrons, plasmids and resistance islands (1). The acquisition of these elements can result in the rearrangement of a bacterial genome, imparting a source of adaptability (27). Acquired resistance

can also arise through mutations to core genes, such as those encoding the targets of antimicrobials (26). Efflux pumps encoded in the core genome provide base level resistance to antimicrobials, however when coupled with other resistance mechanisms such as mobile elements and enzymatic degradation their functions can lead to high-level resistance to antimicrobials and emergence of multidrug resistant strains.

1.4 Efflux pumps encoded in the accessory genome

1.4.1 Insertion sequences and transposons

Insertion sequences (IS) are generally considered to be the smallest mobile elements carrying genetic information necessary for their mobilisation, they consist of a transposase gene, which mediates the transposition of IS elements, flanked by short inverted repeat (IR) sequences (27). More than 35 distinct IS have been reported in *A. baumannii* (28) of which IS*AbaI* is the most prevalent (29). Mobilisation of IS can contribute to resistance in many ways, including, insertion of IS element upstream from an ORF leading to a higher level of expression of the resistance gene, which are otherwise poorly expressed (27) such as insertion of IS*AbaI,* within the *adeRS* operon resulting in overexpression of the AdeABC efflux pump (30). Overexpression of the AdeABC efflux pump has been associated with extrusion of several antibiotics including aminoglycosides and beta-lactams (30).

In addition to occurring independently, IS can also mediate mobilisation of other mobile elements (31). IS are known to flank gene cassettes through IR mediated displacement of intervening DNA thereby forming a composite transposon (31). Tn*10* is an example of a composite transposon, it has nine ORFs including *tetA* efflux pump gene which mediates resistance to tetracyclines (32).

1.4.2 Integrons

Genetic elements such as integrons are assembly platforms, which mediate acquisition and insertion of exogenous ORFs through site-specific recombination and transform it to functional genes by directing their correct expression (33). All integrons are composed of three key elements; a main recombination site (attI), gene-encoding integrase (intI) and an outward-orientated promoter (Pc) which controls transcription of the acquired genes (33, 34). The *qacE* gene, which encodes an SMR efflux pump is generally found to be carried by type 1 integrons while four copies of the *qacE* gene has been found on larger mobile elements such as resistance islands, these efflux pumps have been associated with resistance to quaternary ammonium compounds (QACs) (35).

1.4.3 Resistance islands

Resistance islands are distinct clusters of genes with features that suggest horizontal acquisition of the genetic material such as presence of mobility genes (e.g. integrases/recombinases and transposases) and atypical nucleotide composition, findings from several studies have led to the speculation that these regions may serve as 'hot spots' for acquisition of novel genes through horizontal transfer since damage to the cell as a result of insertion is highly unlikely (35-37).

A study by Fournier *et al.*, wherein they compared genomes of multidrug resistant *A. baumannii* strains AYE and SDF, led to the discovery of the largest known resistance island reported thus far in any bacterial strain. Found in strain AYE and termed AbaR1, it exhibits an 86-kb genomic region in which 45 resistance genes are clustered (35). Eight efflux pump genes are reported to be part of resistance island AbaR1, these are associated with mediating resistance to various classes of antibiotics including tetracycline efflux pump gene (*tetA*) and repressor (*tetR*) as well as chloramphenicol efflux pump gene (*cmlA*) (35), as their names imply, these efflux pumps confer resistance to tetracyclines and chloramphenicol. A similar structure to resistance island AbaR1, termed AbaG1 exhibiting a 20 kb-genomic island was identified in the commensal isolate SDF at the homologous ORF, however it was devoid of resistance markers (35). Furthermore, a similar insertion locus was also found in *A. baumannii* strain ATCC 17978 (37).

Collectively, the ability of *A. baumannii* to acquire, retain, and disseminate genes typifies the genetic agility and plasticity by which this organism is able to confer resistance.

1.5 Membrane permeability and role of multidrug efflux pumps in resistance

1.5.1 Membrane permeability

The outer membrane of Gram-negative bacteria serves as a permeability barrier to reduce influx of toxic compounds, while allowing entry of nutrients into the cell (38). The outer membrane includes channel-forming proteins such as porins, which allow movement of molecules across the lipid bilayer of the outer membrane (38). Aside from transport, porins serve multiple functions in Gram-negative bacteria, including, acting as targets for adhesion; evading antimicrobial stress through down regulation of porin expression and modifications in structure (39).

One of the major outer membrane protein (OMP) in *A. baumannii* is the heat-modifiable protein (HMP-AB) (40). HMP-AB belongs to the monomeric OmpA family of porins also referred to as slow porins (40). Monomeric proteins have been reported to be less penetrable to negatively charged β-lactams (41), therefore high levels of intrinsic resistance may be observed where proteins from this family serve as primary porins in bacteria (39). Another OMP of great importance in *A.*

baumannii is CarO, the absence of which, due to loss of gene via insertion elements, has been associated with imipenem resistance in *A. baumannii* (42, 43). Other mechanisms of resistance mediated by OMPs can be attributed to the presence of a small number of small-sized porins. Collectively, these characteristics provide an explanation for the reduced outer membrane permeability to antimicrobials in *A. baumannii* when compared to other Gram-negatives (39, 44).

1.5.2 Families of multidrug efflux pumps

Multidrug efflux systems are generally classified into five different families: the ATP binding cassette (ABC) superfamily, the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family (10, 17, 27). Furthermore, a new family of multidrug efflux pumps has recently been described, namely the Proteobacterial Antimicrobial Compound Efflux (PACE) family, common amongst proteobacterial lineages (45). Members of this novel multidrug efflux protein family have shown to be encoded in the core genome of most encoding species (45).

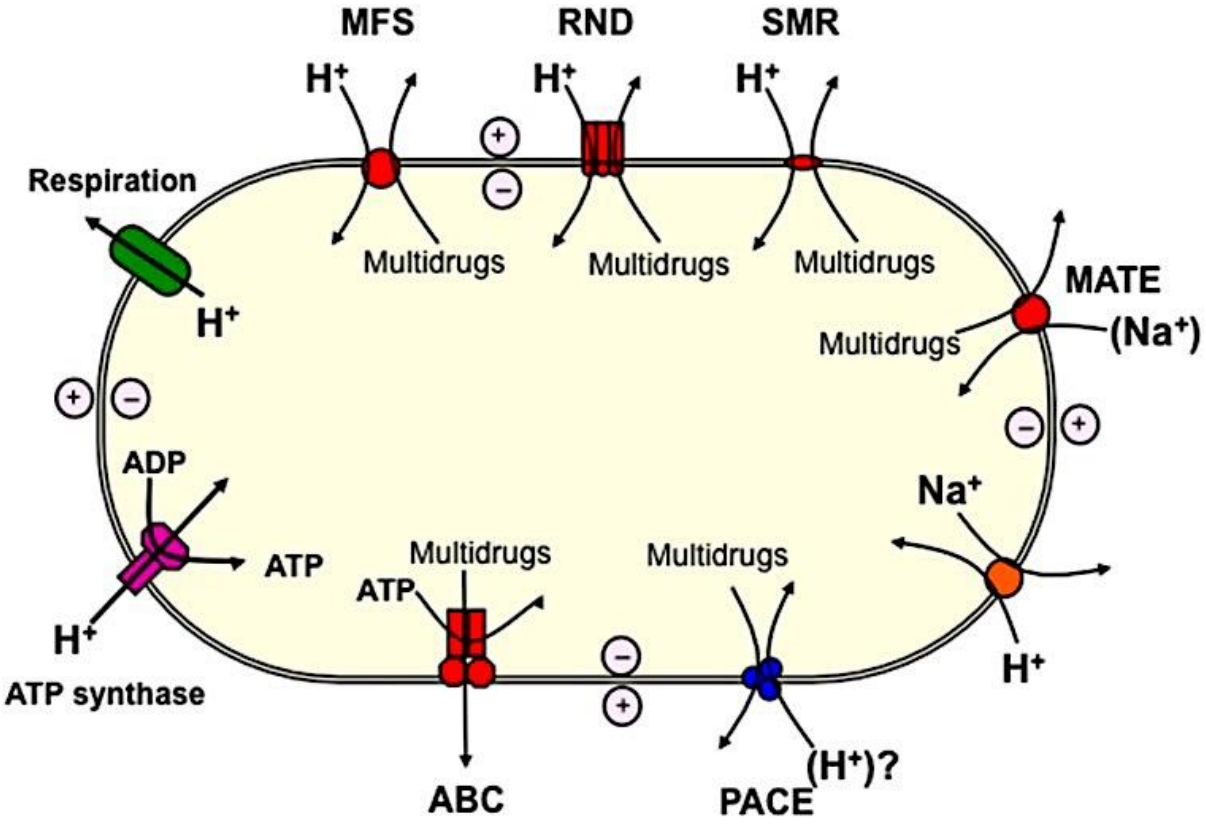

Figure 1.2 (Figure credit: Hassan *et al.*, 2015) Schematic illustration of multidrug efflux families in Gram-negative bacteria (45).

1.5.2.1 The resistance-nodulation-division (RND) family

The RND family of transporters is one of the most clinically significant agents in terms of resistance; they are also ubiquitous in Gram-negative organisms, including species of *Acinetobacter* and confer high-level drug resistance (10, 27). RND efflux pumps are known to export a wide range of variant molecules, such as classes of antibiotics, dyes (10), detergents (10), toxic fatty acids and homoserine lactones affiliated with quorum-sensing (47). Members within this family generally operate as a tripartite system that is composed of an outer membrane protein (OMP), a transport protein (the RND pump) and a periplasmic membrane fusion protein (MFP) that links the other two protein types (10, 17, 46, 48). The first RND efflux system to be described in *A. baumannii* was termed AdeABC, whereby AdeB was the RND multidrug transporter protein and AdeA and AdeC resembled the MFP and OMP, respectively (46). It is suggested that the genes encoding these proteins may constitute an operon due to their contiguous and directly oriented nature (49). The *adeABC* genes are preceded by the *adeR* and *adeS* genes, which are oriented in the opposite direction and encode a two-component regulatory system controlling *adeABC* gene expression (49). The AdeABC efflux pump in *A. baumannii* is responsible for efflux-mediated resistance to aminoglycosides (46). It also lowers susceptibility to a variety of other antimicrobials such as tetracycline, kanamycin, tigecycline, chloramphenicol and meropenem (27). Several genomic studies on *A. baumannii* isolates have illustrated that not all strains carry the AdeABC system (50-52), suggesting that it may not be part of the core genome in *A. baumannii*, yet highly prevalent amongst nosocomial strains (27).

Another RND efflux pump, the AdeIJK system has been shown to be co-transcribed and present in all strains of *A. baumannii* (52, 53), yet, high-level expression of the cloned *adeIJK* operon is found to be toxic to cells, this suggests that it may be associated with low-level intrinsic resistance (52). Furthermore qRT-PCR and transcriptomic microarray analysis have illustrated that levels of *adeIJK* expression are relatively low in comparison to *adeABC*, implying tight regulation of *adeIJK* system (53). Until recently, no regulatory genes had been found within the vicinity of *adeIJK*, however whole genome sequencing analysis conducted by Rosenfeld *et al.*, implicates *adeN* (present in all isolates tested) as encoding a transcriptional regulator from the TetR family that represses expression of the *adeIJK* operon (54).

Finally, the RND efflux pump AdeFGH was found more recently than the other two RND systems and provides a high-level of resistance against chloramphenicol, trimethoprim and fluoroquinolones, when overexpressed (55). A LysR-type transcriptional regulator gene termed *adeL*, was also identified in opposite orientation upstream from the *adeFGH* operon, mutations within the *adeL* gene have been affiliated with overexpression of this efflux system (55).

1.5.2.2 The major facilitator superfamily (MFS)

The major facilitator superfamily (MFS), also referred to as the uniporter-symporter-antiporter family is found across all kingdoms of life (56). These transporters are single polypeptide secondary carriers, and together with the ABC superfamily they account for almost half of the transporters in the genome of prokaryotes (56, 57). Some examples of MFS efflux pumps in *A. baumannii* are the tetracycline efflux systems (58), CmlA (35), AmvA (59) and CraA efflux pumps (60).

TetA and TetB proteins are the main Tet efflux pumps in *A. baumannii*, whereby TetA is implicated in conferring resistance to tetracycline, and TetB in tetracycline and minocycline resistance (58). These efflux systems are generally encoded with a repressor protein, which is responsive to tetracycline (39). In the absence of tetracycline, the repressor proteins binds upstream of the transporter gene and prevents expression (39). However, in the presence of a tetracycline–Mg^{2+} complex, these repressor proteins undergo a conformational change, causing them to be released from the structural genes and thus permitting its transcription (61). The Tet efflux proteins function to catalyse extrusion of tetracycline divalent metal complexes in exchange for protons (61, 62). In *A. baumannii tet* efflux genes can also be present on accessory genome such as transposons, which are inserted into plasmids via conjugation (39). A study conducted by Riberia *et al.,* reported that the location of *tetA* gene together with its transcriptional regulator, was on a Tn*1721*-like transposon, which may in turn be part of a much larger mobile element such as the resistant island - AbR1 described by Fourier *et al.* (35, 63).

CraA, which is another MFS efflux pump protein, has been implicated in chloramphenicol resistance of *A. baumannii* isolates (60). Identified by Roca *et al.,* this efflux protein showed sequence and structural similarities to that of MdfA efflux pump from *E. coli,* yet demonstrated a different substrate recognition profile (60). So far, it has been identified in all *A. baumannii* isolates tested, therefore it can be postulated that CraA may form part of intrinsic resistance against antimicrobials (27).

The third MFS efflux pump protein to be characterised in *A. baumannii* is termed AmvA (59). Again this protein has been identified in all *A. baumannii* isolates studied so far hence it may also form part of intrinsic resistance mechanisms (59). Molecular and functional characterisation of this efflux pump protein by Rajamohan *et al.,* has shown that the AmvA efflux pump confers resistance to agents such as benzalkonium chloride, ethidium bromide, SDS, DAPI and Acridine Orange (59).

Very recently, Li *et al.,* found drug resistance capabilities in two MFS efflux pump proteins ABAYE_0913 and A1S_2795 encoded by *A. baumannii* strains AYE and ATCC 17978 respectively (64).

1.5.2.3 The multidrug and toxic compound extrusion (MATE) family

AbeM is the only MATE family efflux pump to have been described in *A. baumannii* thus far. AbeM reportedly confers resistance to several dyes, chloramphenicol, aminoglycosides, fluoroquinolones, and ethidium bromide (65). Members of this family of efflux proteins are energised through either the proton motive force or the sodium ion gradient (Figure 1.2) (39), this was confirmed by Su *et al.*, which showed that AbeM uses the proton motive force for extrusion of antimicrobials (65).

1.5.2.4 The small multidrug resistance (SMR) family

Efflux pumps that belong to the SMR family are expressed in the inner membrane and consist two polypeptides that each contains four transmembrane helices (66). Some extensively studied SMR proteins are the EmrE efflux pump in *Escherichia coli* and the EbrAB efflux pump found in *Bacillus subtilis* (66). Srinivasan *et al.*, have described a chromosomally encoded SMR efflux pump protein in *A. baumannnii*, termed AbeS. This protein, identified in AYE strain displays 51.8% similarity to that of EmrE protein in *E. coli* (66). Furthermore, analysis of AbeS mutants in *A. baumannii* indicates that it might confer low-level resistance to novobiocin, fluoroquinolones, dyes, detergents and chloramphenicol (66).

1.5.2.5 The ATP binding cassette (ABC) superfamily

Similar to MFS proteins, members within the ATP binding cassette (ABC) superfamily form one of the largest protein families and are found across all living organisms (67). Their ubiquity and conserved structure suggests a fundamental role in survival (67). ABC proteins utilise the energy produced through ATP hydrolysis to mediate movement of substrates, as well as opening and closing of membrane channels. One example of a multidrug exporter within this family namely Sav1866, is found in *S. aureus*. Sav1866 is a bacterial homolog of Mdr1 found in humans, also an ABC transporter (67). Very recently, Li *et al.*, described a resistance function for an ABC efflux pump in *A. baumannii* (64).

