CHAPTER 1
GENERAL INTRODUCTION

1.1 *A. baumannii*; a significant human pathogen with an alarming potential

Acinetobacter baumannii is a Gram-negative non-fermenter that belongs to the group of gamma-proteobacteria. Growth occurs under strictly aerobic conditions and their shape varies from rod to coccoidal under different growth conditions. An increasing prevalence of *A. baumannii* has been observed in the clinical setting throughout the last two decades (Dijkshoorn *et al.* 2007). This has changed its significance from being considered a contaminant in diagnostic specimens, to being a recognised pathogen of great importance. Consequently, *A. baumannii* has become the point of focus in many studies of clinical, epidemiological and molecular nature. *Acinetobacter* species can be isolated from both environmental and clinical samples, with the original source of pathogenic *A. baumannii* still undetermined. Multiple *Acinetobacter* species can be of pathogenic significance, nevertheless, *A. baumannii* is most frequently isolated as a human pathogen (Bergogne-Berezin and Towner 1996; Chuang *et al.* 2011; Diancourt *et al.* 2010; Joly-Guillou 2005; Turton *et al.* 2010). Although uncommon, this Gram-negative bacillus can also be part of the normal flora of the skin and the gastrointestinal and upper respiratory tracts (Berlau *et al.* 1999; Seifert *et al.* 1997). The diversity of these habitats, in which different detoxification and survival strategies are required, has resulted in the presence of a wide variety of resistance and persistence mechanisms. This has given *A. baumannii* the ability to adapt to the hospital environment and is now recognised as one of the most troublesome bacteria in intensive care units (ICUs) worldwide (Cunha *et al.* 1980; Dijkshoorn *et al.* 2007; Neely 2000; Patterson *et al.* 1991).

Various *Acinetobacter* species that are not considered pathogenic have been studied for a diverse range of applications. For example, the DNA uptake system identified and characterised in *A. baylyi* makes this bacterium interesting for use in molecular biology (Busch *et al.* 1999; Palmen *et al.* 1993). Furthermore, having soil as its natural habitat, many *Acinetobacter* species possess a remarkable ability to catabolise toxic compounds. Therefore, the use of *Acinetobacter* in bio-degradation/remediation is also being investigated extensively (Fischer *et al.* 2008; Mishra *et al.* 2004).

1.1.1 *Acinetobacter* classification remains under debate

The genus *Acinetobacter* underwent a turbulent taxonomical history and identification at species level remains difficult (Bergogne-Berezin and Towner 1996). It is only since 1968 that several differently named bacteria, including the first reported *Micrococcus calco-aceticus* by Beijerinck in 1911, were clustered and called the *Acinetobacter* genus (Baumann *et al.* 1968). A number of different DNA-DNA hybridisation groups, or genome species, have been described throughout the following 30 years, but not all have been assigned names (Bouvet and Grimont 1987; Bouvet and Jeanjean 1989; Johnson *et al.* 1970; Tjernberg and Ursing 1989). Besides the well known members of the *A. baumannii-calcoaceticus* cluster; *A. baumannii*, *A. calcoaceticus*, genome species 3 and 13TU (Diancourt *et al.* 2010; Tjernberg and Ursing 1989), other species commonly isolated from clinical specimens include *A. radioresistens, A. ursingii, A. schindleri, A. junii* and *A. parvus* (Nemec *et al.* 2001; Nemec *et al.* 2003; Tjernberg and Ursing 1989). Species isolated from environmental samples include *A. baylyi, A. bouvetii, A. tjernbergiae, A. townreri, A. tandoii,* and *A. gerneri* (Carr *et al.* 2003).

Various amendments have been implemented as similar species were given different names. For example, *A. radioresistence* was found to be part of genome species 12 (Nishimura *et al.* 1988) and *A. lwoffii* of genome species 8 and 9 (Tjernberg and Ursing 1989). Genome species 13 BJ and 14 TU also proved to be of the same DNA-DNA hybridisation group (Janssen *et al.* 1997). More recently, multiple genotypic characterisation methods showed that *A. grimontii* and *A. junii* were indifferent (Vaneechoutte *et al.* 2008). The commonly isolated genome species 3, 13TU, 10 and 11 were recently named *A. pittii, A. nosocomialis, A. bereziniae* and *A. guillouiae*, respectively (Nemec *et al.* 2011; Nemec *et al.* 2010). The remaining unnamed genome species show only minor phenotypic differences to the more common *Acinetobacter* species and often consist only of a limited number of isolates.

The introduction of novel identification techniques, often DNA sequence based, has made the taxonomical classification of *Acinetobacter* species even more complex, as different techniques have varying discriminatory levels. As a result, classification within the *Acinetobacter* genus remains somewhat confusing and lacks consistency. Standardisation of a technique that is practical for diagnostic

laboratories is required to gain insight into the prevalence of different *Acinetobacter* species in the clinical setting.

1.1.2 The global spread of successful *A. baumannii* clonal lineages

A number of studies have indicated that the spread of a limited number of successful clonal lineages has resulted in the globally increased prevalence of *A. baumannii*. These findings specifically concern the multidrug resistant *A. baumannii* (MDRAB) isolates (Dijkshoorn *et al.* 1996; Dijkshoorn *et al.* 2007; Nemec *et al.* 2004a; van Dessel *et al.* 2004). The clonal relationship between *Acinetobacter* isolates can be investigated using techniques such as variable-number tandem repeats (VNTR) analysis, multi-locus VNTR analysis, sequence analyses of the *16S rDNA* gene, multi-locus sequence typing (MLST), restriction analysis of the complete genome using pulsed-field gel electrophoresis (PFGE), amplified *16SrDNA* restriction analysis (ARDRA) and amplified fragment length polymorphism (AFLP) (Bergogne-Berezin and Towner 1996; Dijkshoorn *et al.* 2007; Peleg *et al.* 2008; Pourcel *et al.* 2011). More recently, the accessory genome has been partially determined using the fully sequenced *A. baumannii* strains as a backbone and determination of the presence or absence of genes within this highly variable genomic fraction was utilised as a tool to investigate the clonal relationship of *A. baumannii* strains (Turton *et al.* 2011). The genotypic profiles obtained with the techniques described above can also be used for identification of *Acinetobacter* species when compared to the appropriate reference strain.

The spread of *A. baumannii* is not confined to patient-to-patient transmission, as dissemination has also been reported between medical facilities (Da Silva *et al.* 2007; Nemec *et al.* 1999; Quale *et al.* 2003; Turton *et al.* 2004; van den Broek *et al.* 2006). This may have allowed clonal lineages to be distributed between different countries, as such, the clonal spread of *A. baumannii* is also evident on a global level (Dijkshoorn *et al.* 2007). Clonality analysis of three common lineages, named international clone I, II and III, is straightforward (Turton *et al.* 2007) and their distribution has been well documented. Isolates belonging to the clone I lineage are most prevalent in the UK (Turton *et al.* 2007), whereas clone II makes up more than 50% of the *A. baumannii* isolates studied in the Czech Republic (Nemec *et al.* 2008). Although originally described as European clones, intercontinental spread of these lineages is evident. For example, clone I isolates have been found in South Africa (van Dessel *et al.* 2004), both clone I and II in Australia (Post and Hall 2009; Post *et*

al. 2010) and clone I, II and III in the United States (Golanbar *et al.* 2011; Whitman *et al.* 2008). Interestingly, the transmission of *A. baumannii* from a soldier wounded in Iraq to a health care worker in America displayed a direct example of intercontinental spread of an MDRAB isolate (Whitman *et al.* 2008).

1.1.3 *A. baumannii* can cause a wide range of infections

Mortality rates due to *A. baumannii* infections range from approximately 30% in colonised patients, 52% in cases of bacteraemia and up to 73% in patients with ventilator-associated-pneumonia (Fagon *et al.* 1996; Seifert *et al.* 1995). *A. baumannii* is most often isolated as a respiratory tract pathogen, however, it can be found in clinical samples from various organs and tissues. Common *A. baumannii* infections other than pneumonia or bacteraemia include urinary tract infections, skin and wound infections (including burn infections) and meningitis (Bergogne-Berezin *et al.* 1993; Bergogne-Berezin and Towner 1996). Although *A. baumannii* is often isolated from patients admitted to the ICU, there is no evidence that prevalence of *A. baumannii* is higher in this particular hospital ward. The common association of *A. baumannii* with ICUs is most likely related to the pathogenic impact on patients admitted to the ICU, especially those requiring respiratory assistance. Life-threatening *A. baumannii* infections are predominantly seen in patients with serious underlying diseases, highlighting its importance as an opportunistic pathogen.

1.1.4 Cost-related consequences in the clinical setting

Several studies have shown that patients with MDRAB infections undergo prolonged hospitalisation (Lee *et al.* 2007; The Brooklyn Antibiotic Resistance Task Force 2002; Wilson *et al.* 2004). The delay in patient turnover does not only result in extended waiting lists, but also leads to considerable financial burden. For example, the mean expenditure of an MDRAB infected patient with burn wounds was US$98,575 higher than that of the control group with identical severities (Wilson *et al.* 2004). Also an outbreak of an imipenem resistant *A. baumannii* clone in a French hospital resulted in closure of the ICU; the costs of this outbreak were up to half-a-million Euros (Garlantezec *et al.* 2011). As such, *A. baumannii* does pose a significant financial cost to the health care system and these costs will only increase as MDRAB infections appear to become more prevalent in these settings.

