CHAPTER 7

CONCLUSIONS AND DISCUSSION

The success of *A. baumannii* as a nosocomial pathogen can be attributed to its high persistence and resistance levels. Therefore, features such as iron acquisition, surface adherence, motility and drug efflux, which provide resilience in both the hospital environment and human host niches, were examined in this thesis. These characteristics were investigated using a wide range of phenotypic assays and molecular techniques; bioinformatics were an integral part in this study.

A variety of *A. baumannii* isolates were collected from different regions in Australia. The majority of the strains (76%) proved to be part of clonal lineages prevalent in Europe and other parts of the world (Section 4.2.1). These findings reiterated that the increased occurrence of *A. baumannii* is predominantly associated with global dissemination of a limited number of successful *A. baumannii* clones (Section 1.1.2). Nevertheless, various Australian *A. baumannii* strains possessed unique characteristics regardless of their clonal relationship. Furthermore, various non-international clone strains were identified, such as the community-acquired *A. baumannii* isolate D1279779. Therefore, monitoring the prevalence of *A. baumannii* clones is important for identification and characterisation of successful strains in Australia.

The collection of Australian clinical isolates was extended by including various fully sequenced strains from other countries. Furthermore, a hyper-motile variant of *A. baumannii* ATCC 17978, called *A. baumannii* 17978hm, was isolated (Section 5.2.1). The genomes of strain 17978hm, an Australian multidrug resistant clone II isolate (WM99c) and the Australian community-acquired strain (D1279779) were sequenced during the course of this study in a collaborative project.

7.1 *A. baumannii* persistence mechanisms

Life in a multicellular structure provides protection against desiccation and antimicrobial stress. Therefore, biofilm and pellicle formation facilitates prolonged persistence of *A. baumannii* in the hospital environment. The ability of *A. baumannii* to adhere to eukaryotic cells is a persistence characteristic more directly related to disease potential. These features were studied using 54 clinical *A. baumannii* isolates (Section 4.2). Furthermore, in depth examination of these features was conducted by comparative analysis of strain 17978hm and its parental strain ATCC 17978 (Section 5.2).

7.1.1 Biofilm formation

Although all eight *A. baumannii* strains belonging to the international clone I lineages formed limited biofilms as determined by a static microtiter tray biofilm assay, highly variable levels were observed between international clone II and non-international clone isolates (Section 4.2.3). International clone I isolates are successful in survival in the hospital setting and spread at a global level is evident. This indicates that biofilm formation by *A. baumannii* strains may not be essential for environmental persistence. Instead, the role of biofilms in disease potential of *A. baumannii* could be examined by correlative studies of the data on biofilm formation described here and future examination of virulence, in for example a nematode killing assay. Alternatively, isogenic insertion disruption mutants displaying impaired biofilm forming abilities can be assessed for their pathogenicity, using either a nematode or mouse model system.

Investigation of biofilm formation by strain 17978hm and its parental strain did not reveal significant differences (Section 5.2.2.1). However, when incubated with sub-inhibitory levels of the antimicrobial peptide colistin, the WT strain displayed a significant increase in biofilm formation (Section 5.2.8.3). This was not observed when examining strain 17978hm.

Biofilm formation requires a series of complex processes, from initial adherence to establishing intercellular interactions, which assist in generating a comprehensive biostructure. In this study, bioinformatic analyses showed that strain ATCC 17978 could not express functional Csu type I pili (Section 4.2.5). Strain ATCC 17978 also showed low levels of biofilm formation, this suggests that Csu pili may play a role in biofilm formation, which corroborates observations made by Tomaras *et al.* (2003). Various other extracellular structures, involving OmpA, Bap and PNAG, have previously been shown to play a role in *A. baumannii* biofilm formation. These proteins (OmpA and Bap) and polysaccharide structures (PNAG) are likely to be differentially represented in distinct *A. baumannii* isolates explaining the major phenotypic variation observed in this study.

7.1.2 Pellicle formation

A. baumannii strain 17978hm was shown to display an enhanced ability to form pellicles compared to its parental strain (Section 5.2.2.2). Although a link between motility and pellicle formation could not be established in a study by McQueary and

Actis (2011) using non-isogenic strains, the increased motility and pellicle formation observed in strain 17978hm suggested that an association between these phenotypes may exist in some strains. Additionally, when investigation of the cell surface hydrophobicity using the MATH was perfromed, strain 17978hm was also found to possess more hydrophobic characteristics than strain ATCC 17978 (Section 5.2.3), which may have resulted in enhanced intercellular interactions. Pellicle formation and pellicle stability in *A. baumannii* strain 17978hm appeared to be independent of extracellular proteins or DNA structures (Section 5.2.2.2). The findings obtained in this study indicate that hydrophobicity, pellicle formation and motility may be linked. It was also determined that iron-replete conditions were required for pellicle formation by strain 17978hm (Section 5.2.8.4). Iron plays a crucial role in cellular respiration, consequently, a low-iron concentration, achieved by supplementation of the iron-chelating compound 2,2'-dipyridyl to the growth medium, may have resulted in reduced levels of available oxygen; oxygen has also previously been shown to be important for pellicle formation (Liang *et al.* 2010).