1.5.2.6 The proteobacterial antimicrobial compound efflux (PACE) family

Recently, a multidrug efflux pump designated AceI (Acinetobacter chlorhexidine efflux) was identified to be part of a new family of bacterial multidrug efflux systems, the proteobacterial antimicrobial compound efflux (PACE) family (68). AceI protein is composed of 150 amino acid residues and has two tandem bacterial transmembrane pairs (BTP) (68). It was found that in diverse bacterial species, particularly species with proteobacterial lineages have genes that encode BTP

domain proteins homologous to AceI protein (69). Resistance studies against chlorhexidine showed that genes encoding BTP domain proteins were upregulated in bacterial species *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* J2315 (69). Additionally, expression of BTP domain protein genes from *Acinetobacter baylyi* ADP1 and *Pseudomonas protegens* Pf-5, in *E. coli* resulted in increased susceptibility to chlorhexidine (69). The PACE family represents the first new family of multidrug efflux pumps to be described in 15 years (68). In recent unpublished work, members of the Paulsen group have shown that PACE family members are able to transport one or more polyamines, which could be natural substrates for these proteins (Liu *et al*, unpublished).

1.6 Efflux pumps play important roles in biofilm formation

1.6.1 Biofilms in persistence and virulence

In addition to molecular mechanisms of antimicrobial resistance, structural advantages as seen with many other Gram-negative organisms have allowed *A. baumannii* strains to survive on inanimate surfaces for an extended period of time. *Acinetobacter* spp. in comparison with other Gram-negative species are impervious to dry conditions (70, 71) and are capable of growing across varying temperature, pH and with varying nutrient availability, therefore maximising their survival rates and potential for cross-contamination in hospital settings (1, 2, 4, 36). The persistence and resistance of *A. baumannii* to desiccation has been directly correlated with its ability to form biofilms (71), additionally, studies have shown that, *A. baumannii* are able to survive desiccation much better than other *Acinetobacter* species (70, 71), possibly accounting for its emergence in hospital settings. Furthermore, biofilm forming strains of *A. baumannii* have been reported to be more resistant to antimicrobials than non-biofilm-forming counterparts (70).

A. baumannii has been reported to form biofilms on both abiotic surfaces in hospitals including catheters and respiratory equipment (72) and biotic surfaces, such as during infection (73, 74). It has also been shown to persist and mediate apoptotic death of eukaryotic cells such as *Candida albicans* and A549 human alveolar epithelial cells (9) which could serve as a survival strategy of *A. baumannii* and its ability to impart virulence. Specific genes have been associated with encoding virulence factors in *A. baumannii*, including the *ompA* gene which encodes outer membrane protein OmpA (9). Mutation in the *ompA* gene shows a loss in the ability of *A. baumannii* to attach to eukaryotic cells as well as reduced biofilm formation (9). Subsequent studies have since confirmed that attachment of OmpA is through specific binding to fibronectin in eukaryotic cells (75).

While studies of biofilm formation in *Acinetobacter* spp. have largely been focused on biofilms formed on solid surfaces, biofilm formation at the liquid-air interface has been found to be more

prevalent in pathogenic species (76-78). It has been suggested that the air-liquid interface may be a favourable niche since bacteria can acquire nutrients from liquid, and oxygen from air (79).

1.6.2 Biofilm structure and development

In nature, bacteria usually do not exist as single cells but rather as clusters or aggregates known as biofilms (80). Biofilms are structured microbial communities that are composed of cells sheathed in a self-produced polymeric matrix composed of polysaccharides, extracellular DNA, proteins and fibres (80-82). The matrix provides structural stability and serves as a protective barrier, which allows bacteria to survive in hostile environments, rendering them more resistant to antimicrobials (70, 81). Biofilm initiation and development is a highly orchestrated series of molecular events, which cells maintain under tight regulation (83). General factors, which influence biofilm formation, are nutrient availability, bacterial appendages such as pili and flagella, surface components such as OMPs, macromolecular secretions such as polysaccharides (83) and quorum sensing (QS) systems (84).

Several factors have been reported to play a role in various aspects of biofilm development in *A. baumannii*. It has been shown that in *A. baumannii* the CsuA/BABCDE chaperone-usher pili assembly system is required to promote adherence and biofilm formation on abiotic surfaces, however this system is not required for biotic adherence (23, 85). A two component regulatory system has been found to regulate expression of the chaperone–usher assembly system, whereby inactivation of regulator component results in biofilm deficiency whereas inactivation of the sensor component results in partial loss of biofilm (86).

In addition to the chaperone–usher assembly system, biofilm formation on polystyrene has been found to be dependent on biofilm-associated protein (Bap), inactivation of which resulted in the inability of *A. baumannii* to sustain biofilm thickness, three-dimensional tower structure and water channel formation (73, 74). Another factor that has been found to play a role in biofilm thickness and volume, is polysaccharide poly-β-(1-6)-*N*-acetylglucosamine (PNAG) encoded by the *pgaABCD* gene (87). Other more recent factors which have been found to be associated with biofilm formation in *A. baumannii*, include acidic transcription factor A (AtfA) (88) and surface-adhesion protein - Acinetobacter trimeric autotransporter (Ata) (89).

In addition to the specific mechanisms mentioned thus far, general molecular mechanisms which regulate biofilm formation and dispersal in several Gram-negative species are, cyclic diguanosine-5'-monophosphate (c-di-GMP), small RNAs (sRNA) and quorum sensing (QS) (90). All of these have been found to be associated with the production of exopolysaccharides which forms part of the biofilm matrix (90).

1.6.3 Quorum sensing in biofilms

Quorum sensing systems are cell-density dependent intercellular signalling systems, which are based on self-produced signalling molecules termed autoinducers e.g. acylated homoserine lactones (AHLs) (91-93). When adequate bacteria are present and autoinducers reach a threshold concentration (driven by quantity of producer cells), the community responses are triggered, which may include biofilm formation (92, 93). Studies concerning analysis of quorum sensing in *Pseudomonas aeruginosa*, a model organism for Gram-negative biofilm formation, have postulated that QS regulons can constitute approximately 10% of the genome (94).

A study by Niu *et al.*, found that *A. baumannii* strain M2 produces an acyl-homoserine lactone molecule, a product of the autoinducer synthase gene *abaI* (95). Mutation in the *abaI* gene resulted in 30-40% reduction in the later stages of biofilm development (95). Furthermore, when the *abaI* mutant was complemented with exogenous M2 acyl-homoserine lactone, biofilm maturation was restored (95).

1.6.4 Quorum sensing signals are substrates of multidrug efflux pumps

It has been speculated that, while short chain autoinducers can diffuse freely across the bacterial cell membrane larger autoinducers may require efflux pump mediated extrusion from the cell (15, 47). Extensive studies of biofilm formation in Gram-negative model organism *P. aeruginosa* have shown that efflux pumps belonging to the Resistance Nodulation Division Efflux (RND) family are involved in the extrusion of several quorum-sensing signals in *P. aeruginosa* (15, 47, 96, 97).

Pearson *et al.*, showed that upregulation of RND multidrug efflux pump MexAB-OprM significantly reduces intracellular concentrations of a major quorum-sensing signalling molecule, 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL), suggesting that it may be a substrate of the MexAB-OprM efflux pump (47). Further studies into MexAB-OprM pump have shown that several non-native N-acylated L-homoserine lactones and related derivatives are also substrates of these efflux pumps (97).

Similar to MexAB-OprM, RND multidrug efflux pump MexEF-OprN has also been found to mediate active extrusion of 3-oxo-C12-HSL (15). Interestingly, MexEF-OprN has also been found to reduce intracellular concentrations of *Pseudomonas* quinolone signal (PQS) (15). PQS has been reported to influence biofilm formation in *P. aeruginosa* (98). PQS together with acyl homoserine lactone has also been found to play a role in the generation of extracellular DNA found in the biofilm matrix (99). RND efflux pumps BCAL1675 and BCAL2821 have also been implicated in mediating extrusion of autoinducers in *Burkholderia cenocepacia*, whereby mutations in these efflux pumps resulted in 30% less accumulation of N-octanoyl homoserine lactone (C8-HSL) in the medium relative to parental strain (100). Very recently, He *et al.*, found a positive correlation

between biofilm formation and the up-regulation of RND efflux pump genes *adeB* and *adeG* genes in *A. baumannii*. Furthermore, upregulation of autoinducer synthase gene (*abaI*) and *adeG* gene resulted in extensive biofilm formation (101).

1.6.5 The biofilm life cycle

Mapping of transitional episodes in the biofilm life cycle of *Pseudomonas aeruginosa* has shown that physiological changes from initial attachment through to detachment and re-entry into planktonic growth are profound and highly complex (102). Furthermore, biofilm cells at different stages of the life cycle were shown to change regulation of motility, alginate production, and quorum sensing (102). Using microscopy and proteomics approaches, the biofilm life cycle of Gram-negative *P. aeruginosa* was classified into five stages: (i) reversible attachment, (ii) irreversible attachment, (iii) maturation-1, (iv) maturation-2, and (v) dispersion (102). Hence, since the molecular events within the biofilm life cycle are dynamic, it can be postulated that efflux pumps may have different roles at different stages of the biofilm life cycle.

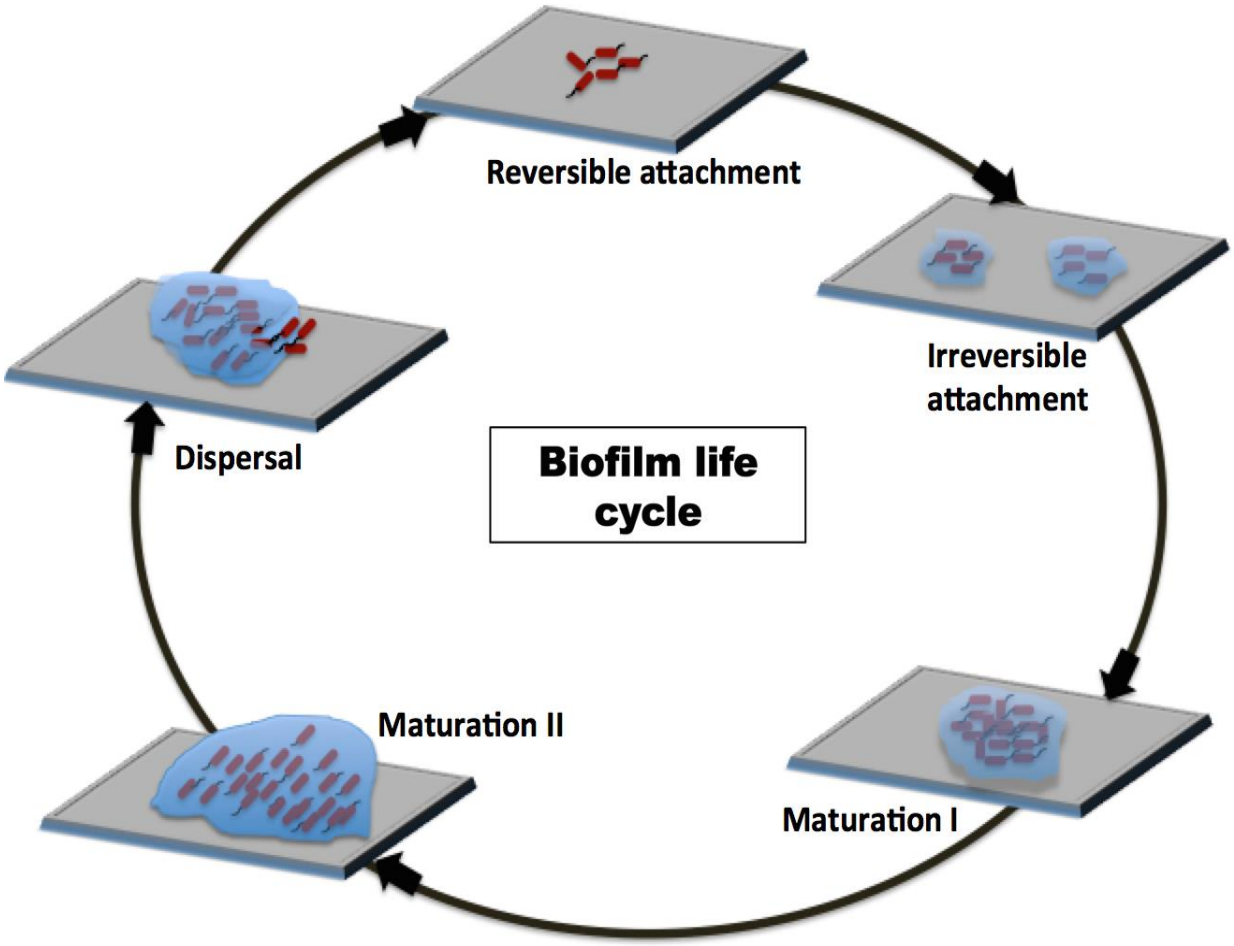

Figure 1.3 Schematic illustration of biofilm development and dispersal in *P. aeruginosa*

1.7 Polyamines: characteristics, role in biofilm formation and association with efflux pumps

Polyamines are low molecular weight organic polycations which are fully protonated at physiological pH and are ubiquitous in bacteria (103, 104). Organic polycations such as polyamines as well as inorganic cations have been implicated in maintaining conformation of nucleic acids (103). The hydrocarbon chain of polyamines encompasses positive charges at regularly spaced intervals, this unique structure allows polyamines to serve as electrostatic bridges between negative phosphate charges on nucleic acids (103).

The most extensive cellular polyamines crucial for cell growth and division are putrescine, spermidine, spermine and cadaverine (103, 104). Putrescine and cadaverine exist as diamines consisting two amino groups, whilst spermidine includes three and spermine has four amino groups. Biosynthesis of polyamines begins with decarboxylation of precursor amino acids arginine and ornithine either directly into polyamines or other intermediates (103). In *E. coli*, *Yersinia pestis* and many *pseudomonas* species, decarboxylation of ornithine leads to the formation of putrescine whilst decarboxylation of arginine leads to agmatine, which is then converted to putrescine (103-105).

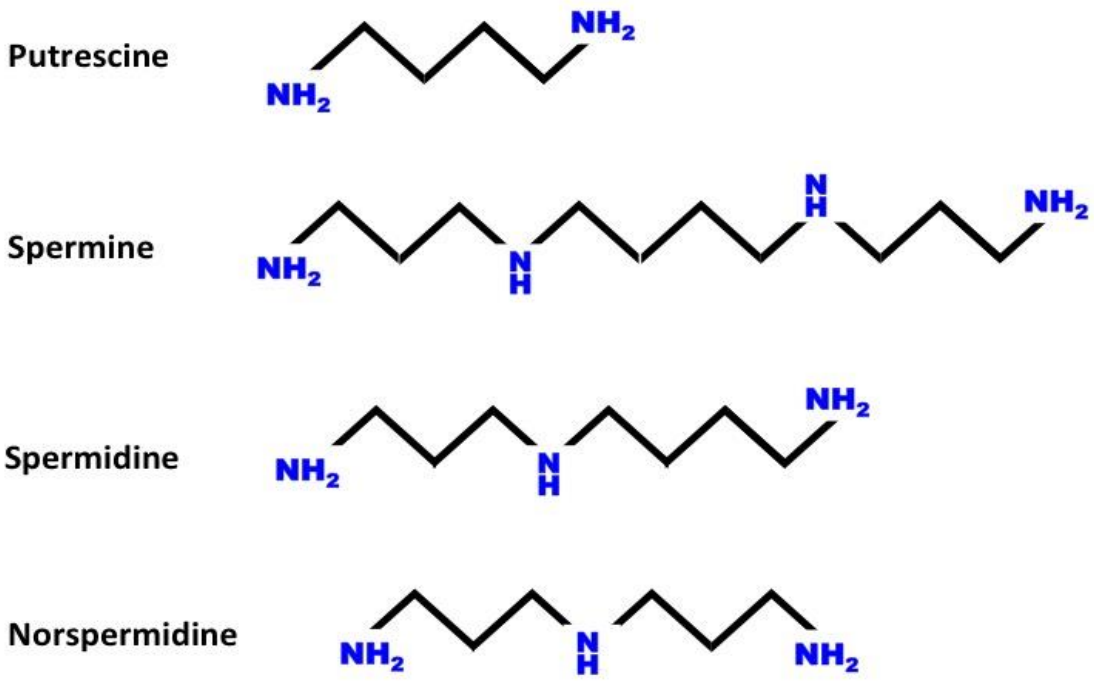

Figure 1.4 Chemical structures of polyamines

As well as biosynthesis of polyamines, bacteria also promote extracellular intake of polyamines through transport systems (103). Although polyamine transporters have only been well characterised in *E. coli,* several studies suggest a high likeliness of conservation across Gram-

negatives and Gram-positives (103). Furthermore, this high-level conservation may indicate importance of these transport systems as an adaptive/survival advantage (103). The major transport systems affiliated with polyamine uptake has been restricted to two ABC (ATP-binding cassette) transporters namely the PotABCD system selective for spermidine, and the PotFGHI system which is putrescine specific (103, 104). Furthermore, antiporters that mediate exchange of putrescine for ornithine and cadaverine for lysine have also been described.