1.1.5 *A. baumannii* as a community-acquired pathogen

1.1.5.1 Carriage of Acinetobacter

Up to 40% of healthy individuals may carry *Acinetobacter* on their skin (Berlau *et al.* 1999; Dijkshoorn *et al.* 2005; Thom *et al.* 2010). This predominantly concerns the less pathogenic members of the species, such as *A. lwoffii*. *Acinetobacter* carriage rates of up to 75% have been observed in hospitalised patients (Seifert *et al.* 1997). However, the species distribution of carriage strains in the hospitalised individuals was similar to that observed in the healthy population, with a high prevalence of *A. lwoffii* and very low prevalence of *A. baumannii*. Of greater concern are patients with previously confirmed *A. baumannii* infections, as they often remain carriers of the strain, with the gastrointestinal tract as a potential reservoir (Gordon and Wareham 2009; Marchaim *et al.* 2007). Therefore, selective decontamination of the digestive tract of ICU admitted patients with an *A. baumannii* infection may be a suitable regimen when eradication of endemic *A. baumannii* strains is required, using the non-absorbent antibiotics polymyxins and tobramycin, to which resistance is uncommon (Agusti *et al.* 2002).

1.1.5.2 Prevalence of community-acquired A. baumannii *is higher in (sub)tropical areas*

Disturbing results were obtained when summarising all published cases of community-acquired *A. baumannii* using a PubMed database search from 1973 to June 2006 with "community AND Acinetobacter" (Falagas *et al.* 2007). A mortality rate of 56% was observed in six case studies described in the review. Pneumonia was the most predominant disease, although co-morbidity was diagnosed for most patients. Concerns have also arisen after reports of multiple cases of community-acquired meningitis caused by *A. baumannii* (Lowman *et al.* 2008; Taziarova *et al.* 2007).

High alcohol consumption and heavy smoking are linked to *A. baumannii* carriage and community-acquired infections (Anstey *et al.* 2002; Anstey *et al.* 1992; Chen *et al.* 2001). *A. baumannii* throat carriage rates of 10% were observed in high-risk groups within a northern Australian community during the wet-season (Anstey *et al.* 2002). Overall, it appears cases of community-acquired *A. baumannii* are more prevalent in regions with tropical or sub-tropical climates and in individuals with health impaired lifestyles (Anstey *et al.* 2002; Anstey *et al.* 1992; Barnes *et al.* 1988; Obaro *et al.* 2011; Seifert 1999).

1.1.5.3 Post-traumatic community-acquired A. baumannii *infections*

A. baumannii wound infections have been diagnosed in both military personnel and civilians in combat zones. MDRAB infections are particularly common in soldiers returning from Iraq or Afghanistan (Davis *et al.* 2005; Hujer *et al.* 2006). Unfortunately, the clonal spread of this genus is not monitored within these regions, therefore, distinction between nosocomial and community-acquired *Acinetobacter* infections in these cases needs further clarification. Regardless, these MDRAB strains do not concern common carriage isolates found in US army soldiers, indicating that the strains have been obtained on location (Griffith *et al.* 2007). *A. baumannii* has also been isolated from wounded soldiers in East-Timor conflicts (Elston *et al.* 2008), victims of the 2004 South-East Asian tsunami (Garzoni *et al.* 2005), various earthquakes (Kang *et al.* 2009; Kiani *et al.* 2009) and the 2002 Bali bombings (Silla *et al.* 2006). The latter caused further complications after patients were admitted to a hospital in Adelaide, South Australia. A particular clone, believed to have originated from a Bali bombings victim, persisted for several months and required frequent screening of patient and environmental samples, in order to monitor its persistence and spread (H. Brettig, personal communication).

1.2 Mechanisms involved in *A. baumannii* virulence and persistence

Significant complications due to an *A. baumannii* infection are most common in patients suffering from serious underlying diseases, an impaired immune system or those that have been subjected to invasive procedures. MDRAB infections are difficult and in some cases impossible to treat, resulting in life-threatening diseases (Dijkshoorn *et al.* 2007). Although most threatening for the critically ill, individuals with minor illness can be involved in cross-contamination of *A. baumannii* between patients during outbreaks. *A. baumannii* is the model organism primarily used for investigation of the mechanisms that play a role in the success of *Acinetobacter* species as human pathogens. Despite being known predominantly for its high level of multidrug resistance, *A. baumannii* has a considerable level of virulence (Bergogne-Berezin and Towner 1996; Gordon and Wareham 2010). Analyses of the fully sequenced *A. baumannii* genomes have provided a wealth of information on the virulence potential of *A. baumannii*. Genes encoding pili, capsule components, iron uptake mechanisms and many other virulence determinants are present in most fully sequenced clinical isolates (Adams *et al.* 2008; Fournier *et al.* 2006; Iacono *et al.* 2008; Smith *et al.* 2007; Vallenet *et al.* 2008). However, there is no evidence for the

production of soluble toxins or cytolysins by *A. baumannii* (Gordon and Wareham 2010).

1.2.1 Iron acquisition mechanisms

1.2.1.1 The importance of iron acquisition for pathogens

Iron plays a crucial role in many cellular processes, such as metabolism, cell-to-cell signalling and biofilm formation (Braun and Braun 2002; Hazan *et al.* 2010). Soluble iron (Fe^{2+}) is available in limited quantities in many environments. Consequently, ongoing competition for this micronutrient takes place, both in human host niches and on abiotic surfaces, such as medical equipment. Therefore, iron acquisition from the environment is crucial for persistence of pathogens such as *A. baumannii*. Most clinical *A. baumannii* strains have the ability to grow under iron-limiting conditions (Dorsey *et al.* 2003a). Immunoblot analysis using serum from patients with an *Acinetobacter* infection showed that the iron acquisition mechanisms are being expressed during a state of infection, suggesting that these mechanisms play a role in virulence (Smith and Alpar 1991).

1.2.1.2 Siderophore-mediated iron acquisition

A. baumannii cells express outer membrane proteins (OMPs) that directly bind iron or heme from the extracellular space (Clarke *et al.* 2001; Koster 2005). However, most *A. baumannii* strains also possess a more active mechanism for iron acquisition, involving siderophores. These molecules are synthesised in the cell and then secreted into the extracellular space where they scavenge for available iron. Once iron has been bound, receptors presented on the cell surface recognise the siderophores allowing for their uptake. Receptors recognising foreign siderophores have also been identified, increasing the competition for iron (Plessner *et al.* 1993; Poole and McKay 2003). The uptake of iron-bound siderophores is mediated by the TonB-ExbB-ExbD energy transduction system (Braun 1995; Braun 2003).

A second very similar iron uptake system, that utilises hemophores instead of siderophores, exists for extracellular scavenging of iron (Cescau *et al.* 2007). However, to date, the importance of hemophores in *A. baumannii* is largely unknown.

1.2.1.3 Iron acquisition in A. baumannii

Although considered adaptable to low-iron conditions, *Acinetobacter* species show major differences in their iron acquisition mechanisms (Antunes *et al.* 2011; Dorsey *et al.* 2003a). The three types of iron uptake mechanisms described above have been identified in the *A. baumannii* genome, including ferrous iron or heme receptor proteins, siderophore-mediated uptake mechanisms and hemophore utilisation mechanisms (Antunes *et al.* 2011; Zimbler *et al.* 2009). The most thoroughly studied *A. baumannii* siderophore biosynthesis gene cluster is responsible for synthesis of acinetobactin, which displays a high affinity for iron (Dorsey *et al.* 2004; Yamamoto *et al.* 1994). A biosynthesis protein (BasD), a receptor (BauA) and an uptake protein (BauD) within the acinetobactin biosynthesis cluster, have been characterised by means of transposon insertion disruption of the respective genes (Dorsey *et al.* 2004). The role of a second siderophore biosynthesis gene cluster in iron acquisition, identified in *A. baumannii* strain 8399, was determined by heterologous expression in *Escherichia coli* (Dorsey *et al.* 2003b). A third putative siderophore biosynthesis cluster was identified in *A. baumannii* strain ATCC 17978 and examination of transcription levels showed that a gene within this cluster was responsive to iron-limiting conditions (Zimbler *et al.* 2009). Although hemophore biosynthesis pathways do not appear to be widely distributed throughout *A. baumannii* strains, exogenous hemophores can most likely be recognised and reduced for iron acquisition by most strains. Despite identification of a ferric uptake regulator (FUR) (Daniel *et al.* 1999), little is known about the regulatory network involved in *A. baumannii* iron homeostasis. The FUR protein dimerises in the presence of iron and in this state is capable of binding to palindromic sequences known as the FUR binding sites or FUR boxes (Stojiljkovic *et al.* 1994). In this dimeric state, FUR acts as a transcriptional repressor when bound in the regulatory region of a gene. In the absence of iron, FUR dimers dissociate from the DNA and can no longer repress transcription; this allows expression of genes, normally repressed by FUR, to occur, which often includes numerous iron uptake related genes.