In *P. aeruginosa*, iron limitation has a negative effect on biofilm formation as a result of increased production of biosurfactant in the form of rhamnolipids (Glick *et al.* 2010). Over-production of biosurfactant induced by iron limitation could have resulted in the diminished pellicle formation seen in *A. baumannii* strain 17978hm as described above (Section 5.2.8.4). However, genomic analyses using Blastp and Mauve showed that *A. baumannii* ATCC 17978 does not produce rhamnolipids. Glick *et al.* (2010) also reported that twitching motility increased under iron-limiting conditions, a finding contradictory to results on motility and iron-limitation obtained during the course of this study (Section 3.2.5). Therefore, a potential role for a biosurfactant in pellicle formation and increased motility of *A. baumannii* strain 17978hm requires further investigation, which will be discussed in Section 7.2.2.

Stress responses of *A. baumannii* strain 17978hm were also investigated by measuring pellicle formation in the presence of the fluoroquinolone antibiotic ciprofloxacin or the cationic antimicrobial peptide colistin. Whereas incubation with sub-inhibitory concentrations of ciprofloxacin did not appear to affect biofilm formation (Section 5.2.8.3), a significant increase in pellicle formation was observed for strain 17978hm under ciprofloxacin stress (Section 5.2.8.4). These findings on stress responses highlighted that different mechanisms or regulatory pathways are possibly involved in biofilm and pellicle formation. Supplementing colistin to the

growth media resulted in fluctuating levels of the pellicle biomass of strain 17978hm (Section 5.2.8.3), showing that *A. baumannii* pellicle formation, in general, is responsive to environmental stress. Investigating the response of *A. baumannii* to antimicrobials is of significance in optimisation of patient treatment and decontamination regimes. Avoiding the use of antimicrobials that may enhance the survival strategies of pathogens is crucial for their successful eradication. The clinical significance of pellicles as investigated in the laboratory setting is difficult to determine. However, the increased ability to form these multicellular structures at room temperature compared to incubation at 37°C indicated that pellicle formation may play a role in environmental persistence, rather than host colonisation, which corroborates previous studies (Section 1.2.2).

7.1.3 Interaction with eukaryotic cells

A. baumannii is commonly associated with lung infections (Section 1.1.3). Therefore, adherence to human epithelial cells of the respiratory tract was examined (Section 4.2.4). The *A. baumannii* strains selected for this investigation (n=11) represented different clonal lineages and displayed differences in biofilm formation and motility. As observed in the biofilm assays, *A. baumannii* strains showed major variation in binding to epithelial cells. Interestingly, two strains (ATCC 17978 and D1279779) adhered better to lung epithelial cells (A549) than to nasopharyngeal cells (Detroit 562). This indicated that these strains possess a higher disease potential compared to their ability to survive as a carriage strain in the upper respiratory tract.

A. baumannii strain 17978hm was found to possess an increased ability to adhere to human lung epithelial cells (A549) compared to its parent strain (Section 5.2.2.3). The approximately 2-fold enhanced binding to eukaryotic cells and increased pellicle formation (Section 5.2.2.2) indicated that, in general, strain 17978hm is better at binding to biotic surfaces. As described above for pellicle formation, the increased hydrophobicity may facilitate stronger interactions between the surface of the bacteria and epithelial cells.

7.2 The mechanisms involved in *A. baumannii* motility

Motility plays a role in both the persistence and virulence potential of pathogens (Section 1.2.4.2). In *P. aeruginosa*, motility is required for biofilm formation, furthermore, enhanced motility has been correlated with increased disease potential (Klausen *et al.* 2003; Stewart *et al.* 2011). Motility also plays a significant role in the virulence of *D. nodosus*, the cause of ovine footrot and of the plant pathogen *X. fastidiosa* (De La Fuente *et al.* 2007; Han *et al.* 2008). In this study, the motility phenotypes of *A. baumannii* strains were partially clonally conserved, e.g. all international clone I isolates displayed a twitching phenotype (Section 4.2.2). Interestingly, all motile international clone II isolates also possessed the ability to form well developed biofilms (Section 4.2.3). Although McQueary and Actis (2011) did not identify a correlation between motility and biofilm formation, results obtained here indicated that these two features may be correlative in strains from certain clonal lineages.