Physiological functions of polyamines in bacteria had been largely unclear until of late; several studies have described the role of polyamines in microbial carcinogenesis (106); defense against oxidative and acid stress (107, 108) and importantly biofilm formation (109, 110). Polyamines have been reported to play a role in biofilm formation and regulation in diverse bacterial species, including gamma-proteobacteria *Yersinia pestis* (implicated in plague) and *Vibrio cholera,* and the Gram-positive *Bacillus subtilis* (110-112).

1.7.1 Role of polyamines in biofilms

In *Yersinia pestis*, *speA* and *speC* genes encode biosynthetic enzymes arginine decarboxylase and ornithine decarboxylase, which mediate decarboxylation of arginine and ornithine, respectively to form putrescine (112). As demonstrated by Patel *et al.*, strains with mutations in *speA* and *speC* genes display reduction in biofilm formation, whereby *speC* mutants exhibited moderate depletion of putrescine and biofilm formation whilst *speA* mutants displayed significant depletion of putrescine and biofilm formation (112). Therefore it can be postulated that a particular concentration threshold of putrescine maybe essential for biofilm formation (112). However, the functional role of putrescine in biofilm formation is currently unknown.

In addition to intrinsic regulation of polyamines in biofilms, modulation of biofilm formation by environmental polyamines has also been reported (113). In *V. cholera*, the most common polyamine is a variant of spermidine known as norspermidine, which contains one carbon less than spermidine (113). The *mbaA* gene thought to regulate biofilm formation, forms part of a three-gene operon, which also includes the gene *nspS*, which encodes a periplasmic protein that acts as a norspermidine sensor (113). It has been shown that an elevation of environmental norspermidine triggers biofilm formation in the presence of *mbaA* and *nspS* genes in *V. cholera* (113). Furthermore deletion of *nspS* gene exhibits a reduction in biofilm formation and transcription genes essential for exopolysaccharide synthesis and biofilm formation (113). Furthermore, interaction of NspS and norspermidine complex with the periplasmic domain of MbaA impedes its ability to suppress biofilm formation in *V. cholera*, thus implying that norspermidine, serving as a signalling molecule, may promote adherence to surfaces (113).

1.7.2 Polyamines may be natural substrates of efflux pumps

As mentioned before, polyamines were recently identified as substrates of multidrug transporters from the PACE family (Liu *et al*., unpublished). Polyamines have also been found to be natural substrates of several other multidrug efflux pumps including MFS efflux pump Blt in *B. subtilis* (13); SMR efflux pump MdtJI in *E. coli* (114) and RND efflux pump BpeAB-OprB in *B. pseudomallei* (24) In Gram-negatives *B. pseudomallei* and *E. coli*, accumulation of intracellular spermidine can be toxic to cells therefore spermidine is either catalysed by acetyltransferases to N-acetylspermidine (an inert form) or extruded from the cell (24, 114). In *B. pseudomallei,* acetylation detoxifies the cell of spermidine and also mediates extrusion of acetylated spermidine through RND efflux pump BpeABOprB (24). Interestingly, BpeAB-OprB multidrug efflux pump also mediates extrusion of acylhomoserine lactones (AHLs) involved in quorum sensing, furthermore inhibition of intracellular spermidine synthesis has also been found to inhibit production of AHLs required for quorum sensing as well as reduce biofilm formation (24).

1.6 Fatty acids: physiological role and association with efflux pumps

As described previously, efflux pumps are widely implicated in the extrusion of antimicrobials, however they are also implicated in the efflux of host-derived or natural antimicrobial agents, suggesting that the physiological roles of some multidrug efflux pumps may be to promote survival of bacteria in its ecological niche (17). Equally, bacterial efflux pumps may also mediate extrusion of virulence determinants, which are important for colonisation and infection (17).

A recent study by Truong-Bolduc *et al.,* showed that long chain fatty acids found on the surface of the skin are natural substrates of *Staphylococcus aureus* efflux pump tet38, which is also involved in tetracycline resistance (11). It was found that upregulation of tet38 efflux pump promotes survival and colonisation of *S. aureus* on the surface of the skin (11).

Free fatty acids (FFA) found naturally on the surface of the skin have potent biological activities that are involved in host defense against pathogens (115). They comprise 28% of the sebum and are produced on the skin by lipolytic cleavage of lipids secreted from the sebaceous glands (115). Interestingly structural properties of free fatty acids influences its antibacterial potency, whereby the most potent usually have 14 or 16 carbon atoms, and the antibacterial efficacy seems to be governed by the presence of double bonds, such that unsaturated fatty acids have a higher efficacy as opposed to its saturated counterparts (115).

1.7 Objective of this study

Multidrug efflux pumps recognise a broad range of substrates with different properties including environmental toxins and various classes of antimicrobials which provide a survival strategy for the bacterium (116). However, given the extensive association of these transporters with antibiotics and toxins, they are generally referred to as multidrug resistant (MDR) (116). Nonetheless, it is not clear whether protection against antibiotics is the core physiological function of these efflux pumps (13). Since most efflux pumps are highly conserved in the core genome and have been there prior to the antibiotic era, it may be highly unlikely that conferring resistance against antibiotics is the principle function of these efflux pumps. There is also a growing consensus that multidrug efflux pumps may have physiological roles other than detoxification of cells (13, 45, 116). Furthermore, the ability of multidrug resistant strains of *A. baumannii* to form biofilms and persist in hospital settings, despite being subjected to adverse conditions presents as a survival strategy of this organism, which may also contribute to pathogenesis and virulence. Therefore the aim of this study was to elucidate the physiological functions of *A. baumannii* efflux pumps in mediating resistance to natural compounds such as antimicrobial fatty acids and polyamines, as well as the role of multidrug efflux pumps in biofilm formation and regulation. Furthermore, the role of polyamines in biofilm formation by *A. baumannii* strain AB5075-UW was also investigated

2 Methods

2.1 Bacterial strain, growth and storage

2.1.1 Strains used

A total of eight *A. baumannii* strains were used in this study; D1279779, ATCC 17978, ACICU, AYE, A320, SDF, A1 and AB5075-UW. D1279779, ATCC 17978, ACICU, AYE, A320, SDF and A1 were used to test *A. baumannii* susceptibilities to six fatty acids. AB5075-UW was used for biofilm and polyamine studies (Table 2.1). *A. baumannii* AB5075-UW isolated from a wound infection, has been fully sequenced (NCBI accession: PRJNA243297) and subjected to transposon mutagenesis to generate a mutant library (117). The wild-type strain and isogenic transposon mutants of known and putative efflux pumps (Table 2.1) were obtained from the Manoil laboratory (117).

2.1.2 Storage and growth conditions

All bacterial strains were preserved at -80 °C in 30% glycerol for long-term storage and were routinely cultured in Luria-Bertani (LB) broth or agar at 37 °C unless stated otherwise.

Table 2.1 Transposon mutants from strain AB5075-UW, used in this study.

Transporter Superfamily	Genotype	Putative function of interrupted gene
ABC	ABUW_0965-142::T26	ABC-2 type transporter family
ABC	ABUW_0966-185::T26	Hypothetical protein
ABC	ABUW_1184-123::T26	ABC transporter, ATP-binding protein
ABC	*barB*152::T26	Putative ABC transporter
ABC	ABUW_1247-113::T26	ABC transporter permease protein
ABC	ABUW_1248-108::T26	ABC transporter ATP-binding protein
ABC	ABUW_2280-141::T26	Putative transport protein
ABC	ABUW_2643-171::T26	ABC efflux system and ATP-binding protein
SMR	*rarD*159::T26	Rard protein
SMR	*sugE*168::T26	Suppressor of gro
SMR	ABUW_1343-187::T101	Small multidrug resistance protein
MATE	ABUW_0064-177::T26	MATE family drug transporter
MATE	ABUW_0109-152::T26	MATE efflux family protein
MATE	*abeM*184::T26	Multidrug efflux pump

MFS	ABUW_0041-135::T26	Major facilitator family transporter
MFS	ABUW_0196-129::T26	Major facilitator family transporter
MFS	ABUW_0213-101::T26	Major facilitator family transporter
MFS	*mdfA*173::T26	Chloramphenicol resistance pump cmr
MFS	ABUW_0368-107::T26	Tetracycline resistance protein teta
MFS	ABUW_0701-105::T26	Drug resistance transporter, Bcr/cfla subfamily
MFS	ABUW_1003-152::T26	MFS family drug transporter
MFS	ABUW_1078-192::T26	Major facilitator family transporter
MFS	ABUW_1523-145::T26	Transporter, major facilitator family
MFS	ABUW_1596-105::T26	MFS superfamily permease
MFS	*smvA*169::T26	Methyl viologen resistance protein
MFS	ABUW_1931-111::T26	Transporter, major facilitator family
MFS	ABUW_1950-104::T26	Multidrug resistance protein vceb
MFS	ABUW_2187-173::T26	Transporter, major facilitator family
MFS	ABUW_2403-159::T26	MFS permease
MFS	ABUW_2683-175::T26	Transporter, major facilitator family
MFS	*fsr*125::T26	Fosmidomycin resistance protein
MFS	*emrB*180::T26	Multidrug resistance protein B
MFS	ABUW_3124-177::T26	Major facilitator superfamily MFS_1
MFS	ABUW_3152-127::T26	Major facilitator superfamily
MFS	ABUW_3286-194::T26	Major facilitator superfamily MFS_1
MFS	ABUW_3317-173::T26	Major facilitator superfamily MFS_1
MFS	ABUW_3697-186::T26	Inner membrane transport protein yieo
PACE	ABUW_1673-156::T26	Bacterial transmembrane pair family protein
PACE	ABUW_2319-171::T26	Putative membrane protein
PACE	ABUW_2319-143::T26	Putative membrane protein
RND	ABUW_0035-172::T26	RND efflux transporter
RND	ABUW_0645-181::T26	Quaternary ammonium compound-resistance protein
RND	*nolG*129::T26	Multidrug efflux protein
RND	*adeJ*122::T26	Multidrug efflux protein adej
RND	ABUW_0922-152::T26	Multidrug efflux protein
RND	ABUW_1335-195::T26	Efflux transporter, RND family
RND	*adeB*150::T26	Multidrug efflux protein adeb
RND	ABUW_3769-110::T26	Polyketide cyclase/dehydrase family

2.2 Quantitative biofilm assay

A. baumannii AB5075-UW and all mutants (listed in table 2.1) were streaked onto LB agar from glycerol stocks. Single colonies of each mutant and the wild type strain were inoculated in 5 ml of Mueller-Hinton (MH) broth and the cultures grown for 16 hours overnight at 37 °C with shaking (200 rpm). The overnight cultures were diluted 1:100 in fresh MH broth and 150 µl of each diluted culture dispensed into the wells of a sterile 96-well, flat-bottom microtiter plate (CELLSTAR, Greiner Bio-One, Australia). The plates were incubated at 37 °C with shaking at 200 rpm, which did not disturb biofilm formation due to the small size of wells. Subsequent to incubation the cell density in all wells was measured at 600 nm using PHERAstar FSX (BMG LABTECH, Germany) microplate reader. Next, planktonic cells were aspirated, and all the wells were washed twice with 150 µl of 0.01 M phosphate-buffered saline (PBS). Biofilms were stained by adding 100 µL of 0.1% v/v crystal violet solution to each well and allowed to stand for 15 min. Following staining, wells were washed twice with 150 µl of 0.01 M phosphate-buffered saline (PBS) to remove any excess crystal violet. Crystal violet bound to the biofilm was solubilised with 100 µl of a 4:1 v/v mixture of ethanol and water. Solubilised crystal violet was diluted 1:10 in fresh 4:1 v/v mixture of ethanol and water in a new sterile 96-well flat-bottom microtiter plate and the absorbance was measured at 570 nm using a PHERAstar FSX (BMG LABTECH, Germany) microplate reader. All biofilm assays were performed with a minimum of eight replicates for each mutant and the wild-type strain. Negative controls (wells with media only) were setup to measure background binding of crystal violet to wells. All absorbance readings were normalised against that of negative control. Unequal variance *t*-tests were used to evaluate statistical significance between the absorbance readings of the wild-type and mutant strains.

2.3 Antimicrobial Susceptibility Testing.

Single colonies of mutants and the wild type strains were inoculated in 5 ml of MH broth and the cultures grown for 16 hours overnight at 37 °C with shaking (200 rpm). Overnight cultures were used for antimicrobial susceptibility testing. The minimum inhibitory concentrations (MICs) of antimicrobials, including fatty acids; palmitic acid, palmitoleic acid, undecanoic acid, lauric acid, linoleic acid and oleic acid, and polyamines; putrescine, spermidine and spermine were determined using the broth microdilution technique. Stock solutions of antimicrobials were prepared at 50 mg/ml and stored temporarily as per the supplier's instructions. Stock solutions of antimicrobials were diluted in cation adjusted (10 mg/L Mg^{2+} and 20 mg/L Ca^{2+}) MH (CA-MH) broth for antimicrobial susceptibility testing. Two-fold serial dilutions were performed across the wells of flat bottom polystyrene 96 well microtitre plates, to achieve geometrically decreasing concentrations of each antimicrobial. The highest concentration of antimicrobial used was 5000 µg/ml (column 1) and

the lowest concentration was 9.76 µg/ml (column 10), some wells were reserved as positive controls (no antimicrobial; column 11) and negative controls (liquid media only; column 12) (Figure 2.1). In parallel with setting up antimicrobial plates, overnight cultures of each strain were diluted 1:50 in CA-MH broth using corning cell culture flasks and the sub-cultures grown to an OD_{600} of 0.6 at 37 °C, with shaking (200rpm). The cells were diluted 1:15,000 into the wells containing antimicrobial and positive control wells. Microtitre plates were incubated for 24 hours at 37 °C with shaking (100 rpm). Following 24 hours, absorbance was measured at 600 nm using PHERAstar FSX (BMG LABTECH, Germany) microplate reader to measure cell growth and determine minimum inhibitory concentrations (MIC) for each antimicrobial, defined as the lowest concentration required to fully inhibit bacterial growth.