1.2.2 Adherence to abiotic surfaces and biofilm formation

1.2.2.1 Persistence of A. baumannii *in the hospital environment*

A. baumannii is a common coloniser of surfaces in the hospital environment, including keyboards, pillows, gloves, beds and medical equipment (Bureau-Chalot *et*

al. 2004; Cefai *et al.* 1990; Neely 2000; Weernink *et al.* 1995). Prolonged survival on dry surfaces and high levels of resistance contribute to persistence of *A. baumannii* and complicates eradication during outbreaks. Cross-contamination is predominantly linked to inadequate hand hygiene practices by medical staff (Dijkshoorn *et al.* 2007; Garlantezec *et al.* 2011; Markogiannakis *et al.* 2008). Therefore, as well as ward decontamination, implementation of stringent hygiene regimes is required for successful eradication of *A. baumannii* outbreaks, as previously described in a neonatal ward (Chan *et al.* 2007). After implementing these measures, all environmental samples in this ward were negative, except for ventilator tubing used for a patient with *A. baumannii* positive sputum. Adherence of *A. baumannii* on surfaces of medical equipment, such as ventilators or catheters, is common and can be related to pneumonia, urinary tract infections and bacteraemia (Jang *et al.* 2009; Raad *et al.* 2008). Colonisation of these surfaces may form reservoirs, as these niches are not exposed to the antimicrobial agents administered to the patients. Therefore, intubation or catheterisation can result in prolonged exposure of the patient to the pathogen.

It has been proposed that a specific form of biofilm, known as the pellicle, is most relevant in persistence of *A. baumannii* in environments other than human host niches (Marti *et al.* 2011). Pellicle formation apparent on top of a liquid is more pronounced at room temperature (RT) as compared to incubation at 37°C. It has also recently been shown that biofilm formation and pellicle formation are not directly correlated when investigating different strains (McQueary and Actis 2011).

Secretion and over-production of extracellular compounds by cells in a biofilm provide protection against environmental stress factors. A variety of studies have shown a correlation between biofilm formation and increased drug resistance. For example, MDRAB isolates often show higher levels of biofilm formation than their susceptible counterparts (King *et al.* 2009; Lee *et al.* 2008; Rajamohan *et al.* 2009; Rao *et al.* 2008; Rodriguez-Bano *et al.* 2008). Furthermore, proteins involved in drug resistance, including beta-lactamases and aminoglycoside acetyltransferases, were found to be overexpressed in biofilm cells (Shin *et al.* 2009). This indicates that expression of virulence and resistance mechanisms may be controlled by similar regulatory pathways. Accordingly, increased biofilm formation as a result of exposure to sub-inhibitory concentrations of antimicrobial compounds has also been observed (Nucleo *et al.* 2009).

Two *A. baumannii* regulatory mechanisms involved in biofilm formation have been identified; BmfRS, a two-component regulatory system (Gaddy and Actis 2009; Shin *et al.* 2009; Tomaras *et al.* 2008), and acyl homoserine lactones which are intercellular communication molecules of the quorum-sensing (QS) signalling pathway (Niu *et al.* 2008). AbaI, an autoinducer synthase, is the only protein involved in *A. baumannii* QS that has been functionally investigated (Niu *et al.* 2008). In the much more widely studied model biofilm organisms, such as *P. aeruginosa*, regulators, such as GacAS, PsrA and CbrA, function in multiple processes including biofilm formation, resistance and chemotaxis (Gooderham *et al.* 2008; Hassan *et al.* 2010; Yeung *et al.* 2011). Considering the importance of colonisation, persistence and disease initiation, the regulatory pathways of *A. baumannii* virulence determinants are severely understudied compared to that of *P. aeruginosa*.

1.2.2.2 Various mechanisms contribute to abiotic surface adherence and biofilm formation

The life of a bacterial biofilm is extremely complex and goes through multiple stages, from initial surface adherence to biofilm maturation and seeding dispersal (Webb *et al.* 2003). Initial adherence to abiotic surfaces is often mediated by pili or other protein structures, indeed, insertion disruption of either a type I pili clusters or outer membrane protein A (OmpA) results in altered biofilm levels (Gaddy *et al.* 2009; Gohl *et al.* 2006; Tomaras *et al.* 2003). The biofilm-associated protein (Bap) plays a role in maturation of the *A. baumannii* biofilm, which has been investigated using confocal microscopy (Loehfelm *et al.* 2008; Rahbar *et al.* 2010). Bap was first identified in *Staphylococcus aureus*, where it was shown to also play a role in virulence (Lasa and Penades 2006). Finally, production of poly-β-(1-6)-*N*-acetylglucosamine (PNAG), a polysaccharide, appears critical for *A. baumannii* biofilm formation (Choi *et al.* 2009). Disruption of the *pgaABCD* gene cluster resulted in an almost complete loss of biofilm mass, potentially due to lack of intercellular interaction as a result of the loss of this polysaccharide. Clinical *Acinetobacter* strains show major differences in their levels of biofilm formation (Cevahir *et al.* 2008; de Breij *et al.* 2010; Lee *et al.* 2008). This strain-to-strain variation has complicated investigation of the molecular mechanisms behind the formation of *A. baumannii* biofilms and the mechanisms responsible for driving

populations into these complex lifestyles. Whether the molecular mechanisms described above are conserved across most isolates remains to be examined.

1.2.3 The interaction between *A. baumannii* and eukaryotic cells

Adherence to eukaryotic cells is a crucial step for colonisation of host tissues and can therefore be considered one of the first steps in disease progression. The ability of clinical *A. baumannii* isolates to adhere to biotic surfaces has been investigated in various cell culture experiments. Human lung epithelial cells, such as A549 pneumocytes (Talbot *et al.* 1996), are commonly used to study interactions of *A. baumannii* with eukaryotic cells. Lung epithelial cells are the primary colonisation site for *A. baumannii* when developing a respiratory tract infection (Gaddy *et al.* 2009; March *et al.* 2010; McConnell and Pachon 2011). Major differences in adherence potential exist across clinical isolates, as seen in biofilm formation assays (Section 1.2.2). Interestingly, the binding of an *A. baumannii csu* type I pili cluster mutant to lung epithelial cells was unaltered despite an observation of reduced biofilm formation by this strain (de Breij *et al.* 2009; Tomaras *et al.* 2003). Furthermore, a direct correlation between adherence to abiotic and biotic surfaces could not be established in clinical *A. baumannii* isolates (Costa *et al.* 2006; de Breij *et al.* 2010; Lee *et al.* 2008). Differences have also been observed between the level of biofilm formation and the hemaglutination of human group AB erythrocytes by clinical *A. baumannii* isolates (Braun and Vidotto 2004). These results strengthen the notion that different molecular mechanisms are involved in adherence to distinct surfaces.

A rat pneumonia and soft tissue model was established to investigate tissue damage and survival of a variety of *A. baumannii* strains (Russo *et al.* 2008). Both penicillin binding protein-7/8 and production of K1 capsule in *A. baumannii* were shown to play an important role in protection against antimicrobial compounds present in human ascites fluid (fluid from the peritoneal cavity in pathologic state), human serum and soft tissues in rat (Russo *et al.* 2010; Russo *et al.* 2009). Diminished growth in human serum has also been observed in a phospholipase D *A. baumannii* mutant strain (Jacobs *et al.* 2010). These results indicated that penicillin binding protein-7/8, K1 capsule and phospholipase D assist in survival in the host environment. Intranasal inoculation of mice with wild-type (WT) or phospholipase D mutant strains did not result in differential levels of bacterial burden in the lung. However, the number of *A. baumannii* phospholipase D mutant cells in

blood, liver and heart tissues was significantly lower, showing that phospholipase D may also play a role in crossing the blood-lung barrier (Jacobs *et al.* 2010).

Lipopolysaccharides (LPSs) are involved in many different bacterial processes, including resistance, adherence and virulence. In *A. baumannii*, LPS has been shown to provide resistance against human serum and therefore potentially promoting *in vivo* survival (Luke *et al.* 2010). The structure of various *A. baumannii* LPSs have been determined (Haseley *et al.* 1998; Haseley and Wilkinson 1996; MacLean *et al.* 2009) and appear to be well conserved within clonal groups, such as the international clone I strains (Pantophlet *et al.* 2001). Nevertheless, selective pressure can result in structural alteration of LPSs and may subsequently affect susceptibility patterns (Section 1.3.5.1). To date, there is scare evidence about the virulence potential or immunostimulating properties of the *A. baumannii* LPS.

Another potential virulence factor is the type VI secretion system, however, its function remains somewhat obscure (Jani and Cotter 2010). Although originally investigated in relation to pathogenicity by means of host cell invasion assays (Mougous *et al.* 2006), the ubiquitous presence in both pathogenic and non-pathogenic bacteria indicates that these protein structures fulfil multiple functions (Jani and Cotter 2010). Recently, type VI secretion systems have been proposed to play a role in type I pili regulation and inter-bacterial communication (de Pace *et al.* 2010; Schwarz *et al.* 2010a; Schwarz *et al.* 2010b). Although gene clusters encoding type VI secretion systems can be found in most *A. baumannii* genomes, to date, their function in *A. baumannii* has not been described.

One of the most extensively studied virulence proteins in *A. baumannii* is OmpA, which was also found to be involved in adherence to abiotic surface, as described in Section 1.2.2.2. OmpA does not only facilitate adherence to eukaryotic cell surfaces, it also mediates invasion, as seen in an *A. baumannii* OmpA deletion mutant which showed 30% reduction in its ability to invade eukaryotic cells (Choi *et al.* 2008; Gaddy *et al.* 2009). OmpA has also been shown to promote cell death of lung epithelial cells by induction of interleukin-8 and other cytokines (Choi *et al.* 2005; McConnell and Pachon 2011; Ofori-Darko *et al.* 2000).