A limited number of studies have been conducted to investigate the molecular mechanism responsible for *A. baumannii* motility. *Acinetobacter* motility has previously been associated with the presence of surface structures, such as type IV pili (Section 1.2.4.3). Recently, the correlation between phenotypic properties of various distinct *A. baumannii* isolates to the presence of the CsuA/B type I pili tip adhesin and cell surface hydrophobicity was studied (McQueary and Actis 2011). However, neither biofilm formation, pellicle formation nor motility could be linked directly to either Csu type I pili or increased cell surface hydrophobicity.

7.2.1 Pili-mediated motility

In this study, iron-replete conditions were found to be required for motility of strain ATCC 17978 (Sections 3.2.5, 5.2.8.2 and 5.2.9.2). A potential role for type IV pili in motility was identified as various genes encoding type IV pili were down-regulated under iron limitation, conditions resulting in inhibition of motility. However, genes and gene clusters encoding the type IV pili were found in most *A. baumannii* genomes, including that of non-motile isolates. Therefore, the sequence similarity of the highly variable major fimbrial subunit, PilA, was investigated. ClustalW2 and neighbour-joining analyses showed separate clustering of PilA from motile and non-motile strains (Section 4.2.5). Interestingly, various PilA sequences from motile *A. baumannii* strains possessed a higher level of

homology to PilA sequences from motile strains not part of the *Acinetobacter* genus, such as *X. fastidiosa* (52% identity), than to PilA from non-motile *A. baumannii* isolates, such as ATCC 19606 (27% identity). Similar observations of PilA sequence homology have been described for type IV mediated motility in *P. aeruginosa* (Stewart *et al.* 2011).

An insertion disruption of *pilA* in twitching and swarming isolates is desired to confirm the role of PilA in motility. Complementation with distinct PilA variants from different *A. baumannii* strains allows investigation of the correlation observed between motility and the distinct PilA sequences. These mutants can also be employed to examine the significance of motility in disease potential by, for example, using an animal model system.

Transcriptomic analysis of *A. baumannii* strain ATCC 17978 and 17978hm revealed that a novel type I pili cluster (A1S_1510-1507) and the type VI secretion system (A1S_1292-1311) may play a role in the phenotypic alterations of strain 17978hm. Although heavily up-regulated in motile 17978hm cells compared to non-motile WT cells (Section 5.2.4.2), these surface structures are more likely to play a role in adherence to either abiotic surfaces or eukaryotic cells (Sections 1.2.2 and 1.2.3). This can be investigated in biofilm formation experiments, eukaryotic cell adherence/invasion assays and possibly mouse model experiments using type I pili and type VI secretion system null mutants. The Gateway-based knockout strategy designed in this study (Section 6.2.5) can be used to generate the required mutant strains.

7.2.2 Hydrophobicity and biosurfactant production

Examination of the cell surface hydrophobicity showed that strain 17978hm possessed a more hydrophobic cell surface when compared to WT cells (Section 5.2.3). Changes in hydrophobicity can be associated with over-production or structural alteration of extracellular material, such as extracellular polysaccharides. These polymers, which can function as potential biosurfactants, can be present as unbound ‘slime’, in the form of LPS or as part of the capsule and may play a role in motility and adherence (Burch *et al.* 2010; Glick *et al.* 2010). In general, motility appears to be more pronounced as a result of increased biosurfactant production (Glick *et al.* 2010). Conversely, biosurfactants may have an inhibitory effect on adherence, as biosurfactants can disrupt the interaction between the bacteria and the

surface (Glick *et al.* 2010; Rosenberg *et al.* 1983; Rosenberg *et al.* 1981). In this study, strain 17979hm displayed both increased motility and adherence, indicating that over-production of polysaccharide is unlikely to be the cause of the phenotypic changes observed. Therefore, the increased hydrophobicity, motility and adherence characteristics of strain 17978hm is potentially a result of structural alteration of extracellular material, such as the addition or repositioning of a polysaccharide side-chain. These types of structural changes of extracellular material and subsequent effects on motility and/or cell surface hydrophobicity have been observed in various other bacteria (Abeyrathne *et al.* 2005; Cong *et al.* 2011; Kearns 2010; Norman *et al.* 2002).

Polysaccharides, especially in the unbound form, have been shown to enhance solubilisation of aromatic compounds, facilitating more efficient catabolism (Prabhu and Phale 2003). Therefore, the major transcriptional differences observed in metabolic pathways between the WT and strain 17978hm, such as metabolism of the aromatic amino acid phenylalanine (Section 5.2.4.2), could be a result of changes in the cell's hydrophobicity and/or biosurfactant production. Nonetheless, comparative analysis of carbon source utilisation using a Phenotype MicroArray™ (Biolog) did not reveal major differences in the catabolism of aromatic compounds between the WT and variant strain (Section 5.2.5.2). The analysis of carbon source utilisation did reveal a noticeable difference between the WT and 17978hm cells in the presence of L-threonine or D-malic acid. Further analysis is required to determine if efflux of threonine by the product of the overexpressed gene A1S_3397 (Appendix D) may have resulted in decreased metabolic activity of strain 17978hm in the presence of L-threonine as the sole carbon source. Interestingly, A1S_3397 may be under regulatory control of the global transcriptional repressor H-NS, of which the encoding gene (A1S_0268) is insertionally disrupted in *A. baumannii* strain 17978hm (Section 5.2.7.2).