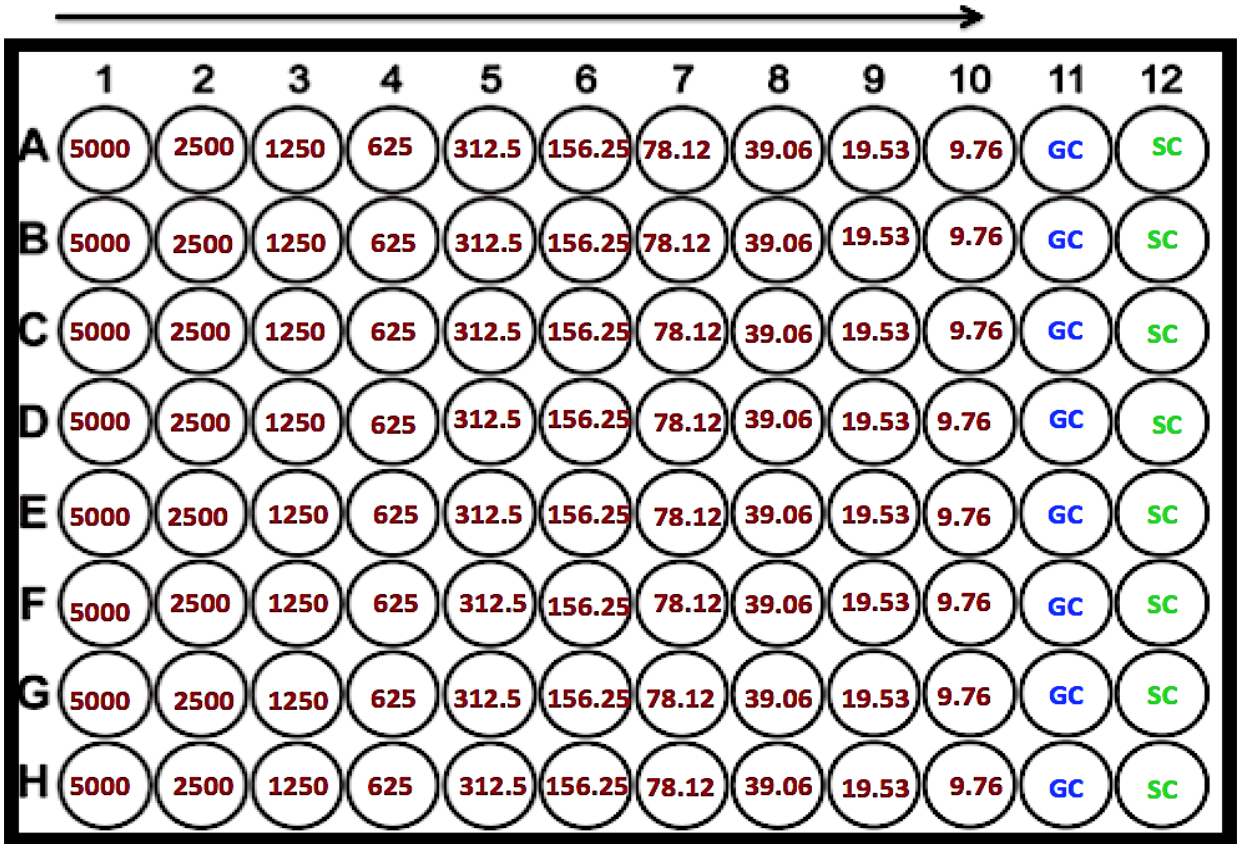

Figure 2.1 Outline of 96 well plate for antimicrobial susceptibility testing. The highest concentration of antimicrobial used was 5000 µg/ml (column 1) and the lowest concentration was 9.76 µg/ml (column 10). GC; growth control (no antimicrobial; column 11). SC; sterility control (media only; column 12). Concentration units: µg/ml.

2.4 Quantitative biofilm assay with exogenous polyamines

Once the MIC of polyamines was determined using antimicrobial susceptibility testing, quantitative biofilm assays were employed to evaluate the role of exogenous polyamines on biofilm regulation in *A. baumannii* AB5075-UW. The polyamines tested were putrescine, spermidine and spermine. Stock solutions of polyamines were prepared at 50 mg/ml and stored temporarily as per supplier's instructions. Stock solutions were diluted in MH broth, to a concentration twice the desired concentration and a serial dilution was performed across wells to achieve a geometrically decreasing concentration of polyamine. Overnight culture of strain AB5075-UW was diluted 1:100 in fresh MH broth, and diluted culture was dispensed into each well, so that each well contained a 1:1 v/v mixture of culture and polyamine, yielding a starting polyamine concentration below MIC (2500 µg/ml of Putrescine, 625 µg/ml of Spermidine and 312.5 µg/ml of Spermine), and a starting cell density 1:200 from the overnight culture. Inoculated plates containing each polyamine treatment were incubated for different time points (8, 24 and 48 hours) and at 37 °C with shaking (200 rpm), as described above. The crystal violet assay was performed as described (section 2.2). All biofilm assays were performed with a minimum of eight replicates for each polyamine concentration. Some wells were reserved as positive controls (no antimicrobial; column 11) and negative controls (liquid media only; column 12). All absorbance readings were normalised against sterile control wells.

2.5 Colony PCR for screening transposon insertion

The mutant strains used in this work were obtained from a large collection of mutants. Therefore, colony PCR was used to amplify DNA fragments to confirm the locations of transposon insertions in mutants showing significantly different biofilm formation to the wild-type strain; ABUW_0035-172::T26, ABUW_0109-152::T26 and ABUW_0213-101::T26. Primers were designed using Primer3, a web-based application (118). Primers used in this study are shown in Table 2.2. Primer pairs were suspended in nuclease-free water to make working stocks of oligonucleotide primers. The following components were combined in a 0.5 ml PCR tube to a final volume of 12 µl:

- 2.5 µl of 5X Green GoTaq Flexi Buffer (Promega)
- 1.5 µl of 25 mM $MgCl_2$ solution
- 0.25 µl of 10 mM PCR nucleotide mix
- 0.063 µl of 5 u/µl GoTaq DNA Polymerase
- 0.8 µl of ~28 nM forward and reverse primers

Cells from a single colony of either a mutant or the wild-type strain were added to PCR tubes containing the above components using sterile pipette tips. Thermal cycling conditions included, a 2

minute initial denaturation at 95 °C, a second 1 minute denaturation at 95 °C, a 30 second annealing step at 42-65 °C (25 -35 cycles) and a 4 minute extension at 72 °C (typically 1 minute/kb of template), followed by a final extension at 72 °C for 5 minutes.

Subsequent to amplification, PCR products were verified using agarose gel electrophoresis and Easy ladder II (Bioline, UK) was used as a standard. Gel images were captured using Gel Logic 2200 PRO (Carestream, USA).

Table 2.2 Oligonucleotides used in present study

Primer name	Sequence	Use in the present study
ABUW_0035F	CAATGACATTCGCCATGAAC	Confirm disruption of gene ABUW_0035-172::T26
ABUW_0035R	AGAACGGCGAATTTCATCAC	Confirm disruption of gene ABUW_0035-172::T26
ABUW_0109F	AATGACTAAACCGGCCATTG	Confirm disruption of gene ABUW_0109-152::T26
ABUW_0109R	TTTTAGTGGGTCAGGCTTGG	Confirm disruption of gene ABUW_0109-152::T26
ABUW_0213F	AGAAACCAATGGCAAACAGG	Confirm disruption of gene ABUW_0213-101::T26
ABUW_0213R	TCTTTCCCGTGAGTGGATTC	Confirm disruption of gene ABUW_0213-101::T26

2.6 Isolation of mRNA from AB5075-UW for qRT-PCR

Six samples, corresponding to planktonic and sessile cells from biofilms were obtained at 3 different time points (8, 24 and 48 hours). Cells were disrupted using a lysis reagent (QIAzol). Total RNA was isolated using a miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total mRNA was treated with DNase to remove any DNA using a TURBO DNA-free kit (Ambion). The concentration and purity of samples were determined using a Qubit RNA Assay kit (Life Technologies) following the manufacture's instructions.

2.7 Scanning electron microscopy

A. baumannii AB5075-UW and mutants (ABUW_0035-172::T26, ABUW_0109-152::T26 and ABUW_0213-101::T26) were grown in overnight cultures and diluted 1:100 in fresh MH broth. Five millilitres of diluted culture was dispensed into the wells of a sterile 6 well plate, with each well containing a sterilised 12 mm round coverslip (ProSciTech, Australia). The plates were incubated for different lengths of time (8, 24 and 48 hours) at 37 °C with slow shaking (100 rpm), due to the larger size of the wells and greater potential for surface movement, to promote biofilm formation. The coverslips were removed from the wells, washed with 0.01 M phosphate buffered saline (PBS) and fixed with 3% (v/v) glutaraldehyde in PBS for 24 hours. Following fixation, samples were stored in 0.01 M PBS for no longer than 4 days. Dehydration of samples was achieved by a series of sequential ethanol dehydration steps (30%, 50%, 70%, 80%, 90% and 100% ethanol) for 10 minutes each. Next, samples were further dehydrated with 1:1 v/v mixture of hexamethyldisilazane and 100% ethanol for 10 minutes, then with 100% hexamethyldisilazane 3 times for 10 minutes. All samples were left to air-dry overnight and sputter gold-coated using an EMITECH K550X Sputter Coater (Quorum Technologies, UK). Samples were analysed using a JSM-6480LV (JOEL, Japan) scanning electron microscope at an accelerating voltage of 10 kV.

3 Results

3.1 Resistance to fatty acids in *A. baumannii*

A recent study reported that the minimum inhibitory concentration (MIC) of certain fatty acids against *S. aureus* ranged between 10 μg/mL - 50 μg/mL. Moreover, overexpression of *tet38*, which encodes a tetracycline efflux pump, resulted in a 4- to 8-fold increase in the MIC (11). Since *S. aureus* is a nosocomial human pathogen and causes similar range of infections to *A. baumannii*, it was predicted that fatty acids might also be substrates of *A. baumannii* efflux pumps as well as play a role in protection against *A. baumannii* infections. Therefore, based on this study, a range of concentrations was selected to assess the influence of fatty acids (linoleic, lauric, palmitic, palmitoleic, undecanoic and oleic) on seven different strains of *A. baumannii.* The strains, D1279779, ATCC 17978, ACICU, AYE, A320, SDF and A1 chosen for this study were isolated from various geographic locations. They include hospital pathogens, an isolate from a community-acquired infection, and a commensal isolate. They are known to cause a range of diseases and have different levels of resistance.

Following MIC tests it was found that none of the seven strains of *A. baumannii* were susceptible to any of the fatty acids at concentrations as high as 5000 μg/mL (data not shown). It was particularly notable for SDF, which has undergone genome reduction and in general shows lower resistance to antimicrobials. Moreover, disc diffusion antimicrobial susceptibility test of fatty acids, showed no zone of inhibition against any *A. baumannii* strain, thus indicating a high-level of resistance to fatty acids (data not shown). These results suggest that, fatty acids are not likely to control *A. baumannii* related infections as they may for *S. aureus*, furthermore, it can be speculated that efflux pumps may be a factor in the low susceptibility of *A. baumannii* to fatty acids, however due to the lack of susceptibility it was not feasible to pursue studies examining fatty acids as substrates of *A. baumannii* efflux pumps.

3.2 Role of multidrug efflux pumps in biofilm formation

Efflux pumps of opportunistic pathogens related to *A. baumannii*, such as *P. aeruginosa* have been found to play important roles in biofilm formation, however this phenomenon remains largely unexplored in *A. baumannii*. Therefore, to determine if efflux pumps in *A. baumannii* have physiological roles in biofilm formation and regulation, a quantitative biofilm assay using crystal violet was used to screen a library of known and putative efflux pump mutants in *A. baumannii* strain AB5075-UW (Table 2.2). A total of 48 multidrug efflux pumps were chosen for this study, they were identified using the TransAAP pipeline and originate from seven different families of

efflux pumps, these include MFS, ABC superfamily, MATE family, SMR family, PACE family and RND family.

3.2.1 Initial screen of biofilm formation at 24 hours

Each of the 48 chromosomally encoded efflux mutants were inoculated into LB broth and grown for 24 h in twenty-four replicate microtiter wells. A positive correlation (P = 0.24) between cell density and biofilm formation was found at 24 h (data not shown). Population density dependent cellular signalling has been found to be essential for biofilm formation and maturation (84). Thus, biofilm formed by each mutant was normalised against its cell density to dismiss growth deficit as a factor in biofilm-forming capacity of mutants. Biofilm formation of each mutant when normalised against its cell density, varied greatly between different mutants, ranging from 0.0352 to 0.0068 normalised units (Figure 3.1). Interestingly, mutations in 16 and 9 multidrug efflux pumps resulted in a statistically ($p > 0.05$) higher or lower biofilm formation than the parental strain, respectively (Figure 3.1).

3.2.2 The effect of incubation time on biofilm formation

After the initial screen, the quantitative biofilm assay was also employed to monitor the biofilm-forming capacity of mutants and the parental strain over three time points (8 h, 24 h and 48 h) (Figure 3.2). At the 8 h time point, the cell density ($OD_{600\ nm}$) of all mutants, except three (ABUW_0109-152::T26, ABUW_0035-172::T26 and smvA169::T26) was between 1.6 and 2, however biofilm formation amongst these 45 mutants varied greatly (Figure 3.2A). Generally, biofilm formation was found to be the highest at 8 and 24 h interval, and lowest at 48 h. Interestingly, a negative correlation between biofilm formation and cell density was observed at 48 h (P = -0.213), i.e. cell density of mutants at 48 h was similar to that of 24 h, however the amount of biofilm formed by most mutants at 48 h was reduced relative to the 24 h interval (Figure 3.2) possibly due to nutrient limitation.

Some mutants displayed unique growth and biofilm forming capacity under the conditions of the assay. For example, RND efflux mutant ABUW_0035-172::T26 had a much higher cell density and biofilm forming capacity in comparison to other mutants and the parental strain at 8 h and 24 h, followed by decline in both biofilm formation and cell density at 48 h (Figure 3.2). This may suggest that the population density of RND efflux mutant ABUW_0035-172::T26 expands more rapidly, possibly allowing for better biofilm-forming capacity, however rapid biofilm formation may have led to earlier nutrient limitation and therefore potential dispersal of biofilm at 48 h, however, this hypothesis needs to be confirmed by additional experiments.

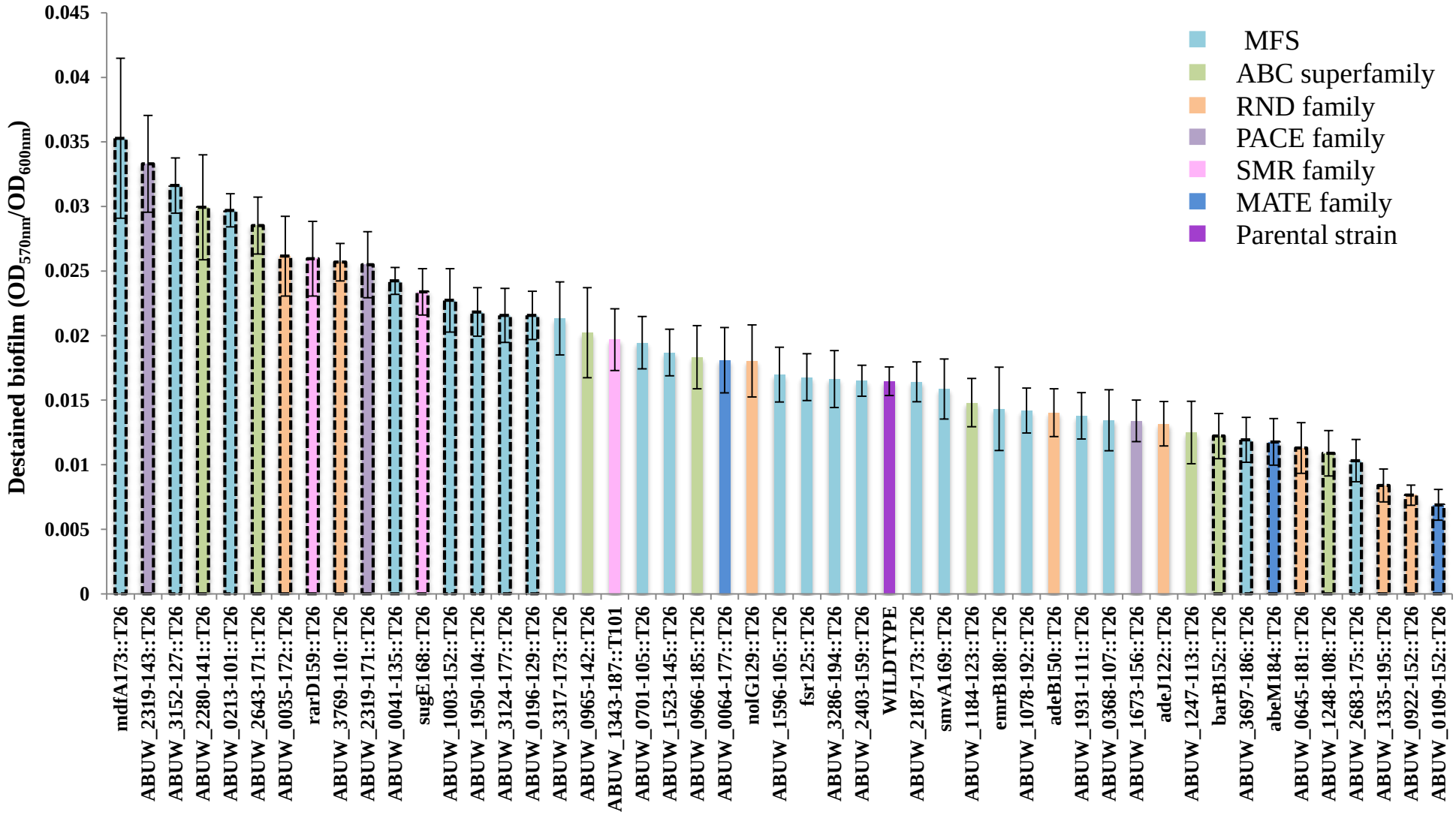

Figure 3.1 Quantitative biofilm assay of *A. baumannii* strain AB5075-UW and multidrug efflux mutants incubated in LB broth at 37 °C for 24 hrs**.** Bars indicate the means of 24 wells. Error bars indicate standard errors of means. Bars encased in broken lines indicate statistically significant difference in comparison to parental strain ($p<0.05$).