Interestingly, cytokine production by either human bronchial cells or type I and II macrophages was in most cases found to be significantly lower when cells were incubated with *A. baumannii* compared to *A. junii* clinical isolates (de Breij *et al.*

2010). Furthermore, pan-resistant *A. baumannii* isolates showed lower levels of cytotoxicity as compared to susceptible *A. baumannii* strains in human lung cells (Smani *et al.* 2011). The immune evasion of *A. baumannii* cells observed in these studies may contribute to its success as a human pathogen.

1.2.4 *Acinetobacter* motility characteristics

1.2.4.1 The different forms of bacterial motility

The five different types of bacterial motility currently recognised are swimming, swarming, twitching, gliding and sliding (Kearns 2010). Swimming motility is known as flagella-mediated migration through liquid media. Propulsion of the bacterium is facilitated by the motor-like movement at the membrane anchor end of the flagella. Swimming is required for bacteria to be classified as motile. The other forms of motility take place on surfaces (Henrichsen 1972; Kaiser 2007; Kearns 2010; Mattick 2002; Semmler *et al.* 1999). The most widely applied classification scheme is based predominantly on the molecular mechanism responsible for movement, rather than phenotypic characteristics. Briefly, these forms of motility can be specified as; swarming is mediated by flagella (Henrichsen 1972; Kearns 2010; Overhage *et al.* 2007), twitching by pulling using type IV pili (De La Fuente *et al.* 2007; Mattick 2002; Nudleman and Kaiser 2004), gliding by focal-adhesion complexes (Mauriello *et al.* 2010; Mignot 2007) and sliding by the production of surfactant (Henrichsen 1972). Kaiser (2007) proposed using the term swarming for all forms of bacterial translocation in the fluidic layer that forms at the surface/air interface (Kaiser 2007). The definition of twitching proposed by Semmler *et al.* (1999) was also based on phenotypic characteristics. Instead of migration on top of a surface as seen in swarming motility, twitching was defined as migration in the surface/plastic interface of solid agar plates (Semmler *et al.* 1999).

1.2.4.2 The role of motility in virulence

The role of flagella-mediated motility in bacterial pathogenesis has been well documented (Josenhans and Suerbaum 2002). However, the role of the other forms of motility in virulence has not been studied as extensively. We can learn from knowledge gained about plant pathogens, such as *Acidovorax citrulli*, which require the type IV pili for twitching motility, biofilm formation, and subsequently virulence (Bahar *et al.* 2009). Flow-chamber experiments confirmed that *A. citrulli* twitching motility facilitates migration against the flow of xylem, allowing the bacterium to colonise different parts of the plant (Bahar *et al.* 2010). Similar observations of

twitching motility and virulence have been made in the non-flagellated plant pathogen *Xylella fastidiosa* (Meng *et al.* 2005).

A direct correlation between the lack of twitching motility and reduced virulence has been shown for *Dichelobacter nodosus*, the cause of ovine foot rot. Mutations in either the *pilU* or *pilT* determinants, which encode type IV pili subunits, resulted in lack of virulence (Han *et al.* 2008). PilT plays a role in retraction of the type IV pili and is therefore responsible for the pulling force required for twitching motility, the role of PilU remains largely unknown. A link between *P. aeruginosa* twitching motility and corneal infections has also been recognised, in which the highly variable major fimbrial subunit, PilA, appears to play a role (Winstanley *et al.* 2005; Zolfaghar *et al.* 2003). Amino acid sequence homology analyses have been performed on PilA from a wide range of isolates, including various strains isolated from corneal infections. Interestingly, the PilA sequences from *P. aeruginosa* isolates that caused corneal infections were found to be highly homologous (Stewart *et al.* 2011). Type IV pili mediated migration has also been shown to be essential for the mushroom-like structure of the *P. aeruginosa* biofilm (Klausen *et al.* 2003); type IV pili mutants showed underdeveloped biofilms, as the non-motile cells only create the lower section of the biofilm structure.

Comparing swarming and non-motile *P. aeruginosa* cells by means of transcriptome analysis revealed low expression of genes functioning in metabolism and virulence in the swarming cells compared to their non-motile counterparts in the centre of the agar plate (Tremblay and Deziel 2010). Migrating cells were considered as scouts, which express antigenic features at lower levels to avoid recognition by the host and allow successful colonisation of new sites.

1.2.4.3 Motility of Acinetobacter species

Acinetobacter species are traditionally known as non-motile bacteria, in fact, ‘acineto’ is derived from the Greek work for akinetic, or non-motile. This is related to the inability of *Acinetobacter* to actively migrate through media, due to the lack of flagella (Baumann *et al.* 1968). However, other forms of *Acinetobacter* motility have been described (Barker and Maxted 1975; Henrichsen 1984; Henrichsen and Blom 1975; Mukerji and Bhopale 1983; Mussi *et al.* 2010). A large gap exists between earlier reports on *Acinetobacter* motility and more recently published studies. In the 1970s, Henrichsen published several articles on flagella-independent bacterial

translocation, including that of *Acinetobacter* (Henrichsen 1972; Henrichsen 1975; Henrichsen and Blom 1975; Henrichsen *et al.* 1972). Difficulties determining *Acinetobacter* to species level at that time made it unclear whether *A. baumannii* was considered motile or if this feature was restricted to *A. calcoaceticus*. Only recently, motility of the fully sequenced *A. baumannii* strain ATCC 17978 was shown (Mussi *et al.* 2010). In this study, Mussi *et al.* (2010) showed that blue light had an inhibitory effect on motility, which appeared to be regulated by the blue light sensing protein BlsA (Mussi *et al.* 2010). A more recent attempt at linking cell surface hydrophobicity, presence of the Csu-type I pili, biofilm formation, pellicle formation and motility by several *A. baumannii* isolates showed that the molecular mechanisms responsible for motility by *A. baumannii* remains unknown (McQueary and Actis 2011). From earlier studies it appeared that a correlation existed between the presence of surface structures, such as pili or fimbriae, and motility exhibited by *Acinetobacter* (Henrichsen and Blom 1975). For that reason, *Acinetobacter* translocation was classified as twitching motility by Henrichsen. A recent study, using an insertion disruption mutant of *pilT*, has confirmed the role of type IV pili in *A. baumannii* twitching motility (Clemmer *et al.* 2011).

1.3 *A. baumannii* contains a broad arsenal of resistance mechanisms

Acinetobacter species are notorious for having an exceptionally high level of resistance to antimicrobial compounds. Epidemiological studies and investigation of the prevalence of multidrug resistant isolates has also revealed that this problem is growing worldwide (Garnacho-Montero and Amaya-Villar 2010; Goel *et al.* 2011; Michalopoulos and Falagas 2010; Neonakis *et al.* 2011). As seen in other bacteria, *A. baumannii* employs multiple mechanisms for dealing with foreign toxic compounds, such as antibiotics, disinfectants and antiseptics (Bonomo and Szabo 2006). Throughout the last two decades, many studies have been conducted to delineate these mechanisms and their contribution to resistance in clinical isolates (Gordon and Wareham 2010; Zavascki *et al.* 2010).

1.3.1 Antibiotic modifying enzymes

Enzymatic modification of an antimicrobial agent often results in a high level of drug resistance. Most clinical *A. baumannii* isolates harbour multiple genes encoding these resistance determinants. For example, the aminoglycoside modifying enzymes

are associated with high levels of resistance to widely used antibiotics, such as gentamicin and amikacin. Different classes of aminoglycoside modifying enzymes, such as acetyltransferases, phosphotransferases and nucleotidyltransferases, have been identified in *A. baumannii* (Nemec *et al.* 2004b). The serine-active-site cephalosporinase (Class C) and oxacillinase (Class D) beta-lactam hydrolysing enzymes, and the metallo-beta-lactamases (Class B) are a common cause of multidrug resistance in *A. baumannii* (Bonomo and Szabo 2006; Nordmann and Poirel 2002; Poirel *et al.* 2010; Poirel and Nordmann 2006). The extended-spectrum beta-lactamases have also been commonly identified and can either be mutationally derived from narrow-spectrum beta-lactamases or horizontally acquired via mobile genetic elements (Falagas and Karageorgopoulos 2009). The increasing prevalence of *A. baumannii* isolates harbouring extended-spectrum beta-lactamases is a major concern in the clinical setting (Bogaerts *et al.* 2010; Bonnin *et al.* 2011a; Bonnin *et al.* 2011b; Moubareck *et al.* 2009).

1.3.2 Resistance as a result of genetic mutations

Fluoroquinolones, such as norfloxacin and ciprofloxacin, can be rendered ineffective by genetic mutations of the bacterial drug target sites. A correlation between fluoroquinolone resistance and mutation of serine-83 to leucine in GyrA and serine-80 to leucine in ParC has been established, as these mutation are commonly identified in fluoroquinolone resistant *A. baumannii* isolates (Golanbar *et al.* 2011; Higgins *et al.* 2010; Higgins *et al.* 2004; Sheng *et al.* 2009; Vila *et al.* 1995; Wisplinghoff *et al.* 2003). Overexpression of *adeB*, a gene encoding a major *A. baumannii* efflux pump, as a result of mutations in a regulatory protein (Section 1.3.4.5), has also been linked to fluoroquinolone resistance (Higgins *et al.* 2004; Pannek *et al.* 2006).