Motility of *A. baumannii* strain 17978hm was found to be inhibited by high-salt concentrations (supplementation with 100 mM NaCl to MH media containing 0.25% agar) (Section 5.2.8.2), which corroborates previous findings on increase salinity and inhibition of motility and decreased metabolic activity in *Acinetobacter* (Abouseoud *et al.* 2010; Bazire *et al.* 2007; Jung *et al.* 2011; Kang and Park 2010). Interestingly, supplementation with colistin to high-salt MH media resulted in expression of the motility phenotype by strain 17978hm, as seen for this strain under untreated

conditions (MH media containing 0.25% agar) (Section 5.2.8.2). Noteworthy, the antimicrobial peptides of the polymyxin family, such as colistin, have the ability to increase the wettability of the agar (Gooderham *et al.* 2008; Kearns 2010). This suggests that inhibition of motility under highly saline conditions may have been mediated by interference of salt with the cell's biosurfactants.

It is of great interest to conduct a correlative investigation of cell surface hydrophobicity, adherence and motility characteristics. The cell surface hydrophobicity of the extensive collection of *A. baumannii* isolates, of which the clonal relationship, motility and adherence has been examined, could be determined to identify potential correlations between these distinct phenotypic characteristics. This also allows identification of clone specific trends. Using the information available from the genomic and transcriptomic analyses performed during the course of this study, comparative genetic and transcriptional studies can be employed to delineate the significance and correlative nature of the hydrophobicity, adherence and motility at a molecular level.

7.2.3 Distinct *A. baumannii* strains employ different mechanisms for motility

Various molecular mechanisms involved in motility of *A. baumannii* strain M2 were described in a study published at the final stages of writing this thesis (Clemmer *et al.* 2011). Various *A. baumannii* M2 motility-impaired mutants confirmed the results obtained here, such as the involvement of quorum-sensing (QS) in *A. baumannii* motility (Section 7.3.1). However, major differences between strain M2 and ATCC 17978/17978hm were also observed, therefore, the mutants described by Clemmer *et al.* (2011) will be discussed; a *pilT* null mutant, M2-4, M2-2, M2-11, M2-10 and M2-5.

The type IV pili gene *pilT* was found to be essential for a full motility phenotype, as motility by the *pilT* mutant strain (a directed mutant) was reduced by approximately 50%. Transposon disruption of A1S_3027 (strain M2-4) also resulted in reduced expression of the motility phenotype. The product of A1S_3027, a putative transglycosylase, was suggested to function in assembly of surface structures, such as type IV pili (Clemmer *et al.* 2011). The results obtained by Clemmer *et al.* (2011) corroborate the results described in this thesis, such as the the involvement of type IV pili in motility based on transcriptional data of strain ATCC 17978 under iron-limiting conditions (Sections 3.2.5 and 5.2.9.2) and investigation

of the sequence homology of a the major type IV fimbrial subunit PilA (Section 4.2.5).

Two other motility-affected mutants, M2-2 and M2-11, were found to possess the introduced transposon in A1S_0113 and A1S_0115, respectively (Clemmer *et al.* 2011). These genes are located in a putative operon (Section 5.2.6.1), which may facilitate production and secretion of secondary metabolites, such as lipopeptides. This cluster is likely to be regulated by QS-signals and was also found to be heavily overexpressed in strain 17978hm in a motile state (Sections 5.2.6.1 and 7.3.1). The mode of action of the products of this gene cluster remains unknown.

A *bmfS* mutant (M2-10) was also found to be impaired in its ability to migrate over semi-solid media (Clemmer *et al.* 2011). The encoded protein is part of the two-component regulatory system BfmRS. BfmRS appears to function at a genome wide scale, as gene disruption has also shown to affect expression of the *csu* type I pili cluster in *A. baumannii* strain ATCC 19606 (Tomaras *et al.* 2008). Interestingly, expression of *bfmR* was down-regulated by more than 2-fold in strain 17978hm under motile conditions as compared to non-motile ATCC 17978 cells (Appendix E). Therefore, the role of this regulatory mechanism in strains ATCC 17978 and 17978hm requires further investigation, by for example conducting a comparative transcriptome analysis of *bfmRS* null mutant and WT cells.