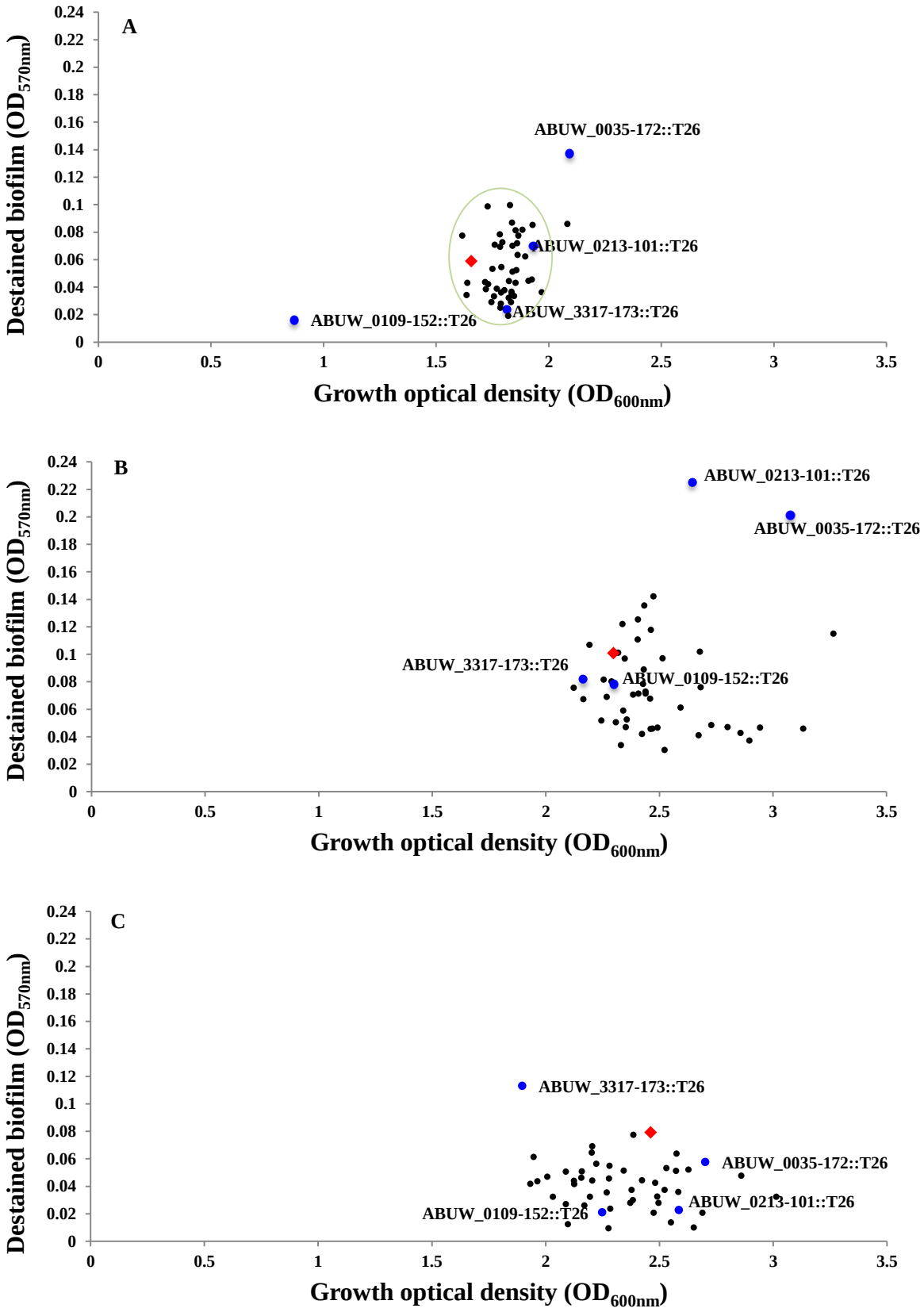

Figure 3.2 Scatter plot of cell density and de-stained biofilm for *A. baumannii* parental strain and multidrug efflux mutants at 8 h(A), 24 h(B) and 48 h(C). Each data point represents the average reading from eight replicates. (◆) Parental strain, (•) multidrug efflux mutants and (•) multidrug efflux mutants showing significant difference.

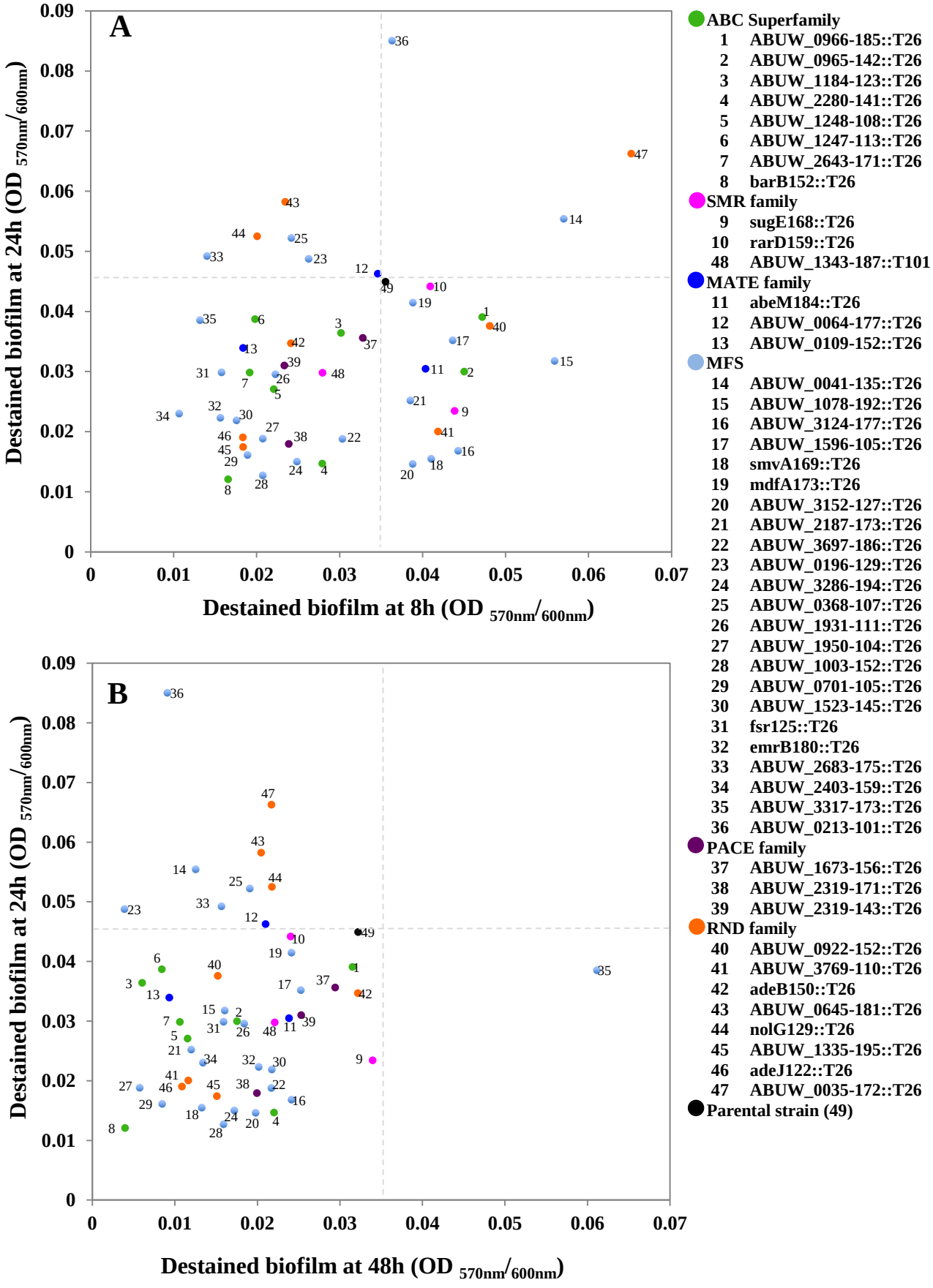

Figure 3.3 Scatter plot of de-stained biofilm. (A) 8 h versus 24 h and (B) 24 h versus 48 h. Each data point represents average from eight microtitre wells

Contrarily, growth and biofilm-forming capacity of MATE efflux mutant ABUW_0109-152::T26 was significantly reduced at 8 h, however it was on par with the other mutants at 24 h (Figure 3.2). Following normalisation, three mutants (ABUW_0041-135::T26 (MFS), ABUW_0213-101::T26 (MFS) and ABUW_0035-172::T26 (RND)) had higher biofilm forming capacity at both 8 h and 24 h in comparison to parental strain (Figure 3.3).

Biofilm quantification using the crystal violet assay is not a highly sensitive technique due to adsorption of crystal violet to the PVC plate, and thus many replicates are required to be confident in phenotypes observed. Hence, in the scope of this study only mutants which showed a high statistically significant ($P > 0.05$) difference in biofilm formation when compared to parental strain were chosen for additional crystal violet assays, these included ABUW_0041-135::T26, ABUW_0213-101::T26, ABUW_0035-172::T26, ABUW_0109-152::T26, smvA169::T26, ABUW_2319-143::T26, ABUW_3152-127::T26 and ABUW_0922-152::T26.

3.2.3 In depth analyses of biofilm formation in selected mutants by crystal violet assays and SEM

Of the eight mutants selected for additional crystal violet assays three showed significant and invariable differences in their biofilm forming capacity compared to the parental strain. The efflux mutants ABUW_0213-101::T26 (MFS) and ABUW_0035-172::T26 (RND) had a higher biofilm-forming capacity. Whereas, the cell density and ability of MATE efflux mutant ABUW_0109-152::T26 to form biofilm was significantly lower than parental strain (data not shown). Therefore, these three mutants were examined in more detail using biofilm-imaging techniques. Furthermore, AB5075-UW parental strain was grown for 8 h, 24 h and 48 h under conditions mentioned (section 2.6). RNA from cells embedded in the biofilm matrix was harvested for qRT-PCR analysis, however due to time constraints this analysis could not be performed.

Prior to conducting any further experiments with the three selected efflux mutants, the chromosomal disruptions in the mutant strains were confirmed through PCR (Figure 3.4). PCR was performed using primers flanking the transposon insertion site, whereby the presence of a larger band indicated successful disruption of gene by transposon (Figure 3.4). The PCRs confirmed the disruption of the respective efflux genes in all of the mutants tested. However, in efflux mutant ABUW_0109-152::T26 no band was observed using primers flanking the Tn26 insertion site (Figure 3.4A). An explanation for this could be that transposon insertion may have overlapped or affected one of the primer binding sites. Nonetheless the PCR demonstrated that the targeted gene had been disrupted in some way; however, due to time constraints the nature the mutation could not be investigated further.

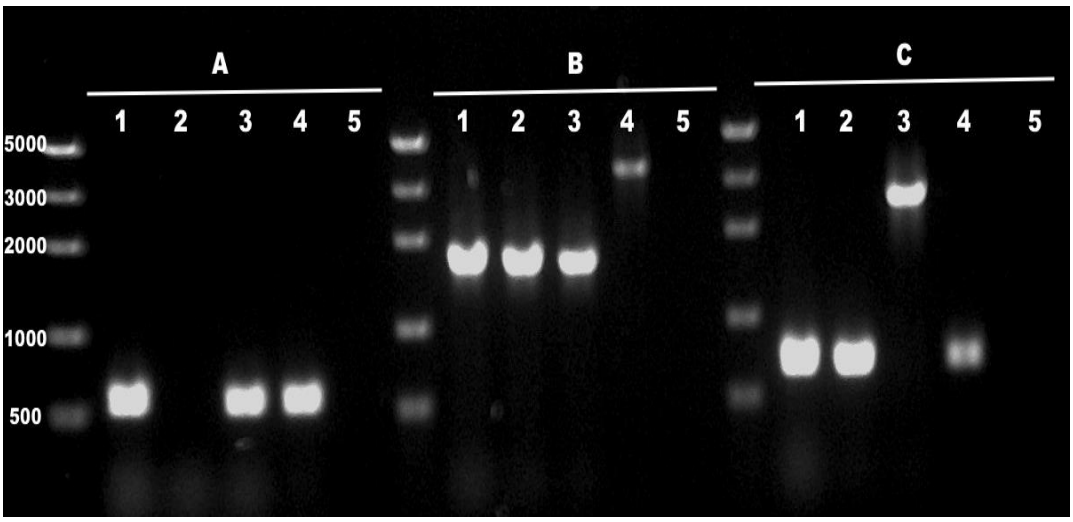

Figure 3.4 Confirmation of chromosomal disruption in mutants through colony PCR. (1) Parental strain, (2) ABUW_0109-152::T26, (3) ABUW_0213-101::T26, (4) ABUW_0035-172::T26 and (5) Negative control. Primer pair used, (ABUW_0109F & ABUW_0109R; A), (ABUW_0035F & ABUW_0035R; B) and (ABUW_0213F & ABUW_0213R; C)

Following confirmation of chromosomal disruption, scanning electron microscopy (SEM) was performed on the three selected mutants to analyse the biofilms formed directly. Biofilms were grown for 8 h, 24 h and 48 h in 6 well plates containing a submerged glass coverslip. Biofilms on the coverslip were dehydrated by a series of sequential ethanol dehydration steps followed by imaging with SEM. Biofilm formation did not appear to be dense under the assay conditions used. Nonetheless, images obtained from scanning electron microscope showed that both efflux mutants ABUW_0213-101::T26 and ABUW_0035-172::T26 adhered to surface of the coverslip better and formed more cell aggregates than parental strain (Figure 3.5). Furthermore, the RND efflux mutant ABUW_0035-172::T26 had the highest biofilm forming capacity (Figure 3.5 and Figure 4.1). These observations follow the results of crystal violet assays.

Contrarily, the ability of MATE efflux mutant ABUW_0109-152::T26 to attach to the coverslip at 8 h was reduced to an extent, that locating cells proved to be challenging (Figure 3.5), this could be due to reduced cell density of this mutant at 8 h, as observed previously in the microplate assays. Furthermore, morphological differences, such as absence of bacterial appendages in the efflux mutant ABUW_0109-152::T26 relative to wild-type AB5075-UW was observed (Figure 3.6). Morphological differences were also observed in RND efflux mutant ABUW_0035-172::T26, whereby these mutant cells appeared more elongated relative to the parental strain (Figure 3.5), however it can be speculated that the elongated nature of their appearance could just be mutant cells encased in extracellular polymeric substance (EPS) secreted by these mutant cells (Figure 3.5).