1.3.3 Resistance due to loss of porin proteins

There are two primary pathways for antimicrobial compounds to pass through the outer membrane of Gram-negative bacteria. Lipid-mediated translocation for hydrophobic compounds or diffusion through porin proteins for hydrophilic compounds. The latter plays an important role in antibiotic resistance, as absence of these membrane proteins or transcriptional down-regulation of the respective genes results in decreased permeability (Delcour 2009). The first report in which an *A. baumannii* outer membrane porin protein was associated with drug resistance

involved a 33 - 36 kDa protein (Clark 1996). Despite being imipenem resistant, beta-lactam hydrolysis was not observed in the strains investigated and further analysis of proteins in the outer membrane indicated absence of a porin protein. Similarly, loss of a 29 kDa porin protein was linked to imipenem resistance in strains lacking detectable carbapenemase activity (Limansky *et al.* 2002). Molecular characterisation of various *A. baumannii* porins has since been carried out and indeed, a significant role predominantly in imipenem resistance has been established (Bou *et al.* 2000; del Mar Tomas *et al.* 2005; Dupont *et al.* 2005; Limansky *et al.* 2002).

1.3.4 Efflux-mediated resistance

Efflux systems function in numerous processes in the cell, such as transport of secondary metabolites, metabolic waste products or foreign toxic compounds (Martin *et al.* 2005). Membrane transport proteins that expel antimicrobial compounds from the cytosol to the periplasm or extracellular space are important resistance mechanisms for many bacteria (Poole 2004). These membrane proteins may have specificity for a particular compound, like the tetracycline transporters (Guay and Rothstein 1993). However, many have an extended substrate profile, where both structurally and chemically distinct compounds can be substrates of the same protein and can include antiseptics, disinfectants, dyes, antibiotics, detergents and antimicrobial peptides. Throughout evolution, efflux pumps have adapted to the introduction of novel antibiotics. Therefore, it is often only a matter of time until clinical strains are discovered that harbour efflux pumps with the ability to expel novel drugs. Drug efflux pumps have been divided into five families based predominantly on their phylogenetic differences (Paulsen *et al.* 1996; Saier and Paulsen 2001) (www.tcdb.org). These include the multidrug and toxic compound extrusion (MATE) family, small multidrug resistance (SMR) family, resistance-nodulation-cell division (RND) superfamily, major facilitator superfamily (MFS) and ATP-binding cassette (ABC) superfamily (Figure 1.1).

Membrane transporters of the same family may exhibit different substrate profiles, regardless of the level of sequence identity observed (Yu *et al.* 2003). Therefore, individual putative transporter proteins require functional characterisation using experimental approaches such as insertional inactivation or heterologous overexpression. Analysis of the genome of a number of fully sequenced MDRAB strains revealed a high number of putative drug efflux pumps. For example, 52 have

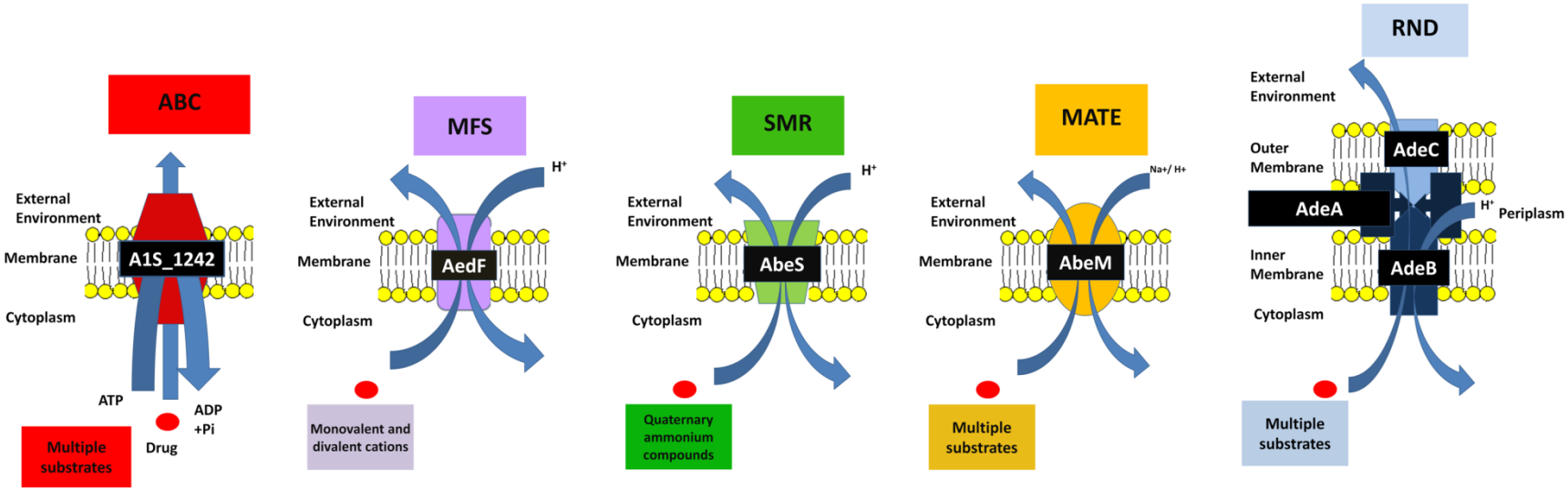

Figure 1.1: The five families of drug transporters

The drug transporter proteins are inserted in the lipid bilayer (yellow circles with tails) representing the cell membrane. The transporters can be divided into five different families based on sequence identity and secondary structure prediction; ATP binding cassette (ABC), major facilitator superfamily (MFS), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE) and resistance-nodulation-cell division (RND). Examples of *A. baumannii* drug transporters have been used in the figure and common substrate characteristics have been specified under the respective efflux system. Although A1S_1242 has not been characterised, ABC transporters from other bacterial species often possess a broad substrate spectrum. Members of the MFS, SMR, MATE and RND families utilise the ion gradient (H^+ or Na^+) across the inner membrane for substrate translocation. The ABC transporters use energy obtained from ATP hydrolysis. The RND protein complex is comprised of an inner membrane protein (AdeB), an outer membrane protein (AdeC) and a membrane fusion protein (AdeA). Figure modified with permission from S Baltzer.

been identified in *A. baumannii* ATCC 17978 (www.membranetransport.org). Some of these *A. baumannii* drug transporter proteins have been functionally characterised, including; AbeS, an SMR member (Srinivasan *et al.* 2009), AmvA (Rajamohan *et al.* 2010), CraA (Roca *et al.* 2009), AedC (Hassan *et al.* 2011), AedF (Hassan *et al.* 2011) and Tet(A) (Ribera *et al.* 2003) from the MFS, the MATE pump AbeM (Su *et al.* 2005), and AdeB (Magnet *et al.* 2001), AdeE (Chau *et al.* 2004) and AdeG (Coyne *et al.* 2010a; Coyne *et al.* 2010b) from the RND superfamily. The presence of various genes encoding other tetracycline efflux pumps has been described, however, only limited functional characterisation of the encoded proteins has been carried out (Guardabassi *et al.* 2000; Huys *et al.* 2005).

1.3.4.1 The ATP-binding cassette superfamily

Transport protein members of the ABC superfamily fulfil diverse functions, such as drug efflux and transportation of siderophores. The significant role of ABC transporters in mediating resistance to chemotherapeutic agents in cancerous cells has also been comprehensively studied (Perez-tomas 2006). Structural studies have shown that the group of ABC drug exporters such as the human P-glycoprotein (Crowley and Callaghan 2010) and staphylococcal Sav1866 (Dawson and Locher 2006) are comprised of 12 alpha-helical transmembrane segments (TMSs) and two cytoplasmic nucleotide binding domains. Energy for structural alteration of the protein complex required for substrate translocation is obtained from hydrolysis of adenosine triphosphate (ATP), which is facilitated by the nucleotide binding sites. The ABC transporters can have a broad substrate spectrum and therefore play an important role in multidrug resistant organisms. To date, *A. baumannii* ABC drug transporters have not been functionally characterised.

1.3.4.2 The major facilitator superfamily

The MFS is a large group of transporters comprised of at least 50 families, which are involved in many different cellular processes. The MFS subfamily of drug:H^+ antiporter (DHA) proteins are mainly responsible for resistance to antimicrobials (Hassan *et al.* 2007; Lewinson *et al.* 2006). As the name indicates, the proton gradient across the membrane is required for active transport of substrates. Proteins in the DHA1 and DHA3 MFS subfamilies contain 12 TMSs and those in the DHA2 subfamily contain 14 (Hassan *et al.* 2007). Various well characterised DHA members have an extensive substrate profile, such as the staphylococcal QacA protein (Hassan *et al.* 2007). However, the tetracycline specific efflux systems, such

as Tet(A) and Tet(B), are also part of the DHA subfamily. All six DHA2 members identified in the currently available *A. baumannii* genome sequences have been functionally examined (Hassan *et al.* 2011; Rajamohan *et al.* 2010). Two of these, AedC and AedF (and its orthologue AmvA), are confirmed multidrug transporters, as both proteins confer resistance to multiple distinct compounds. The DHA1 member CraA showed specificity for chloramphenicol, although complementation in a CraA null mutant also resulted in increased resistance to tetracycline (Roca *et al.* 2009). The *tet(A)* gene and its regulator *tetR(A)* were identified in an *A. baumannii* genomic segment which was examined in *E. coli* by tetracycline resistance analysis (Ribera *et al.* 2003). Interestingly, *tet(A)* and *tetR(A)* were located in a mobile genetic element within the analysed DNA fragment. The significance of the tetracycline resistance proteins has been investigated mainly by correlative studies in which predominantly *tet(A)* and *tet(B)* were linked to tetracycline resistance in clinical *A. baumannii* isolates (Guardabassi *et al.* 2000; Huys *et al.* 2005).