Rhamnolipids, functioning as potential biosurfactants, were found to be involved in motility of *A. baumannii* strain M2, as strain M2-5, a *rmlB* insertion disruption mutant, was shown to be impaired in motility (Clemmer *et al.* 2011). As mentioned in Sections 3.2.5 and 7.1.2, the gene cluster essential for biosynthesis of dTDP L-rhamnose can not be found in strain ATCC 17978. Furthermore, genetic and physiological proof for the involvement of a biosurfactant in motility of strain ATCC 17978 is lacking. Biochemical analysis of extracellular biomaterial, such as lipopeptides or polysaccharides, using liquid-chromatography or gas-chromatography, may shed light on the presence of potential biosurfactants secreted by strain ATCC 17978. Screening the transposon insertion library of *A. baumannii* strain ATCC 17978 or 17978hm, available in the laboratory, for impaired motility may provide details about the requirement of certain genes in the production of biosurfactants.

Overall, the findings on the involvement of type IV pili and QS in motility of *A. baumannii* strain ATCC 17978 described in this thesis were supported by Clemmer *et al.* (2011). However, other mechanisms appeared to be different, including the biosynthesis of dTDP L-rhamnose. Furthermore, the *A. baumannii* M2 mutant strains described did not display differences in cell surface hydrophobicity compared to the WT cells (Clemmer *et al.* 2011). In conclusion, these results indicated that distinct *A. baumannii* isolates employ different molecular mechanism for motility and potentially other persistence and virulence mechanisms.

7.3 Regulatory mechanisms involved in *A. baumannii* persistence

7.3.1 Quorum-sensing is essential for *A. baumannii* motility

QS-signalling plays a crucial role in regulation of many different bacterial persistence and virulence mechanisms. For example, *A. baumannii* QS has previously been shown to be involved in biofilm and pellicle formation (Niu *et al.* 2008). Furthermore, QS-signal production, facilitated by the homoserine lactone synthase AbaI, was shown to be crucial for motility of strain M2 (Clemmer *et al.* 2011). Motility of this *abaI* mutant strain could be restored by supplementation with homoserine lactones to the medium (Clemmer *et al.* 2011). In the transcriptomic analysis described here, the QS-biosynthesis cluster was found to be heavily up-regulated in motile populations as compared to non-motile populations (Sections 5.2.4.2 and 5.2.9.1). The regulatory pathway between QS-signals and up-regulation of the relevant molecular mechanisms is largely unknown.

Using a bioinformatics approach, three putative binding sites for AbaR, the *A. baumannii* regulator for QS-biosynthesis genes, were identified in this study (Section 5.2.6.2). One of the AbaR-binding sites is located upstream from an ORF encoding a hypothetical protein and the divergently transcribed gene *abaR*, indicating an auto-regulatory function for AbaR. The upstream region of *abaI*, the gene encoding homoserine lactone synthase, also possessed a putative AbaR-binding site as described previously (Niu *et al.* 2008). The third putative AbaR-binding site was identified upstream of A1S_0112, which is the first ORF of a putative operon that encodes proteins putatively involved in lipopeptide production and secretion (Section 5.2.6.1). This cluster is also involved in motility of *A. baumannii* strain M2 as discussed in Section 7.2.3.

Transcriptional profiling of *abaI* under different conditions showed that QS is directly correlated to motility of strain 17978hm. The gene *abaI* was expressed at high levels when semi-solid MH media were supplemented with either colistin or ciprofloxacin, conditions that allow motility to occur. In contrast, *abaI* was expressed at low levels under high-salt or low-iron conditions, conditions that both resulted in inhibition of motility.

The gene coding for Lon protease (A1S_1030) was significantly down-regulated in strain 17978hm compared to the WT strain (Appendix E). Lon proteases have previously been shown to play a role in the regulation of QS-signal production in *Pseudomonas* species, by proteolysis of the QS-signal biosynthesis proteins or their regulators (Bertani *et al.* 2007; Marr *et al.* 2007; Takaya *et al.* 2008). Therefore, down-regulation of *lon* in strain 17978hm may have resulted in increased QS-signal levels.

7.3.2 The involvement of H-NS in expression of the *A. baumannii* persistence features

Using whole genome sequencing, an insertion element was identified in strain 17978hm, which disrupted A1S_0268, an ORF intact in the WT strain (Section 5.2.7.2). Interestingly, when analysing the sequence of this region in *A. baumannii* strain B23, a hyper-motile clone isolated from an *A. baumannii* ATCC 17978 transposon library, A1S_0268 was also found to be disrupted by the same insertion element as that identified in strain 17978hm, but in a different position. The insertion element appears to have been derived by a duplication event, as the sequence is fully conserved with a region that includes A1S_0628, a putative transposase. The position of the mini-Tn*10* transposon in the genome of *A. baumannii* strain B23 is currently being investigated.