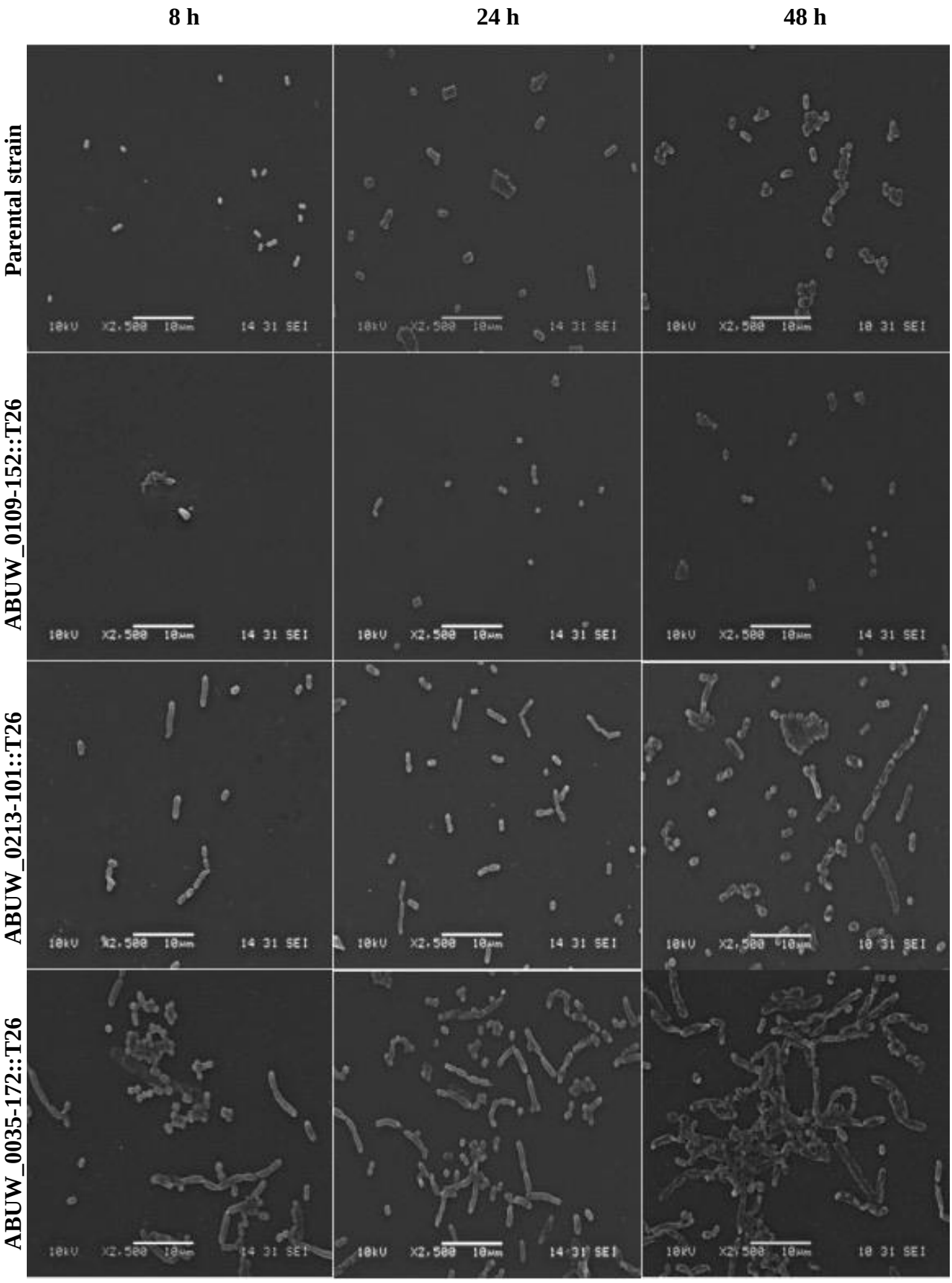

Figure 3.5 Scanning electron microscopy images of parental strain and mutants ABUW_0213-101::T26, ABUW_0035-172::T26 and ABUW_0109-152::T26 at 8 h, 24 h and 48 h. All cells were cultured on 12 mm glass coverslips in LB broth with shaking (100 rpm) at 37 °C. Scale bar = 10 μm.

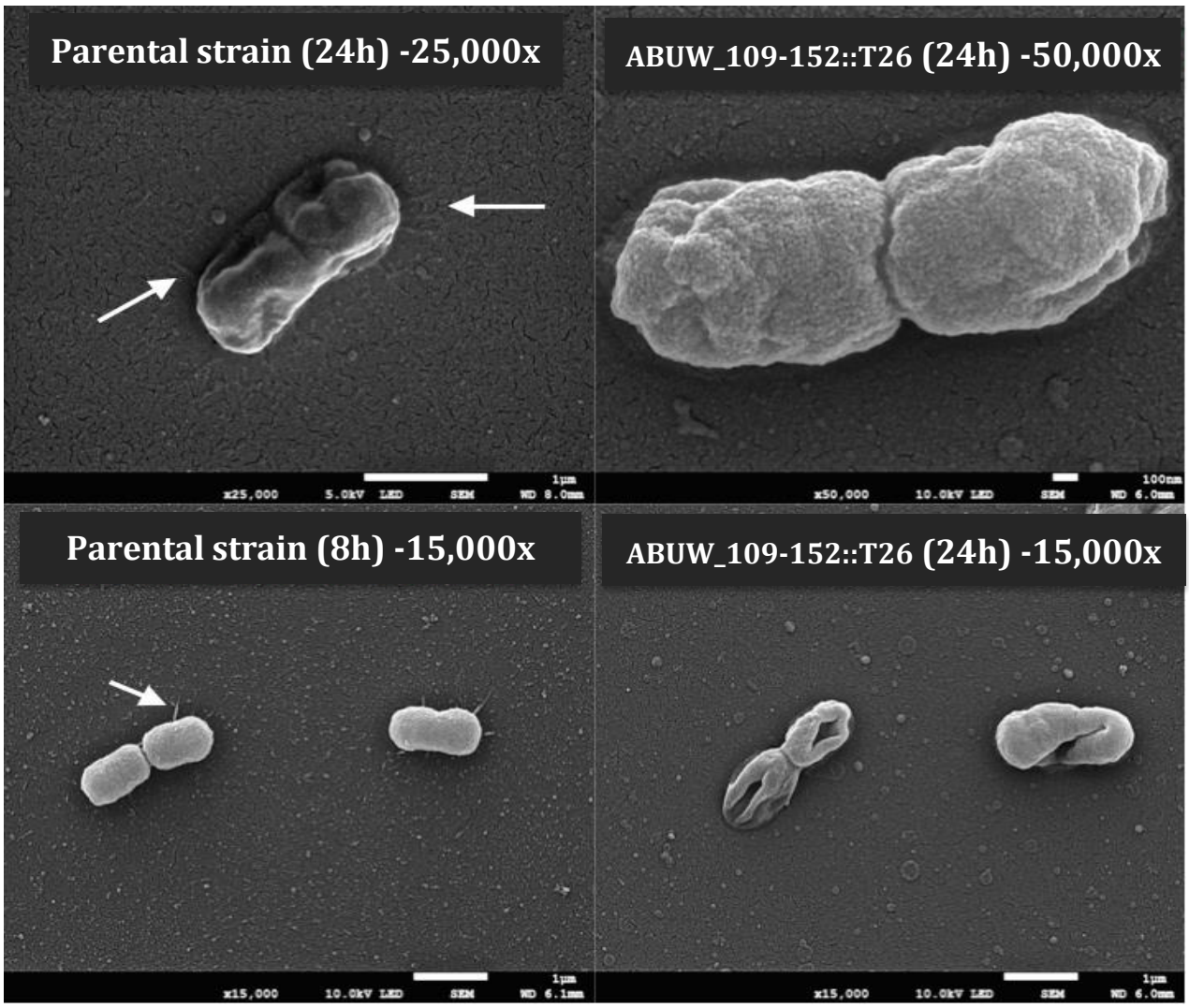

Figure 3.6 Scanning electron microscopy (SEM) images of *A. baumannii* AB5075-UW AT 8 h and 24 h, and MATE efflux mutant ABUW_0109-152::T26 at 24 h. Absence of bacterial appendages is notable in ABUW_0109-152::T26 in comparison to parental strain

3.3 Role of polyamines in biofilm regulation and as potential substrates of multidrug efflux pumps in *A. baumannii* AB5075-UW.

Polyamines, produced by bacteria and host cells, are pleiotropic in nature. These compounds have a number of important cellular functions in bacteria, and have been found to be natural substrates of several efflux pumps, as well as play a role in biofilm formation, possibly as signalling molecules. Hence, it can be speculated that efflux pumps, which influence biofilm formation, may also have polyamines as natural substrates and mediate their extrusion from the cell.

Furthermore as described previously, the role of exogenous polyamines in biofilm formation can differ depending on the bacterial species being tested. For example, increasing concentrations of spermidine (from 0.1 mM to 5 mM) in *E. coli* cultures reduced biofilm production whereas in *S. enterica* cultures, a concentration shift of spermidine from 0.1 mM to 1 mM caused slightly higher biofilm production (119).

3.3.1 MIC of polyamines against AB5075-UW and selected efflux pump mutants

Prior to conducting any experiments with polyamines on biofilms, the influence of three polyamines – putrescine, spermidine and spermine (known to be present at high concentrations in bacterial cells), at concentrations ranging from 9.76 μg/mL to 5000 μg/mL were tested for the ability of *A. baumannii* strain AB5075-UW as well as it's efflux mutants; ABUW_0213-101::T26 (MFS), ABUW_0035-172::T26 (RND family) and ABUW_0109-152::T26 (MATE family) to tolerate these compounds (Table 3.1). All polyamines had varying MIC.

Putrescine was found to have no impact on the viability of the parental strain or the efflux mutants at concentrations as high as 5000 μg/mL. The MIC of spermine and spermidine in efflux mutants was 2- to 4-fold lower than the parental strain. Differences in the MIC were particularly apparent in efflux mutant ABUW_0109-152::T26, disruption of which decreased the MIC of spermine by a factor of four (Table 3.1). Therefore it can be speculated that spermine may be a substrate of MATE efflux mutant ABUW_0109-152::T26.

Table 3.1 Effect of polyamines on *A. baumannii* strain AB5075-UW and MDR efflux mutants after 24 h incubation.

	MIC (μg/mL)		
A. baumannii AB5075-UW	Putrescine	Spermidine	Spermine
Parental strain	> 5000	1250	625
ABUW_0213-101::T26	> 5000	625	312.5
ABUW_0035-172::T26	> 5000	625	312.5
ABUW_0109-152::T26	> 5000	625	156.25

3.3.2 Influence of polaymines on biofilms

Subsequent to MIC determination, different concentrations of polyamines (spermidine, spermine and putrescine) were added to cultures of *A. baumannii* strain AB5075-UW (parental strain only) in 96 well microtitre plates and incubated for 8, 24 and 48 h (Figure 3.7). The influence of these three polyamines on the ability of AB5075-UW to form biofilms was then analysed using crystal violet assays. Cell densities at the point of biofilm sampling were also measured at OD_{600nm}.

It was found that the cell density of AB5075-UW in the presence of putrescine, spermidine and spermine was generally highest at 48 h and lowest at 8 h. However, cell viability as a result of different concentrations of both putrescine and spermidine was largely unaffected (Figure 3.7 A, B, C). Contrarily, the biofilm forming capacity of AB5075-UW decreased as a result of increasing concentrations putrescine and spermidine. While the results of the spermine experiment had a large error, a dose dependent effect of spermine on biofilm formation could also be seen (Figure 3.7 D, E, F).

Spermidine had the strongest effect on the biofilm forming capacity of AB5075-UW, whereby an apparent dose dependent effect can be seen (Figure 3.7E). Furthermore, at 48 h biofilms formed by AB5075-UW in the presence of spermidine were completely abolished, as represented by the negative bars in Figure 3.7E, which shows that the ODs of treatment wells were lower than the ODs of negative control wells which contained MH media only. Interestingly, spermine which was found to be the most toxic polyamine in resistance assays against AB5075-UW (table 3.1), did not show a strong inhibition of biofilm formation when compared to spermidine (Figure 3.7F).

Putrescine, which had no inhibition activity against AB5075-UW at concentrations up to 5000 µg/mL (Table 3.1), and did not result in a significant difference in cell density of AB5075-UW (Figure 3.7A), led to a dose dependent decrease in biofilm formation, whereby higher concentrations of putrescine resulted in lower biofilm formation (Figure 3.7 D). Collectively, these results suggest that the polyamines putrescine, and mostly spermidine, have no effect on cell viability yet are able to prevent biofilm formation in AB5075-UW.

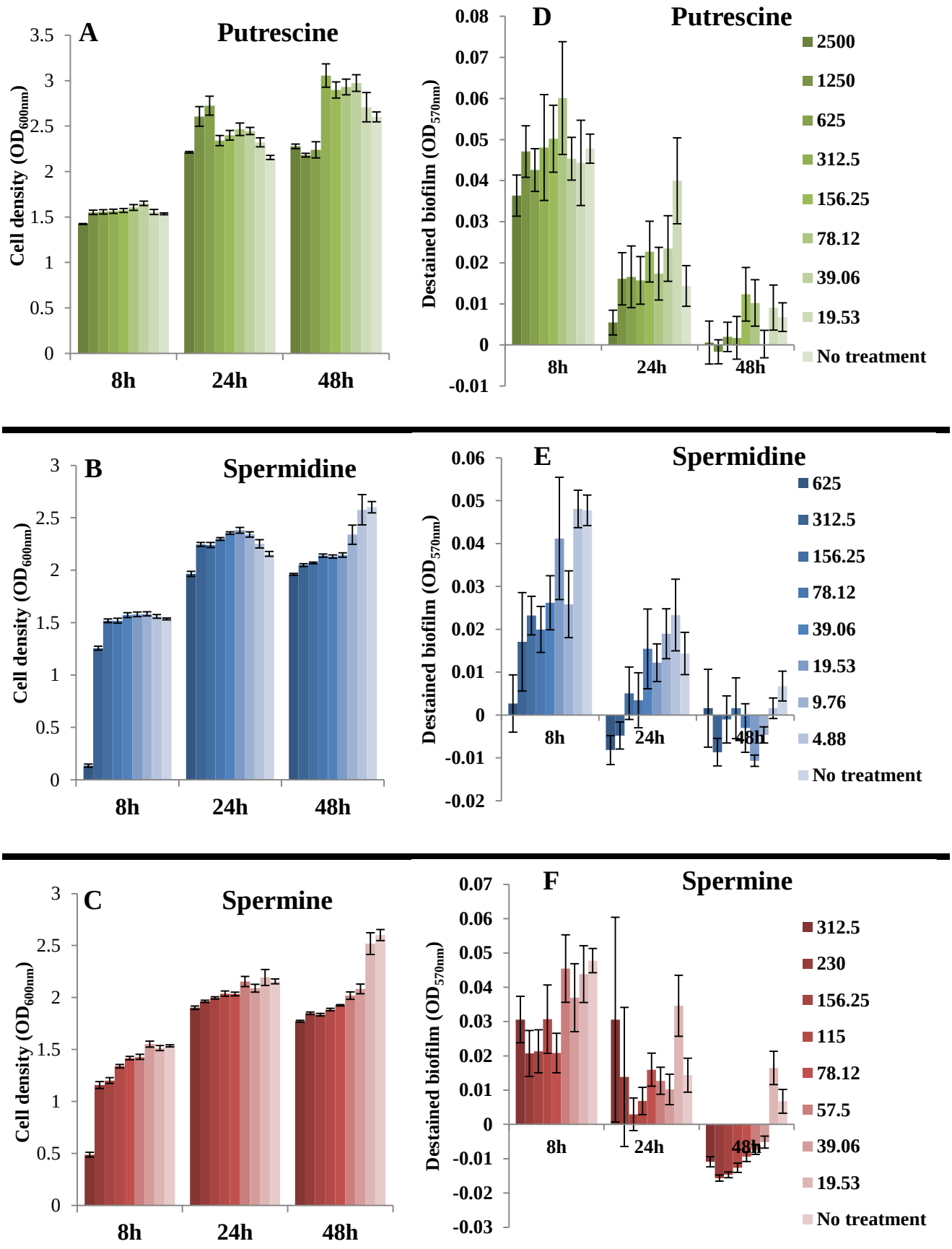

Figure 3.7 (A-C) Cell density and (D-F) de-stained biofilm with and without polyamine at 8 h, 24 h and 48 h. Each bar represents eight replicates normalised against negative control wells. Error bars indicate the standard error of the mean.

4 Discussion

Overexpression of multidrug transporters in bacteria has widely been associated with resistance to antibiotics. As a result, studies of efflux pumps have primarily concentrated on antibiotics as substrates of efflux pumps, neglecting other potential physiological functions of these transporters.

Fatty acids as well as other host-derived compounds have been found to be substrates of multidrug efflux pumps in several bacterial species. The ability to confer resistance to host-derived substrates allows bacteria to colonise and cause infections in hosts. In addition to promoting survival, multidrug efflux pumps are also known to maintain intracellular homeostasis by promoting extrusion of toxic compounds such as polyamines. Polyamines have also been found to play a role in biofilm formation and dispersal in many bacterial species. For example in *B. pseudomallei* inhibition of intracellular spermidine has been found to inhibit production of AHLs (QS signal) and cause a reduction in biofilm formation (24). Interestingly, both spermidine and AHLs are substrates of RND efflux pump BpeAB-OprB in *B. pseudomallei* (24). The objective of this study was to gather preliminary data on physiological functions of known and putative multidrug efflux pumps in *A. baumannii*.