1.3.4.3 The small multidrug resistance family

SMRs are the smallest known bacterial drug transporters (100 – 140 amino acids) and the alpha-helices transverse the inner membrane 4 times. Substantial evidence exists for oligomerisation of SMRs, which creates a protein complex containing of up to 16 TMSs (Bay and Turner 2009; Chung and Saier 2001; Korkhov and Tate 2008; Nara *et al.* 2007; Poulsen *et al.* 2009). The topology of the SMR oligomers is, after many years of debate, still unclear (Fleishman *et al.* 2006; Nara *et al.* 2007). Various SMR efflux pumps have been functionally characterised and substrates include primarily antiseptics and quaternary ammonium compounds, such as chlorhexidine and benzalkonium, respectively. Like members of the MFS, substrate translocation in SMR proteins is facilitated by the proton motive force. Three SMR subgroups have been described; the suppressor of *groEL* (Sug)-like proteins, the small multidrug proteins (SMP) and the paired-SMRs (PSMR). These groups possess differences in structure and function and can be distinguished using well defined amino acid signature sequences (Bay *et al.* 2008). The *E. coli* SMR member SugE expresses a chaperonic system that plays a role in suppressing mutations of *groEL* (Greener *et al.* 1993). The Sug-like proteins play a limited role in drug resistance. Although SMRs of both other subgroups have shown to play a significant role in drug resistance, they differ in their oligomerisation characteristics. Whereas SMPs form homo-dimers, the PSMRs are transcribed from two different

genes and form hetero-dimers. *A. baumannii* AbeS, which was functionally characterised by insertional inactivation and heterologous expression in *E. coli*, is a member of the SMP subgroup and confers resistance to various compounds including sodium dodecyl sulphate (SDS) and ethidium (Srinivasan *et al.* 2009).

1.3.4.4 The multidrug and toxic compound extrusion family

The MATE pumps were initially characterised as proteins belonging to the MFS, as they typically possess 12 TMSs (Morita *et al.* 1998). However, amino acid sequence alignments and phylogenetic analysis showed significant dissimilarity of NorM from *Vibrio parahaemolyticus*, and YdhE, a homologue in *E. coli*, which resulted in the proposal of the MATE family (Brown *et al.* 1999). Sequences homologous to members of the MATE family can be found in all kingdoms of living organisms (Omote *et al.* 2006).

MATE proteins play an important role in the adaptation of bacterial multidrug resistance which has been confirmed in numerous knockout studies (Kuroda and Tsuchiya 2009). Several studies have shown that MATE proteins have a wide range of substrates, which comprises antibiotics from different classes (often including quinolones), antiseptics and cationic dyes, such as ethidium and Hoechst 33342 (He *et al.* 2004; Su *et al.* 2005). Interestingly, a study on the rat rMATE1 protein showed that this protein, which shares a low level of homology with bacterial MATE proteins, also has the ability to secrete quinolones (Ohta *et al.* 2009).

At first, only Na^+ antiporter MATE proteins were identified, including NorM, YdhE and all characterised *Vibrio* MATE members (Begum *et al.* 2005; Chen *et al.* 2002; Huda *et al.* 2003; Huda *et al.* 2001; Xu *et al.* 2003). Later, two MATE proteins, PmpM of *P. aeruginosa* and AbeM of *A. baumannii*, were found to be H^+ antiporters (He *et al.* 2004; Su *et al.* 2005). A molecular differentiation between using either H^+ or Na^+ as a driving forces has not been established. The high-resolution structure of NorM from *Vibrio cholerae* suggested that structural plasticity in the protein during antiportation allows active secretion of substrates (He *et al.* 2010).

AbeM from *A. baumannii* strain ATCC 19606 was studied in recombinant *E. coli* (Su *et al.* 2005). AbeM shares 77%, 76% and 75% similarity with PmpM from *P. aeruginosa* (He *et al.* 2004), YdhE from *E. coli* (Morita *et al.* 1998) and NorM from *Vibrio paraheamolyticus* (Morita *et al.* 2000), respectively. The

recombinant *E. coli* cells expressing AbeM exhibited increased resistance to a wide range of compounds and thus suggested that AbeM is a multidrug efflux pump. Similar to the substrate profiles of other characterised MATE pumps, substrates of AbeM are chemically and structurally distinct and include fluoroquinolones, several dyes, such as 4',6-diamidino-2-phenylindole (DAPI), Hoechst 33342, acriflavine and ethidium, and other toxic compounds, such as doxorubicin and daunorubicin. A link between drug resistance in clinical *A. baumannii* strains and *abeM* expression levels was not established (Bratu *et al.* 2008). Therefore, the significance of AbeM as a drug transporter in *A. baumannii* requires further investigation.

1.3.4.5 The resistance-nodulation-cell division superfamily

RND efflux systems are considered the most significant multidrug transporters, especially in Gram-negative bacteria. The extended substrate profile and the ability to transport toxic compounds to the extracellular space contribute to their importance. Furthermore, mutations in the regulators or regulatory elements have been associated with high expression levels of RND genes. In clinical *A. baumannii* isolates, AdeB has arisen as the most significant transporter, as high levels of *adeB* expression have been correlated with resistance to ciprofloxacin, cefepime and tigecycline (Bratu *et al.* 2008; Higgins *et al.* 2004; Pannek *et al.* 2006; Ruzin *et al.* 2007; Sun *et al.* 2010).

In Gram-negative bacteria, the genes encoding RND proteins are often found in an operon with two other genes encoding a membrane fusion protein (MFP) and an OMP. The functional protein complex is an assembly of the three different components spanning across both the inner and outer membranes (Figure 1.1). The RND and OMP are believed to form trimers (Nikaido and Takatsuka 2009; Pos 2009b). The periplasmic terminus of the RND is connected to the OMP and together forms a channel-like structure. The connection between the RND and OMP is stabilised by the MFP (Figure 1.1). However, the MFP is also involved in the conformational changes during active transport (Murakami 2008). The fully assembled complex is often simply called an RND pump, however, MFP/OMP complexes with members of the ABC superfamily or MFS have also been identified (Tanabe *et al.* 2009; Tikhonova *et al.* 2009). Many studies have proven that these efflux systems play an important role in bacterial multidrug resistance (Damier-Piolle *et al.* 2008; Magnet *et al.* 2001; Poole 2004; Srikumar *et al.* 1998).

After publication of the crystal structure of AcrB (Murakami *et al.* 2002), a major RND member of *E. coli*, the focus shifted to elucidation of the mode of transport behind this complex efflux system (Misra and Bavro 2009; Murakami 2008; Nikaido and Takatsuka 2009). Unliganded AcrB forms symmetrical trimers, however, different conformations of AcrB were observed in crystal structures with bound ligands (Murakami 2008; Seeger *et al.* 2006; Sennhauser *et al.* 2007) and were defined as tight, loose and open (Seeger *et al.* 2006), or access, binding and extrusion (Murakami 2008). Both research groups described a functional rotation of the AcrB monomers during drug transportation, from tight > loose > open > tight > and so forth. The MFP plays a role in determination of substrate specificity, potentially by structurally altering the substrate-binding vestibule in the pore domain of the RND protein (Pos 2009b). The entrance of this vestibule is positioned 15 Å above the inner membrane and runs diagonally through the pore domain to the central periplasmic terminus, from where the substrates can be secreted into the extracellular space via the OMP (Murakami 2008; Pos 2009a). The vestibule plays a crucial role in substrate recognition, binding and translocation to the inner channel-like space.

To date, four *Acinetobacter* RND proteins have been characterised, AdeB, AdeE, AdeJ and AdeG (Chau *et al.* 2004; Coyne *et al.* 2010a; Coyne *et al.* 2010b; Damier-Piolle *et al.* 2008; Magnet *et al.* 2001), however, only AdeB, AdeJ and AdeG can be found in *A. baumannii* (Coyne *et al.* 2010b; Lin *et al.* 2009). Although most studies describe the importance of AdeB in multidrug resistant *A. baumannii*, the findings for all three RNDs in knockout and overexpression studies indicate that AdeJ has a resistance potential as great as AdeB (Coyne *et al.* 2011).

1.3.5 The need for development of novel antimicrobial therapies

The current knowledge about the effectiveness of drugs against MDRAB is predominantly based on published case studies and *in vitro* examination of the minimal inhibitory concentration (MIC) values. Unfortunately, controlled clinical trials have never been performed (Dijkshoorn *et al.* 2007). The definition of multidrug resistance varies between studies, which leads to confusion when summarising results (Falagas *et al.* 2006). Regardless, drug resistance of *A. baumannii* isolates against a number of different classes of antibiotics is common. Although resistance mechanisms against practically all antibiotics have been identified, pan-resistant *A. baumannii* strains are rarely isolated. Prediction of the

appropriate antibiotic therapy is required when adequate and prompt treatment is crucial for survival of the patient. Monitoring the resistance profiles of the *A. baumannii* strains isolated regionally assists in optimised prediction of successful treatment options (Bergogne-Berezin 1997).

1.3.5.1 'Last resort' antibiotics

Antibiotics with a high level of activity against *A. baumannii* are tigecycline (glycylcycline) and colistin (polymyxin E). Tigecycline was approved by the US Food and Drug Administration (FDA) in 2005 and appeared promising for use against many bacteria including *A. baumannii* (Seifert *et al.* 2006; Slover *et al.* 2007). Since its release, contradicting reports have emerged about the effectiveness of tigecycline in treatment of MDRAB (Al-Sweih *et al.* 2011; Jamal *et al.* 2009; Kulah *et al.* 2009; Peleg *et al.* 2007). To date, active efflux has been associated with tigecycline resistance and in particular overexpression of *adeB* (Section 1.3.4.5) (Ruzin *et al.* 2007; Sun *et al.* 2010).