A1S_0268 encodes a H-NS protein, which belongs to the histone-like family of proteins. These proteins bind DNA, where they function as transcriptional repressors. H-NS proteins have been found to bind AT-rich DNA sequences preferentially, which often includes horizontally-acquired genetic material (Lang *et al.* 2007). The loci encoding the type VI secretion systems of various bacteria are generally AT-rich and have been shown to be regulated by H-NS proteins (Bernard *et al.* 2010). This may explain the high levels of up-regulation of the type VI secretion system in strain 17978hm (Section 5.2.4.2).

Interestingly, the *E. coli* type VI secretion system is involved in regulation of a type I pili cluster (Section 1.2.3). The comparative transcriptomic analysis conducted here revealed up-regulation of one such type I pili cluster in strain 17978hm (A1S_1510-1507; Appendix D). This type I pili cluster appeared to be well conserved between different *A. baumannii* strains (Section 4.2.5) and is for that reason an unlikely target of H-NS. Instead, this cluster may have been overexpressed as a result of the up-regulation of the type VI secretion system in strain 17978hm, which corroborates the observation made in *E. coli* (de Pace *et al.* 2010).

Various other potential targets of H-NS were identified by comparing the transcriptomic data of *A. baumannii* strains ATCC 17978 and 17978hm to whole genome alignments, which included *A. baumannii* strains AYE, ACICU and WM99c. The whole genome alignments assisted in identification of non-conserved regions, which may represent horizontally-acquired genetic material, which are likely targets of H-NS as described above. Of particular interest were three outer membrane proteins (A1S_0745, A1S_1032 and A1S_1033) that were significantly overexpressed and not conserved between all genomes (Section 5.2.7.2). The encoded proteins may be involved in the phenotypic alterations of strains 17978hm and B23.

An *Acinetobacter*/*E. coli* shuttle vector harbouring A1S_0268 amplified from strain ATCC 17978 was constructed for complementation of the altered phenotypes observed in strains 17978hm and B23. Indeed, expression of A1S_0268 in the hyper-motile variants returned the phenotypes to that observed for the WT ATCC 17978 strain, *viz*. non-motile on semi-solid MH media, lack of pellicle formation and reduced hydrophobicity (Section 5.2.7.3). Furthermore, transcriptional repression of the type VI secretion systems and type I pili cluster was observed. These results indicated that the insertion element disrupting A1S_0268 is solely responsible for the phenotypic alterations observed in strains 17978hm and B23.

Comparative investigation of strain ATCC 17978 and 17978hm using chromatin immunoprecipitation and subsequently gel-shift assays or DNase protection assays may prove useful for confirmation of the putative H-NS target sites described here. These analyses in combination with the transcriptomic data provide a comprehensive depiction of the molecular mechanisms that are regulated by this important global repressor protein.

7.4 The importance of iron acquisition in *A. baumannii*

The significance of iron in virulence and persistence of *A. baumannii* was examined in various parts of this study. Three siderophore biosynthesis gene clusters were found to be heavily up-regulated in the transcriptomic analysis of strain ATCC 17978 under iron-limiting conditions, of which one had not been described previously (Section 3.2.4). Despite differences in the number of siderophore biosynthesis clusters present in the fully sequenced *A. baumannii* isolates, a direct correlation with growth at different iron levels could not be established (Section 3.2.6). This indicated a level of redundancy in the *A. baumannii* siderophore-mediated iron acquisition mechanisms.

The *A. baumannii* FUR box was investigated using a bioinformatics approach together with the obtained transcriptomic data (Section 3.2.3). The novel *A. baumannii* FUR box motif was generated by MEME and MAST analyses of the experimentally determined *E. coli* FUR binding sequences followed by iteration using the predicted *A. baumannii* FUR binding sequences. The resulting motif, consisting of 22 sequences (Figure 3.4; Table 3.2), assisted in identification of an additional 59 putative *A. baumannii* FUR binding sequences (Appendix C). The majority of the overexpressed genes under iron-limiting conditions seen in the transcriptomic data were found to be under regulatory control of FUR, highlighting the importance of FUR in *A. baumannii* iron homeostasis. Nevertheless, titration assays of FUR and these putative regulatory DNA targets need to be conducted to experimentally confirm the bioinformatic results described here. Furthermore, the role in regulation of genes encoding proteins functioning in iron acquisition for the second putative FUR protein encoded by ABAYE1887 in *A. baumannii* strain AYE (Section 3.2.7) requires further investigation. Insertion disruption studies and gel-shift assays can be utilised to examine the biological relevance and putative binding sites of this protein, respectively.

Iron-replete conditions were found to be essential for both motility and pellicle formation (Sections 3.2.5, 5.2.8.2 and 5.2.8.4). These phenotypic characteristics demonstrated the importance of iron in persistence and virulence of *A. baumannii*. However, the molecular mechanism responsible for impaired expression of these features under iron limitation appeared to be different. At a transcriptional level, iron was found to be crucial for expression of type IV pili and subsequently motility

(Sections 3.2.5 and 5.2.8.2). However, protein structures, such as type IV pili, were not essential for pellicle formation (Section 5.2.2.2). Instead, the reduced biomass of these multicellular structures under iron-limiting conditions appeared to be a result of respiratory restrictions due to a reduced abundance of iron or iron-containing molecules (Section 5.2.8.4).