4.1 Role of multidrug efflux pumps in fatty acid resistance

The surface of the skin has high concentrations of antimicrobial fatty acids, which play a role in host defense (115). However, some opportunistic human pathogens have been shown to colonise the surface of the skin - a necessary step prior to infection (11). In an effort to understand the ability of *S. aureus* to survive on the skin, despite the presence of fatty acids, led Truong-Bolduc *et al.*, to postulate that efflux pumps of *S. aureus* may have fatty acids as natural substrates and therefore provide resistance against these compounds (11). Indeed, following resistance assays with fatty acids they found that several fatty acids were natural substrates of the Tet38 multidrug efflux pump in *S. aureus*, even though these efflux pumps had previously thought to specifically recognise tetracyclines (11).

Consequently, it was suspected that *A. baumannii* may have efflux pumps, which recognise fatty acids, since it is a nosocomial pathogen that is similar to *S. aureus* and may encounter fatty acids during infection. In the present study, resistance assays of six different fatty acids were carried out on seven different strains of *A. baumannii*, however none of these strains was susceptible to any fatty acids at concentrations as high as 5000 μg/mL – this concentration was also approaching the solubility limit for these compounds. Given the scope of this project, and that none of the strains were susceptible to fatty acids at high concentrations, further investigations into the role of efflux

pumps could not be easily performed within the context of this project. Nonetheless, there is ample scope for future investigation, in particular, strains with null mutations in several efflux genes could be generated and their susceptibility to these fatty acids tested. Additionally, efflux pump genes implicated in the resistance of fatty acids may be found by shocking bacteria with sub-inhibitory concentrations of the compound and analysing genes whose expression is induced in the presence of these compounds using qRT-PCR. Another tool that can be employed to quantify differential gene expression in response to fatty acids is whole transcriptome shotgun sequencing also known as RNA sequencing; however this technique is more costly than those mentioned previously.

4.2 Role of multidrug efflux pumps in biofilm formation of *A. baumannii* AB5075-UW

Understanding the ability of *A. baumannii* biofilms to persist on a wide range of surfaces, despite being subjected to adverse conditions such as desiccation, nutrient starvation and eradication by disinfectants, may provide an explanation into its persistence and virulence mechanisms (120). Many studies investigating biofilm formation in *A. baumannii* have particularly focused on bacterial appendages, quorum sensing, cell surface components including outer membrane protein (OmpA) and polysaccharides (9, 95). Other studies have been concentrated on regulatory networks including two-component regulatory systems such as BfmRS, which initiates expression of the usher-chaperone pili assembly system required for adherence and subsequent biofilm formation (23). However, the physiological role of multidrug efflux pumps in biofilms of *A. baumannii* remains under-explored.

It has been suggested that the biofilm lifestyle may require an efficient waste management system through increased efflux pump activity (121). Since efflux pumps are known to confer resistance through enhanced efflux pump activity, disruption of these efflux pumps and subsequent impedance of activity during biofilm growth may provide insights into the physiological role of multidrug efflux pumps in biofilm formation and regulation. Baugh *et al.*, have recently shown that disruption in any of the nine multidrug efflux pump genes, belonging to the RND, MATE, MFS and ABC class of multidrug efflux systems in *Salmonella enterica* serovar Typhimurium resulted in reduced ability to form mature biofilms (14).

A study by Sauer *et al.*, in which they monitored the biofilm life cycle of *P. aeruginosa* under flow conditions, in a flow cell over 12 days found that, biofilms undergo five stages of development (102). Furthermore, through whole cell protein analysis and microscopic observation they discovered that each stage of the biofilm development cycle have distinct protein expression profile and phenotype (102). Therefore, in an effort to understand the influence of multidrug efflux pumps during various stages of the *A. baumannii* biofilm life cycle, biofilm-forming capacity of each

mutant and parental strain was quantified at three time points, 8, 24 and 48 h (Figure 3.2 and Figure 3.3), in microtitre plates. The present study results showed that, although cell densities between 24 h and 48 h remained similar, biofilm formation was generally the highest at 24 h, while the highest biofilm forming mutants were the MFS efflux mutant ABUW_0213-101::T26 and RND efflux mutant ABUW_0035-172::T26.

Biofilm formation is influenced by several factors, one of which is cell density dependent signalling, otherwise known as quorum sensing, whereby signalling molecules, which are self-generated coordinate expression of genes across a cell population in response to cell density (84, 122). Quorum sensing is widely accepted as being necessary for the formation of mature, differentiated biofilms. Therefore, levels of biofilm formed by respective strains may be simply related to the density of cells at the point of sampling. Consequently, the biofilm-forming capacity of all mutants and the parental strain were normalised against their respective cell densities at the point of biofilm sampling.

4.2.1 Growth-related changes as a result of mutation in multidrug efflux pumps and its effect on quorum sensing

Mutations in efflux pump gene ABUW_0109 and efflux pump gene ABUW_0035 resulted in contrasting cell densities when compared to parental strain; MATE efflux mutant ABUW_0109-152::T26 had a significantly lower cell density, while the cell density of RND efflux mutant ABUW_0035-172::T26 was significantly higher (Figure 3.2). These are interesting observations as efflux pumps have been reported to have overlapping substrate recognition profiles, thus when genes encoding efflux pumps are disrupted, other efflux pumps may counteract for the loss resulting in minimal effect on phenotype (45, 116). Therefore, it can be speculated that perhaps these efflux pumps have a unique substrate.

Mutations in multidrug efflux pumps which present a growth defect have been reported before in *Pseudomonas aeruginosa* (21). Aendekerk *et al.*, found that, mutations in genes which encoded the efflux protein MexI and porin OpmD from the RND MexGHI-OpmD pump, resulted in impaired growth, they also found that quorum sensing signalling (QSS) molecules were substrates of these efflux pumps (21). Several studies have reported QSS molecules to be substrates of RND multidrug efflux pump including MexGHI-OpmD pump as well as the MexAB-OprM and the MexEF-OprN RND efflux systems, which have been affiliated with the efflux of 3-oxo-C12-HSL and pseudomonas quinolone signal (PQS), respectively, in *P. aeruginosa* (15, 47). In the present study, three RND efflux pumps, ABUW_0922-152::T26, ABUW_1335-195::T26 and ABUW_0645-181::T26 showed reduced biofilm-forming capacity (Figure 3.1), however their role in extrusion of

quorum sensing signalling molecules was not directly investigated due to time constraints. Nonetheless, given the association of quorum sensing signalling molecules and RND efflux pumps in *P. aeruginosa*, investigating this phenomenon in *A. baumannii* would provide a better understanding of cell-to-cell signalling and biofilm formation.

The studies mentioned above have shown that mutations in RND efflux pumps can negatively impact cell density and extrusion of QSS molecules in bacteria. Therefore, enhanced growth rate and biofilm-forming capacity of RND efflux mutant ABUW_0035-172::T26 as well as other efflux mutants, relative to the parental strain, observed in the present study, is somewhat novel. Nonetheless, a study by Yang *et al.,* in *E. coli* showed that Δ*acrAB-tolC210,* or Δ*norE* mutants grew to a higher cell density when in stationary phase, additionally overexpression of either efflux pump resulted in lower cell density (16).

Thus it can be speculated that, a few RND efflux pumps may be implicated in cell-to-cell signalling through QSS molecules in more ways than one, however this may not have been apparent prior, as studies into the role of a library of multidrug efflux pumps in biofilm formation as well as cell growth has not been reported previously in any organism.

4.2.3 SEM imaging of biofilm formation in selected efflux mutant strains

The biofilm forming capacity of the parental strain and selected mutants on polystyrene microtitre plates was validated and examined in more detail through direct observation using scanning electron microscopy (SEM) on glass coverslips. SEM provides high-resolution examination of intricate details in microbial biofilms. The SEM data evidently revealed a difference in cell adhesion of mutants in comparison to the parental strain, thus confirming previous findings from microtitre plate analysis. Interestingly, RND efflux mutant ABUW_0035-172::T26 which had a high cell density also had a significantly higher adhesion capacity than parental strain, while MFS efflux mutant ABUW_0213-101::T26 also had a higher adhering capacity, it wasn't as significant. Contrarily, the ability of MATE efflux mutant ABUW_0109-152::T26 to adhere to the glass surface was immensely reduced at 8h, however not completely abolished at other time points. Aside from adherence, clear biofilm formation was only seen in efflux mutant, ABUW_0035-172::T26 whereby a dense mass of cells forming pillars and mushroom like structures as well as dividing cells were seen (Figure 4.1)

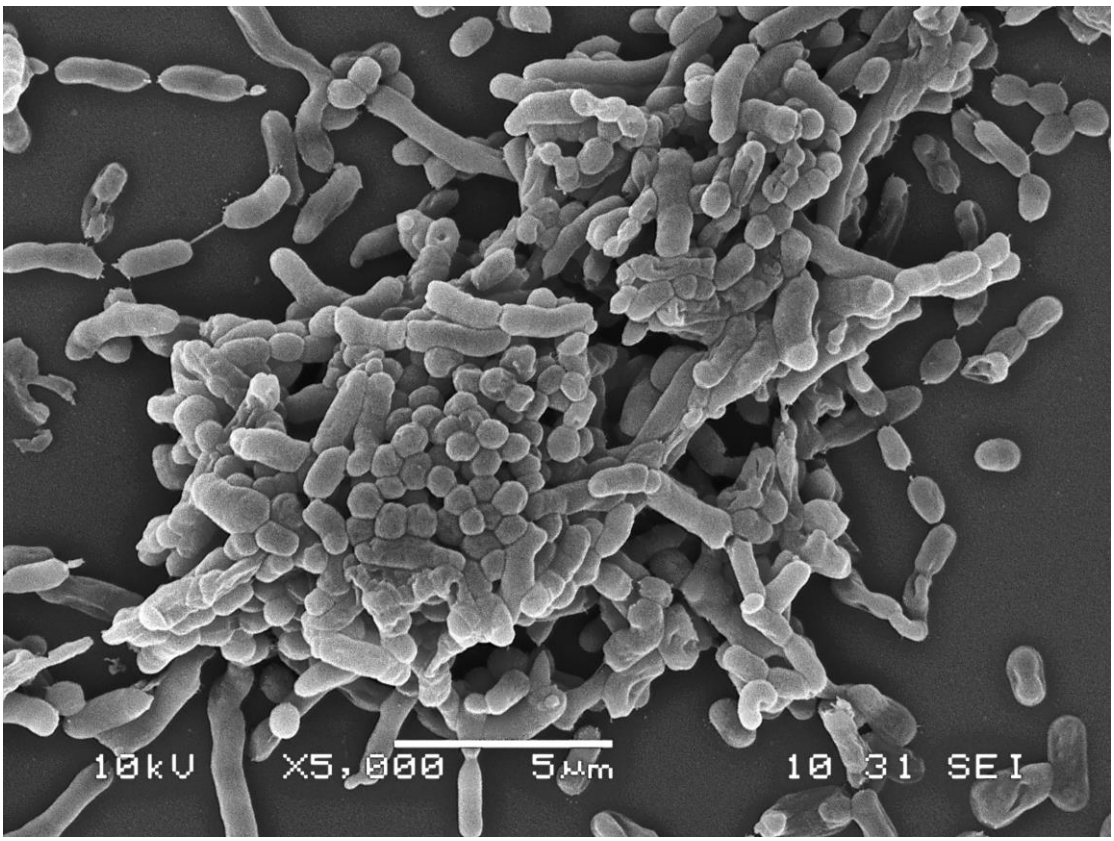

Figure 4.1 Biofilm formation by efflux mutant ABUW_0035-172::T26 at 24h on glass coverslip (magnification x 5,000).

4.2.4 Factors influencing biofilm formation in bacterial cells

The composition and spatial arrangement of biofilm architecture can be very diverse between species, equally, biofilms formed by different strains can also have different properties (81). Furthermore, it has been reported that minor changes in the environment can trigger dramatic variations in the biofilm architecture, which are often reflected in the composition of the extracellular matrix (81). Flow cells, which are often considered the gold standard in biofilm studies, provide an ideal environment for biofilm formation, specifically they provide a constant fresh supply of medium, thus permitting formation of dense biofilm structures consisting of pillars of cells and mushroom-like structures separated by fluid-filled channels (81). Biofilms grown in microtitre plates allow high-throughput screens of multiple strains as well as mutants; it can also be used to test various growth conditions and environmental signals, as well as mapping transitional episodes in the biofilm life cycle. However, these conditions do not promote dense biofilm formation (81). In the present study, biofilms for SEM were grown in 6 well microtitre plates. Hence, this may be plausible partial explanation for the low biofilm density seen, particularly in the ABUW_0109-152::T26 mutant and the parental strain. The apparent lack of biofilm density formed by these strains in SEM images could also be attributed to a number of laboratory conditions. In

particular, biofilm formation in this study was conducted at 37 °C, which is considered an ideal growth temperature for *A. baumannii*, however some studies have reported that biofilm-forming capacity of *A. baumannii* is the highest at 25 °C, these findings may explain, the notable persistence of this strain in hospital equipment's and association with infections (77, 79, 123).

Many researchers have also found that substratum characteristics can influence attachment of bacterial cells and biofilm formation (124-126). It has been suggested that substratum roughness and hydrophobicity can expand bacterial colonisation and biofilm formation, due to minimisation of shear forces and enhanced surface area (127). In this study, the microtitre plate assay was performed on polystyrene surfaces whilst microscopic analysis through SEM was performed on glass coverslips. As highlighted by Costa *et al.*, glass has hydrophilic properties as it is composed of silica, whereas plastics such as PVC and polystyrene have hydrophobic characteristics (124). Many researchers have reported that adhesive properties of bacterial species such as *Salmonella* spp. and *Listeria monocytogenes* are enhanced on hydrophobic surfaces when compared to hydrophilic substrata (125, 126). Furthermore, Costa *et al.*, tested the adhesive properties of five *A. baumannii* strains to different substrata and found that all strains adhered either weakly or did not adhere at all to the surface of glass in comparison to plastic (124). The enhanced ability of *A. baumannii* to adhere to plastics rather than glass has also been reported by Pour *et al.*, (128). Additionally, through microscopic analysis they evidently showed that biofilms formed on polycarbonate surfaces were much greater than those formed on the surface of glass (Figure 4.2) (128). Hence, it is likely that the low biofilm density observed in scanning electron microscopy images of *A. baumannii* strain AB5075-UW is because of a reduced ability of *A. baumannii* to adhere to the surface of glass. Therefore, future microscopy studies could be conducted on samples grown on plastic.

Besides substratum characteristics, bacterial properties such as surface charge, cell surface hydrophobicity and appendages can also influence adhesion (81). It has been reported that cell wall hydrophobicity in conjunction with a nonpolar substrata tends to increase hydrophobic interactions and attachment (81). Pour *et al.*, showed that the biofilm forming capacity of *Acinetobacter* strains with high cell surface hydrophobicity was higher than strains that had reduced cell surface hydrophobicity (128).

Findings from these studies highlight the need to investigate cell surface properties, such as hydrophobicity of the bacterial strain under investigation prior to performing biofilm experiments. Cell surface hydrophobicity of isolates can be determined by performing a microbial adhesion to hydrocarbons (MATH) test (129), whereby the cell density of cell suspensions is measured at 600

nm before and after addition of a hydrocarbon such as xylene. This technique has previously been used to test cell surface hydrophobicity (79, 123).