The polymyxin antimicrobial polypeptides have a relatively high nephro- and neuro-toxicity in humans, making them a less favourable therapeutic option (Lim *et al.* 2010). Although uncommon, colistin resistance in *Acinetobacter* has been documented (Adams *et al.* 2009; Al-Sweih *et al.* 2011; Moffatt *et al.* 2011; Nemec and Dijkshoorn 2010). Colistin resistance can be acquired by insertional disruption of the genes encoding the lipid A biosynthesis proteins, *lpxA* and *lpxC* (Moffatt *et al.* 2011). However, these mutant strains were created under selective pressure in a laboratory and therefore, the viability of these mutants in the clinical setting remains to be determined. Overexpression of the two-component regulatory system PmrAB, as a result of amino acid substitutions, has also been linked to colistin resistance (Adams *et al.* 2009; Park *et al.* 2011). Up-regulation of *pmrAB* results in structural modification of LPS by means of addition of phosphoethanolamine to lipid A (Arroyo *et al.* 2011; Beceiro *et al.* 2011). However, mutations in *pmrB* also results in impaired fitness and virulence, again creating uncertainties about the prevalence and success of these colistin resistant *A. baumannii* mutants in the clinical setting (Lopez-Rojas *et al.* 2011).

In conclusion, resistance of *A. baumannii* to tigecycline or colistin is uncommon compared to resistance to first-line antibiotics such as carbapenems and aminoglycosides. Nonetheless, the presence of tigecycline and colistin resistant

A. baumannii clones is evident. It is most likely a matter of time before other *A. baumannii* clones develop resistance or to see global dissemination of already resistant clones, rendering these 'last resort' antibiotics ineffective.

1.3.5.2 Combination therapies

A number of *in vitro* studies of combination therapies on *A. baumannii* have been conducted in the last few years. Although the success rate is largely strain dependent, various promising results have been obtained. First, the effect of colistin on fluidity of the outer membrane allows use of antimicrobials normally prescribed for treatment of Gram-positive bacteria only. Combinations of colistin and vancomycin show synergy in both *in vitro* and *in vivo* assays, allowing usage of lower dosages of these toxic agents (Gordon *et al.* 2010; Hornsey and Wareham 2011). Colistin has also been used successfully in combination with imipenem, sulbactam, meropenem and azithromycin (Liang *et al.* 2011; Pachon-Ibanez *et al.* 2010; Timurkaynak *et al.* 2006), whereas varying results have been obtained using a combination of colistin and rifampicin (Liang *et al.* 2011; Lim *et al.* 2011; Motaouakkil *et al.* 2006; Pachon-Ibanez *et al.* 2010; Song *et al.* 2009; Tripodi *et al.* 2007). Colistin showed limited efficiency in respiratory tract infections, therefore, a combination of rifampicin and imipenem appears most promising in treatment of pneumonia (Pachon-Ibanez *et al.* 2010; Saballs *et al.* 2006; Song *et al.* 2009; Tripodi *et al.* 2007). Tigecycline exhibits synergistic activity with colistin, polymyxin B, levofloxacin, amikacin and imipenem (Lim *et al.* 2011; Principe *et al.* 2009).

1.3.5.3 Prospective antimicrobial agents

Antimicrobial peptides are widely studied in attempts to identify novel drugs for the treatment of multidrug resistant bacteria. Both the human lactoferrin-derived peptide hLF1-11 and the CPF-C1 antimicrobial peptides from the Eritrea clawed frog have been shown to have activity against MDRAB (Conlon *et al.* 2011; Dijkshoorn *et al.* 2004; van der Does *et al.* 2010). Various other antimicrobial peptides are expected to go into clinical trials in the upcoming years (Michalopoulos and Falagas 2010; Neonakis *et al.* 2011). Finafloxacin is a novel fluoroquinolone with high activity levels in an acidic environment (pH 5.8) and is effective against ciprofloxacin resistant *A. baumannii* isolates containing GyrA and ParC mutations (Higgins *et al.* 2010). Two isolates known to express high levels of *adeB* also displayed lower resistance levels to finafloxacin than to ciprofloxacin. These results indicate that this novel fluoroquinolone exhibits great potential for treatment of

various infections in which an acidic environment can be encountered, such as the lower respiratory tract, urinary tract, skin and stomach mucosa. BAL30072, a monobactum siderophore, shows high levels of both *in vitro* and *in vivo* efficiency in killing *A. baumannii* cells (Mushtaq *et al.* 2010; Page *et al.* 2010; Russo *et al.* 2011). The siderophore chain of this molecule facilitates rapid translocation across the outer membrane, which forms a major barrier for most antimicrobial agents (Mollmann *et al.* 2009). Other examples of novel agents with activity against *A. baumannii* include cyclo(L-tryptophanyl-L-tryptophanyl) produced by a *Streptomyces* species (Lee *et al.* 2010a) and Microbiotix bis-indole molecules (Jacobs *et al.* 2011).

A completely novel approach for the treatment of *A. baumannii* in burn infections was tested in a mouse model (Dai *et al.* 2009). The technique used a non-toxic photosensitiser and red light, together called photodynamic therapy, to show a significant decrease in viable *A. baumannii* cells on the inoculated burn wound. This approach could be a future solution for treating multidrug resistant or even pan-resistant *A. baumannii* burn wound infections. Other prospective agents effective against *A. baumannii* wound infections include nitric oxide releasing nanoparticles (Friedman *et al.* 2011; Mihu *et al.* 2010) and E-101 Solution, a myeloperoxidase based antimicrobial (Denys *et al.* 2011).

1.3.5.4 Targeting virulence determinants

A different approach in combating *A. baumannii* is targeting the expression of virulence factors. Inactivation of regulatory mechanisms, such as QS, may prove useful in eradicating *A. baumannii* from the hospital environment. Azithromycin, a macrolide antibiotic, has been shown to decrease expression of QS-signals in *P. aeruginosa*, which subsequently resulted in decreased virulence levels both *in vivo* and *in vitro* (Kohler *et al.* 2010; Nalca *et al.* 2006). Other antibiotics having similar effects on *P. aeruginosa* QS are ciprofloxacin and ceftazidime (Skindersoe *et al.* 2008). No such reports have been published for *A. baumannii*. Conversely, sub-inhibitory concentrations of antibiotics may induce virulence features such as biofilm formation. Incubation of a multidrug resistant clinical *A. baumannii* isolated with imipenem at a final concentration 50% below the MIC resulted in a significant increase in biofilm formation (Section 1.2.2) (Nucleo *et al.* 2009).

Coating surfaces with antimicrobial agents has been attempted to decrease biofilm formation. Coating of catheters with silver-platinum and carbon, or

chlorhexidine and silver sulfadiazine had no significant effect on *A. baumannii* adherence (Raad *et al.* 2008). However, monocycline and rifampicin coated catheters reduced *A. baumannii* biofilm levels. A recent study described the effect of silver and two antiseptics, gardine and gendine, on *A. baumannii* biofilm formation in endotracheal tubing (Raad *et al.* 2011). Coating with silver resulted in a significant reduction of *A. baumannii* biofilm formation and coating with either gardine or gendine resulted in complete inhibition of biofilm formation. Chlorhexidine has also been used for coating gloves (Reitzel *et al.* 2009). The inoculums contained 10^8 colony forming units (CFUs) of *A. baumannii* and were exposed for 30 seconds, 10 minutes, 30 minutes or 1 hour. Complete killing was observed in all experiments except the shortest exposure time of 30 seconds. Interestingly, the experiments were also performed with other common multidrug resistant nosocomial pathogens, such as *E. coli*, and showed complete killing including the 30 seconds exposure, highlighting the resilience of *A. baumannii*.

1.4 What have genomic analyses taught us so far?

To date, GenBank contains eight 'completed' *A. baumannii* genome projects, 19 projects with 'assembly' genomes and 40 that are 'in progress.' Interestingly, this is more than double the number of sequencing projects available for *P. aeruginosa*. The need for sequencing of many different *A. baumannii* strains comes from the major differences observed in the currently completed genomes, which makes whole genome comparison of significant importance for understanding *A. baumannii* (Adams *et al.* 2010; Fournier *et al.* 2006; Lewis *et al.* 2010; Vallenet *et al.* 2008). In one of the first such comparative studies, major sequence variation was revealed between *Acinetobacter* isolates from distinct sources; *A. baumannii* ACICU (human), *A. baumannii* SDF (louse) and *A. baylyi* APD1 (soil) (Vallenet *et al.* 2008). In total, 2052 orthologous genes were identified, which is the equivalent of 57%, 71% and 63% for strain ACICU, SDF and ADP1, respectively. This indicates that the *Acinetobacter* core genome is small in comparison to its flexible gene pool or accessory genome. Sequence variation is not restricted to isolates of very distinct origins, as similar results have been obtained when comparing the genomes of clinical *A. baumannii* isolates (Adams *et al.* 2008; Fournier *et al.* 2006). In a genome wide analysis of five closely related *A. baumannii* isolates, all part of international clone I and from the same institute, differences were predominantly identified in the resistance islands of the AbaR-type (Section 1.4.1) (Adams *et al.* 2010).