Various analyses performed here have shown that *A. baumannii* is highly responsive to iron limitation, both by means of overexpression of iron acquisition mechanisms and inhibition of energy consuming processes, such as motility and pellicle formation. These adaptations are likely to assist in survival of *A. baumannii* under iron-limiting conditions in the hospital environment and host niches.

7.5 Efflux-mediated resistance

Antimicrobial resistance of *A. baumannii* has been studied extensively. The significance of antibiotic modifying enzymes is evident and the increased prevalence of strains harbouring extended spectrum beta-lactamases raises major concerns. In this study, efflux mediated resistance was examined, due to the limited knowledge available on the role of drug transporters in multidrug resistant *A. baumannii*.

First, the *A. baumannii* genome was analysed for the presence of putative drug transporter proteins using TransAAP (www.membranetransport.org) in a collaborative project with Dr. LDH Elbourne (Macquarie University, Sydney, Australia). A total of 52 predicted drug transporters were identified; 13 ABC transporters, four SMR proteins, four MATE proteins, 22 MFS members and 9 RND proteins. The total number equates to approximately 1.4% of all predicted ORFs of the ATCC 17978 genome, which is significantly higher than the percentage of predicted transporters in for example the *E. coli* K12 genome (1%). The prevalence of these 52 putative *A. baumannii* drug transporters was investigated in 54 clinical *A. baumannii* isolates (Section 6.2.1). Whereas other resistance markers, such as the beta-lactamases, are often located on mobile genetic material (Section 1.3.1), almost 50% of the isolates investigated possessed all 52 transporters. Therefore, most drug transporters appeared to be part of the *A. baumannii* core genome, corroborating previous analyses using comparative genomics (Adams *et al.* 2008). Only a limited number of transporters have previously been functionally characterised, therefore, a cloning strategy, using Gateway-technology, suitable for high-throughput characterisation of these membrane proteins was developed (Section 6.2.3). The

Gateway features from pET-DEST42 were used to construct three novel Gateway-based *E. coli* expression vectors. These plasmids possessed differences in copy-number, the promoter and regulatory elements. All vectors contained a V5-epitope and His_6-tag for purification and identification purposes. Two novel drug transporters with different structural properties, AbeM4 (MATE) and AdeM (RND), were used to examine heterologous expression in *E. coli* using the Gateway-based expression vectors designed here. Major differences were observed in protein synthesis using these different expression vectors (Figure 6.2), which highlighted the importance of optimising heterologous expression of proteins in *E. coli*. Functional analysis of *E. coli* cells expressing either AbeM4 or AdeM indicated that high levels of protein synthesis may result in compromised fitness of the host cell, a phenomenon not unusual for overexpression of membrane proteins (Section 6.2.3). Therefore, the four different expression vectors were divided into two groups; pET-DEST42 and pBADgw for high levels of protein synthesis and $pBSgwP_{lac}$ and $pBSgwP_{T7}$ for lower expression levels, allowing *in vivo* functional characterisation of the recombinant protein.

Five different drug transporters, including all predicted members of the MATE family and a novel RND protein, were functionally characterised using the vectors constructed in this study (Section 6.2.4). By assessing resistance to a panel of antimicrobial compounds, substrates were identified for four drug transporters, three from the MATE family (AbeM, AbeM2 and AbeM4) and one from the RND superfamily (AdeM), confirming their function as drug transporters. This also showed that bioinformatic analysis using TransAAP is a useful tool for prediction of putative drug transporters. The gene *abeM2* is located in a siderophore biosynthesis gene cluster unique to *A. baumannii* ATCC 17978 and may also function as an exporter of siderophores (Section 3.2.4). This is novel as MATE proteins have not been shown to function in iron homeostasis previously. The genes encoding AbeM and AdeM could not associated with other biological functions when examining the adjacent genes and gene clusters. Despite testing a broad spectrum of compounds, substrates for AbeM3 were not identified. Furthermore, *abeM3* is not transcriptionally linked to other genes, hence, a putative function could not be derived.