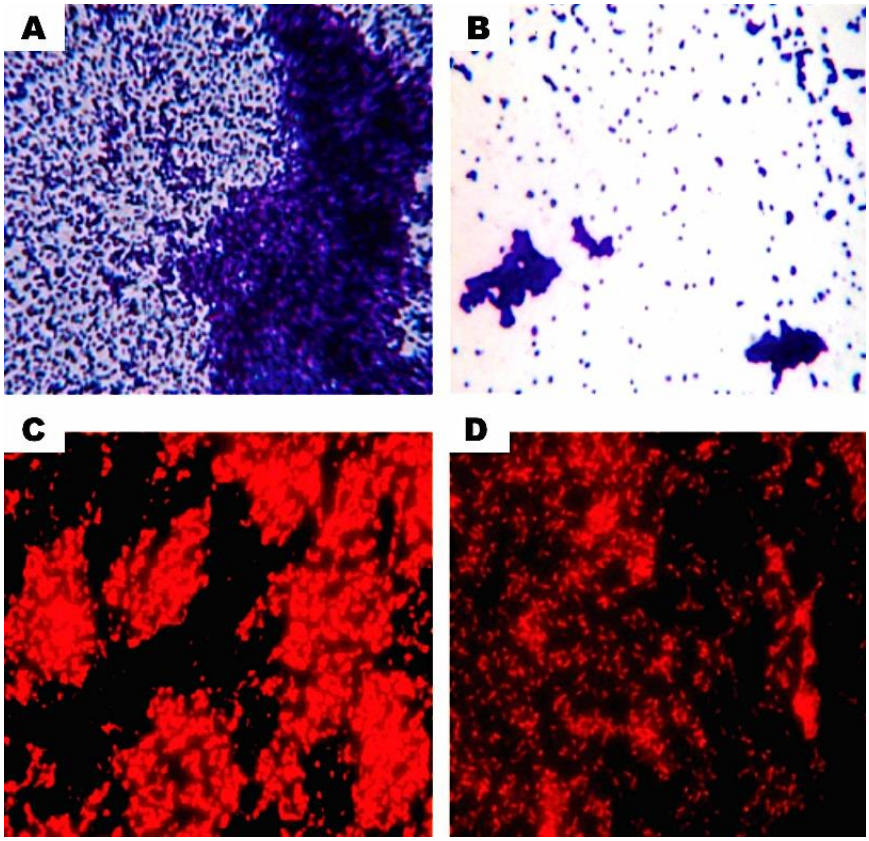

Figure 4.2. Light microscopy study by Pour *et al*., which shows *A. baumannii* A3 cells attached to (a) polycarbonate and (b) glass surface when stained with crystal violet; (c) cells attached to polycarbonate and (d) glass surface stained with 0.02% acridine orange (128).

(Image credit: Pour *et al.*, 2011)

Another biofilm characteristic, which remains unexplored in this present study, is the ability of *A. baumannii* AB5075-UW to form biofilms at the air-liquid interface, otherwise known as pellicles. While most research has been focused on biofilm formation by *Acinetobacter* spp. at the solid-liquid interface, biofilm formation at the air-liquid interface has predominantly been associated with pathogenic species (77-79). For example, in a study, Martì *et al.*, tested biofilm-forming capacity of *Acinetobacter* spp at the solid-liquid and air-liquid interface, and found that only isolates belonging to *Acinetobacter calcoaceticus* - *Acinetobacter baumannii* (ACB) complex were able to form biofilm at the air-liquid interface - a favourable niche (77). Research into pellicle formation by *A. baumannii* has revealed that some isolates in comparison to others form much thicker biofilms at the air-liquid interface (79). Therefore, in an effort to maximise biofilm formation under laboratory conditions to study the structure of the biofilm and genes essential to biofilm formation, it may be necessary to decipher the preferred growth and biofilm-forming model of *A. baumannii* AB5075-

UW. Additionally, since biofilm formation can be highly variable amongst strains, performing experiments on strains that do not form dense biofilms can be challenging. Very recently, Álvarez-Fraga *et al.*, reported that *A. baumannii* strain, MAR002 produces significantly high biofilms in comparison to other strains (76). Hence, this strain could be employed to study phenotypic changes in biofilms as a result of mutations in efflux pump genes, provided it is genetically tractable and appropriate selection markers are available for constructing mutants.

4.3 The role of polyamines in biofilm regulation

Polyamines have been reported to play a role in biofilms in several bacterial species. While some studies have found that polyamines elevate biofilm formation, others have reported that these compounds prevent the formation of biofilms (111). Considering these opposing effects of polyamines on biofilms in different bacterial species, the influence of three polyamines in *A. baumannii* biofilm was investigated. These polyamines included putrescine, spermidine and spermine, each of which is present in high concentrations in bacterial cells.

In the present study, biofilm formed by *A. baumannii* AB5075-UW as a function of exogenous polyamine concentration was measured using the crystal violet staining-based assay. It was found that cell growth as a result of different concentrations of spermidine (excluding 625 μg/mL at 8 h) was largely unaffected (Figure 3.7), however, total biofilm formed by *A. baumannii* AB5075-UW decreased with increasing concentrations of spermidine. Spermidine has been found to regulate biofilm formation in *Vibrio cholera* (110). Deletion of *potD* gene (an ortholog of the periplasmic substrate binding protein of the spermidine uptake system) which results in depletion of endogenous spermidine in *V. cholera* has been found to enhance biofilm formation (110) (Table 4.1). Furthermore when these mutant strains were complemented with >72.63 μg/mL of exogenous spermidine biofilm formation had reduced (110). Interestingly, norspermidine, which is one methylene group shorter than spermidine, has been found to enhance biofilm formation in *V. cholera* (113) (Table 4.1). The authors from these two studies found evidence of a protein termed NspS for norspermidine sensor, which regulates biofilm formation in response to environmental polyamines (110, 113). However, the influence of these polyamines in pre-formed biofilms of *V. cholera* was not reported.

Similar to spermidine, biofilm formation decreased overall as a result of increasing spermine concentration. However, in contrast to spermidine the bacterial cell densities also reduced with higher concentrations of spermine. Studies have reported that exogenous spermine impedes biofilm formation by *Neisseria gonorrhoeae* (109) (Table 4.1). Gotiya *et al.*, found that 65.61 μg/mL or 524.88 μg/mL of polyamines; spermine, spermidine, putrescine, and cadaverine had no antibacterial

activity in *N. gonorrhoeae.* However, spermine was the most successful in preventing biofilm formation (109). In an effort to understand the mechanism by which polyamines are able to exert such effects, they evaluated whether polyamine transport was necessary for prevention of biofilm formation using gonococcal strains lacking *potHI* genes, which form part of the gonococcal polyamine uptake system (109). The authors did not find any association between polyamine uptake and reduced biofilm formation. Furthermore, while they observed that the biofilm architecture as a result of spermine was markedly different, spermine did not induce disassembly of pre-formed biofilm (109) (Table 4.1).

In contrast to spermine and spermidine, putrescine did not exhibit any growth inhibitory effects against the parental AB5075-UW strain (Figure 3.7 and Table 3.1), however biofilm formation had decreased with increasing concentration of putrescine (Figure 3.7). Furthermore, biofilm formation in the presence of putrescine was completely abolished at 48 h, suggesting that putrescine may impede biofilm formation in *A. baumannii* in a dose dependent manner. Contrarily, Patel *et al.*, found that depletion of putrescine resulted in reduced biofilm formation in *Yersinia pestis* (112) (Table 4.1). Furthermore, they discovered that mutants (incapable of producing putrescine) when complemented with exogenous putrescine were capable of recovering their biofilms (112).

While there is growing evidence in the literature supporting the role polyamines in biofilms, it must be noted that different polyamines can exert variable effects on biofilm formation depending on the bacterial species and possibly strain under investigation. The various effects of polyamines in biofilms formed by several bacterial species reported thus far, has been summarised in table 4.1. The present study results show that putrescine, spermine and spermidine might play a role in preventing biofilm formation (Figure 3.7), weather these polyamines induce disassembly of pre-formed biofilms in *A. baumannii* AB5075-UW could not be established as results predicted from the crystal violet assays were highly variable (data not shown). Nonetheless, it can be speculated from the results of the present study that polyamines may be involved in earlier rather than later stages of biofilm formation.

Table 4.1 Role of polyamines in biofilm formation of different bacterial species

Polyamine	Effect on biofilms	Bacteria	Reference(s)
Spermine	Reduces growth and concurrently impedes biofilm formation	*A. baumannii* AB5075-UW	This study
	Impedes biofilm formation, does not affect pre-formed biofilm	*N. gonorrhoeae*	(109)
	Impedes biofilm formation however impact on pre-formed biofilm unknown	*V. cholera*	(110)
	Impedes biofilm formation in a dose dependant manner, does not affect pre-formed biofilm	*E. coli*	(119)
	Promotes biofilm formation in a dose dependant manner, does not affect pre-formed biofilm	*Salmonella enterica*	(119)
Spermidine	Impedes biofilm formation in a dose dependant manner	*A. baumannii* AB5075-UW	This study
Putrescine	Impedes biofilm formation in a dose dependant manner	*A. baumannii* AB5075	This study
	Promotes biofilm formation	*Y. pestis*	(112)
Norspermidine	Promotes biofilm formation however impact on pre-formed biofilm unknown	*V. cholera*	(113)
	Promotes biofilm formation	*B. subtilis*	(111)
	Impedes biofilm formation in a dose dependant manner, does not affect pre-formed biofilm	*E. coli*	(119)
	Promotes biofilm formation in a dose dependant manner, does not affect pre-formed biofilm	*Salmonella enterica*	(119)

4.4 Role of multidrug efflux pumps in polyamine transport

As described previously, polyamines in Gram-negative bacteria are known to play a variety of roles such as regulating translation by interacting with DNA, serve as free radical scavengers and biofilm formation (107, 108, 114). However, high concentrations of polyamines have been reported to be toxic to bacteria since they can suppress protein synthesis and lead to inhibition of cell growth, in the present study MIC results showed that spermine and spermidine were toxic to *A. baumannii* AB5075-UW. To protect against this toxicity, bacteria are known to enzymatically reduce intracellular concentrations of toxic polyamines such as spermidine through acetylation to N-acetylspermidine, a less toxic form (130). In *Escherichia coli*, this reaction is catalysed by spermidine acetyltransferase (130), also it was found that spermidine acetyltransferase-deficient bacteria accumulate spermidine during stationary phase, thus causing decrease in cell viability due to suppression of protein synthesis (131). In addition to enzymatic degradation bacteria also mediate extrusion of polyamines through multidrug efflux pumps, which may protect against polyamine toxicity and possibly contributes to the use of polyamines as signals in biofilm formation. As mentioned before Chan *et al.*, found that in *B. pseudomallei* RND multidrug efflux pump, BpeAB-OprB mediates extrusion of N-acetylspermidine, furthermore this efflux pump was also found extrude AHLs which play a role in biofilm formation (24). Other studies have also found polyamines to be natural substrates of multidrug efflux pumps including Blt in *B. subtilis* (13); SMR efflux pump MdtJI in *E. coli* (114) and PACE efflux pump AceI in *A. baumannii* (unpublished data). In the present study, the susceptibility of polyamines was analysed against AB5075-UW and selected efflux pump mutants. It was found that MATE efflux mutant ABUW_0109-152::T26, which also had reduced biofilm forming ability was 4-fold more susceptible to spermine than parental strain (Table 3.1). Therefore it can be speculated that spermine may be a natural substrate of this efflux pump, additionally, this transport activity may be related to the role of this pump in biofilm formation. Nonetheless, further confirmatory tests are crucial in confirming these observations.

5 Conclusion and future directions

5.1 Role of efflux pumps in biofilm formation

While the assays performed here provide important insights into the potential physiological roles of multidrug efflux pumps in biofilm formation, there is ample scope for further investigation. In particular, further confirmatory tests of the functional relationship between efflux pumps and biofilm formation is crucial. This can be achieved through constructing complementation vectors of these mutants to restore expression of efflux pump genes, and resulting phenotype returned to wildtype biofilm. Use of complementation vectors is central in manifesting that, the observed phenotype was a result of the gene deletion and not some downstream effect or a result of an undetected mutation elsewhere in the genome.

Equally, comparative qRT-PCR, which measures changes in gene expression relative to a housekeeping gene, can be employed to measure the expression of these efflux pump genes in biofilms. This procedure would confirm any efflux pump genes, which may be upregulated during biofilm formation, and may thus play a role in biofilm formation. This confirmatory test was initiated, whereby RNA from cells within the biofilm matrix was extracted and maintained at -80°C however due to time constraints comparative qRT-PCR could not be performed.

Studies have also described the application of efflux pump inhibitors to confirm phenotypic observations through chemically inactivating the efflux pumps under investigation (116). However, efflux pump inhibitors would need to be identified for the efflux pumps under investigation in present study.

5.2 Alternative biofilm imaging approaches

As mentioned above, SEM provides high-resolution images of intricate details in microbial biofilms. However, this imaging method may provide an oversimplified view of the biofilm architecture (82). SEM requires the sample to be dehydrated and so causes the biofilms to collapse as water is removed (82). Contrarily, confocal scanning laser microscopy (CSLM) permits visualisation of hydrated three-dimensional biofilm structures (82). At the time of this research Macquarie University Microscopy department was not able to accommodate live samples of human pathogens, therefore for the purpose of this study SEM was used to visualise biofilms. SEM has some advantages over CSLM in imaging intricate morphological and phenotypic differences between cells grown in biofilms, such as the differences in bacterial appendages observed between the parental AB5075-UW strain and the MATE efflux mutant ABUW_0109-152::T26 (Figure 3.6).

However in future studies, CSLM can be employed to examine variations in the biofilm architecture due to mutations in efflux pump genes.

5.3 Underlying mechanisms in prevention of biofilm formation by polyamines

Biofilms analysed in microtitre plate assays are practical tools in analysing various environmental signals at various concentrations, however the results obtained in the present study requires further confirmatory tests. In particular, the mode of action by which polyamines are able to impede biofilm formation in *A. baumannii* is unknown; furthermore whether these polyamines function via an extracellular mechanism or get taken up by cells is not known either.

Studies investigating the role of polyamines in biofilm formation by other bacterial species, have described using high-performance liquid chromatography (HPLC) to detect the presence of extracellular polyamine in the biofilm matrix, following incubation (111). To detect these polyamines, chemical tags such as AccQ-Fluor Reagent can be attached to polyamines following extraction of polyamines from the biofilm matrix using trichloroacetic acid. Polyamine derivatisation with AccQ-Fluor Reagent would render them visible by fluorescence detection therefore showing localisation of polyamines (i.e. whether polyamines function through extracellular or intracellular mechanisms).

5.4 Confirming spermine as a natural substrate

To confirm spermine as a substrate of MATE efflux mutant ABUW_0109-152::T26, further confirmatory tests will need to be performed. In particular, complementing efflux mutant ABUW_0109-152::T26 with plasmid encoded ABUW_0109 efflux pump gene and proceeding by subjecting it to resistance assays against spermine. Ideally plasmid encoded gene should restore susceptibility of efflux mutant ABUW_0109-152::T26, thus confirming that reduced susceptibility was as a result of disruption of gene and not some downstream effect.

Equally, qRT-PCR could be employed, whereby parental strains could be shocked with sub-inhibitory concentration of spermine and change in gene expression of ABUW_0109 efflux pump gene measured. Other experimental procedures such as using radioactively labelled spermine to perform accumulation and efflux assays on efflux mutant ABUW_0109-152::T26 and ABUW_0109-152::T26 complemented with plasmid encoded ABUW_0109 gene, respectively. In efflux mutant ABUW_0109-152::T26 intracellular localisation of radioactive labelled spermine would confirm spermine as a substrate. Equally, reduced accumulation of radioactive labelled spermine in efflux mutant ABUW_0109-152::T26 complemented with plasmid encoded

ABUW_0109 gene would also confirm spermine as a substrate. These procedures have been used previously to confirm substrates of efflux pumps (69).

Where they are commercially available, stable isotope labelled polyamines could also be considered as alternatives to radioactively labelled polyamines. Stable isotopes would provide signature mass tags to exogenously supplied polyamine rendering these to be identified and quantified by mass spectrometry.

5.5 Conclusion

Collectively, findings from present study and studies mentioned thus far strongly suggests that multidrug efflux pumps may have unidentified physiological substrates other than antimicrobials or environmental toxins. This study shows that chromosomal disruption of multidrug efflux leads to phenotypic difference in biofilms whereby MATE efflux mutant ABUW_0109-152::T26 produced minimum biofilms whereas biofilm forming capacity of RND efflux mutant ABUW_0035-172::T26 was significantly enhanced. Furthermore, it is likely that spermine may be a natural substrate of MATE efflux mutant ABUW_0109-152::T26, however whether any of the multidrug efflux pumps have quorum sensing signals as substrates remains to be explored.

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