The Gateway features from pET-DEST42 were also cloned into pEX18Tc (Hoang *et al.* 1998) to create a *sacB*-based suicide vector for use as a high-

throughput insertion disruption system in *Acinetobacter* (Section 6.2.5). The tetracycline resistance marker located on pEX18Tc is suitable for use in the tetracycline susceptible *A. baumannii* strain ATCC 17978. A gentamicin resistance cassette, isolated from pPS856 by endonuclease digestion with *Xba*I (Hoang *et al.* 1998), was cloned into a native *Xba*I site located in the middle of the *abeM4* ORF. After introduction of the suicide vector containing the *abeM4* knockout construct into *A. baumannii* strain ATC 17978, successfully generated mutants were isolated by supplementing the appropriate selection to the medium (sucrose and gentamicin). PCR analysis of these mutants showed that *abeM4* was disrupted by the gentamicin resistance cassette in all clones. Despite identification of substrates in a recombinant *E. coli* host system expressing AbeM4, increased susceptibility of the *A. baumannii abeM4* knockout mutant was not observed (Section 6.2.5). Other transporters with similar substrate profiles may have overshadowed the potential role of AbeM4 as a drug transporter.

In conclusion, the cloning strategy designed here proved to be successful for the characterisation of *A. baumannii* membrane proteins. The diverse range of vectors are suitable for characterising recombinant proteins and investigation of the biological function of proteins in their native environment in *A. baumannii*. The system was proven successful for characterisation of four novel *A. baumannii* drug transporters, but also displayed potential as a versatile tool for characterisation of structurally and functionally distinct membrane proteins.

7.6 Genome analyses

Major phenotypic differences were observed between clinical *A. baumannii* strains, regardless of their clonal relationship (Section 4.2). This indicated that *A. baumannii* strains have the ability to diversify at a high rate. This level of dissimilarity between strains observed at a phenotypic level was also apparent at a genetic level. Mobile genetic elements have played an important role in shaping the *A. baumannii* genome and may have significant phenotypic consequences, as seen in *A. baumannii* strain 17978hm (Section 5.2.7.2). The disruption of the gene encoding a histone-like protein in strain 17978hm appeared to have resulted in transcriptional activation of various genes responsible for the phenotypic alterations observed.

The molecular mechanisms identified and examined here were analysed in whole genome alignments comprising the available fully sequenced *A. baumannii*

strains. This showed that mobile genetic elements have contributed to both genome expansion and reduction in *A. baumannii*. Both events have resulted in modifications of the siderophore-mediated iron acquisition mechanisms (Section 3.2.6). In *A. baumannii* strain SDF, iron acquisition is limited as the only siderophore biosynthesis cluster is largely deleted by insertion of an insertion element (Figure 3.8). Conversely, *A. baumannii* strain ATCC 17978 has gained a siderophore biosynthesis cluster via horizontal gene transfer. Similarly, the AbaR-like genomic regions exist as a result of insertion of mobile DNA, however, removal of segments within these genomic islands due to recombination events have also resulted in major genomic reductions (Section 1.4.1). Other genome modifications in which mobile genetic elements played a role were identified in the QS-signal biosynthesis cluster in strain SDF and 1656-2 (Section 5.2.6.1). Insertion sequences were also identified in the type VI secretion system of strain 1656-2 and major variation was observed in a genomic region which harbours a siderophore biosynthesis cluster (ABAYE1888-1889) and a type I pili cluster (ABAYE1860-1856 / AB57_2003-2007) (Sections 3.2.6 and 4.2.5).

A different form of genetic variation was examined in the *A. baumannii* type I pili clusters. Differing sizes of homopolymeric tracts in the 5'-end of the first ORF in the type I polycistronic clusters were observed (Section 4.2.5). Frame-shifts introduced by insertion of one nucleotide are likely to result in synthesis of non-functional proteins. This form of sequence dissimilarity may represent a phase variable system. In phase variation, insertion or deletion of a nucleotide in a homopolymeric tract during replication results in switching between functional and non-functional protein synthesis (Henderson *et al.* 1999). Although commonly identified in other organisms such as *Neisseria gonorrhoeae* (Seifert 1996), the sequence sites identified here may represent the first *A. baumannii* phase variation mechanisms. This can be experimentally assessed by constructing a transcriptional fusion between the native ORF downstream from the homopolymeric tract and a reporter gene, such as a (green) fluorescent protein or LacZ. The rate of phase variable switching between on and off can then be determined using the appropriate read-out system.

7.7 Conclusions

The extensive examination of a broad collection of *A. baumannii* isolates at a phenotypic and molecular level conducted throughout this study assisted in identification and characterisation of various mechanisms that play an important role in persistence and resistance of this significant human pathogen. This study also showed that global experimental approaches for investigation of *A. baumannii*, such as genome and transcriptome analysis, provide a wealth of information, which can subsequently be used as guidance for identification and characterisation of novel molecular mechanisms. Significantly, the *A. baumannii* isolates studied here displayed major phenotypic differences, in for example adherence and motility, furthermore, genomic analyses have shown major variation in the genome content of the fully sequenced *A. baumannii* isolates. Therefore, comprehensive understandings about the factors that play a role in the success of *A. baumannii* as a species lies in studying the relevant molecular mechanisms of many distinct isolates.