Whole genome sequencing has also been used for a thorough investigation of an epidemiological pathway (Lewis *et al.* 2010). Conventional molecular typing methods could not discriminate between the *A. baumannii* outbreak isolates from an ICU accommodating both military personnel and civilians. However, single nucleotide polymorphisms (SNPs) were identified in the genome sequences, assisting in drawing a detailed route of patient-to-patient transmittance.

1.4.1 AbaR-type resistance islands

The *A. baumannii* genome contains a large number of mobile genetic elements, of which the AbaR-like genomic islands have been studied most thoroughly (Fournier *et al.* 2006; Iacono *et al.* 2008; Krizova *et al.* 2011; Krizova and Nemec 2010; Post and Hall 2009; Post *et al.* 2010). An 86 Kb genomic island, named AbaR1, was first identified in the genome of *A. baumannii* strain AYE (Fournier *et al.* 2006). AbaR1 contains many genes encoding proteins with a predicted function in resistance, including aminoglycoside modifying enzymes, beta-lactamases, efflux systems and mechanisms for heavy metal detoxification. Various AbaR-like resistance islands have since been identified in both *A. baumannii* strains similar to AYE (international clone I) and those genetically unrelated (Adams *et al.* 2010; Iacono *et al.* 2008; Krizova *et al.* 2011; Krizova and Nemec 2010; Post *et al.* 2010; Thapa *et al.* 2010). The AbaR-like islands show major variation, regardless of the clonal relationship of the strains (Figure 1.2) (Krizova *et al.* 2011). The size, and therefore the number of resistance genes, is dependent on truncations that have taken place within the island, which is often mediated by IS*26* insertion sequences. A direct link between the number of resistance genes within AbaR-like islands and the resistance profile is difficult to establish, as the *A. baumannii* genome often contains other insertion elements harbouring resistance genes (Adams *et al.* 2010; Krizova *et al.* 2011). Interestingly, evolutionary investigation of AbaR-like islands indicated that the existence of particular resistance determinants in AbaR-like islands is related to the antibiotic treatment regimes applied at the time of isolation of the *A. baumannii* strain (Krizova *et al.* 2011). For example, $bla_{\text{TEM-1}}$, *aphA1*, *tetA* and *catA1* were found in AbaR3 or its direct derivatives in international clone I isolates from the 1970s and 1980s (Krizova *et al.* 2011). Antibiotics widely used at the time, such as early generation beta-lactams, aminoglycosides, tetracycline and chloramphenicol, were rendered ineffective by the presence of these genes.

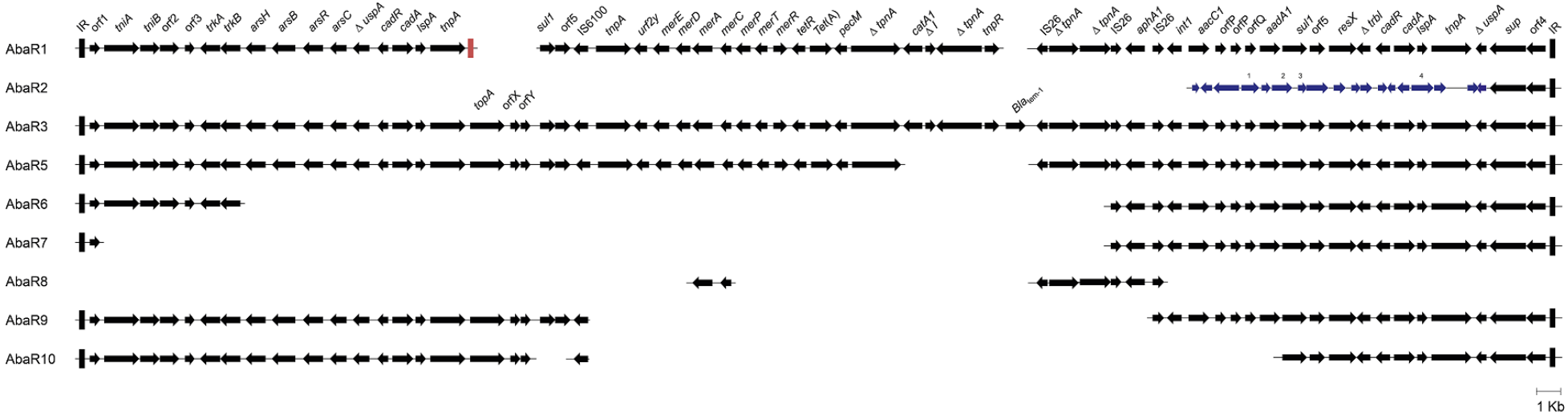

Figure 1.2: Genomic comparison of the *A. baumannii* AbaR resistance islands

The predicted open reading frames (ORFs) of nine AbaR resistance islands are depicted as black arrows. The red square indicates the location of a 29 Kb insertion specific to AbaR1. The AbaR1 and AbaR3 resistance islands are based on published representations and were subsequently used as a template for this figure (Krizova and Nemec 2010). Comparative analyses of AbaR5, AbaR6, AbaR7, AbaR9 and AbaR10 were performed using previously published descriptions (Adams *et al.* 2010; Post *et al.* 2010). Analyses of AbaR2 and AbaR8 were conducted with the sequences available from GenBank, CP000863 and HM590877, respectively. The ORFs represented in blue are specific to AbaR2, some of these are of major significance; *aacA4* (1), $bla_{\text{vim-1}}$ (2), *qacE*Δ1 (3) and a heavy metal detoxification protein (4). The schematic representation of further resistance islands AbaR11-AbaR19 has been published recently (Krizova *et al.* 2011).

Consequently, the international clone I isolates possessed a distinct advantage for survival and dissemination.

AbaR-like resistance islands are not randomly inserted into the *A. baumannii* genome, as genes that possess regions coding for ATPase domains, such as *comM*, form insertional hot-spots (Ramirez *et al.* 2011; Shaikh *et al.* 2009). ComM has been associated with DNA uptake in *Haemophilus influenzae* (Gwinn *et al.* 1998) and in *Acinetobacter* strains other than *A. baumannii*, such as the naturally competent *A. baylyi* strain ADP1 (Bacher *et al.* 2006; Busch *et al.* 1999; Herzberg *et al.* 2000). Interestingly, an *A. baumannii* strain lacking the AbaR-like island, and therefore with *comM* intact, showed natural competency for the uptake of plasmids and linear DNA (Ramirez *et al.* 2010). Selecting naturally competent *A. baumannii* strains, e.g. those with *comM* intact, may provide strains more amendable to molecular modifications.

1.4.2 *A. baumannii* plasmids are highly variable between strains

Plasmids only make up a small proportion of the genomic content of *A. baumannii*. However, many bacteria gain significant benefit from genes located on plasmid DNA. Although highly variable in genetic content and size (from 2.5 Kb up to 94 Kb), plasmids can be found in most *A. baumannii* strains (Adams *et al.* 2010; Fournier *et al.* 2006; Iacono *et al.* 2008). Although most *A. baumannii* resistance markers have been identified on chromosomal DNA, various plasmids carry genes contributing to carbapenem or aminoglycoside resistance (Adams *et al.* 2010; Chen *et al.* 2010; Lu *et al.* 2009; Poirel *et al.* 2010; Rumbo *et al.* 2011; Towner *et al.* 2011). Interestingly, various resistance genes located on plasmids are flanked by insertion sequences, which facilitates their incorporation into the *A. baumannii* genome (Adams *et al.* 2010). Variation in the number and type of plasmids in clinical isolates has also been investigated by comparative analysis of the replication (*rep*) genes (Bertini *et al.* 2010). A total of 19 different *rep* groups have been designated and a multiplex polymerase chain reaction (PCR) method based on plasmid variation has been developed as a novel typing method.

1.4.3 The role of insertion sequences in shaping the *A. baumannii* genome

Mobile genetic elements, such as transposons, are readily found in *Acinetobacter* genomes and play important roles in increasing resistance levels. For example, mobilisation of resistance genes such as the *tet* genes or beta-lactamases is supported by their association with mobile genetic material (Poirel *et al.* 2010). An

alternative approach of increasing resistance is by introducing a strong promoter upstream of a resistance gene located on the chromosome. These events have been linked to increased expression of beta-lactamases and aminoglycoside modifying enzymes (Adams *et al.* 2010; Figueiredo *et al.* 2009a; Figueiredo *et al.* 2009b; Hu *et al.* 2007; Segal and Elisha 1999; Turton *et al.* 2006). Finally, insertional inactivation of genes located on the chromosome may result in increased resistance. For example, *carO*, the gene encoding a major porin protein related to influx of carbapenems, is a common target for insertion sequences, such as IS*Aba825* and IS*Aba10* (Lee *et al.* 2010b; Mussi *et al.* 2005; Ravasi *et al.* 2011). Furthermore, colistin resistance can be mediated by insertional inactivation of the lipid A biosynthesis genes by IS*Aba11* (Section 1.3.5.1) (Moffatt *et al.* 2011).

The genome of the non-pathogenic *A. baumannii* strain SDF has shown to be greatly modified by a wide range of insertion elements (Vallenet *et al.* 2008). Many virulence determinants have either been insertionally inactivated or completely removed by intra-chromosomal recombination events. Insertion sequences have also resulted in advantageous phenotypic outcomes in many clinical strains. Their mobility and selective pressure by environmental stress, such as drug treatment, allows prolific propagation of clones containing insertions with beneficial effects.

1.5 Scope of this thesis

A. baumannii is a known coloniser in hospital wards and its role as a significant human pathogen has been established throughout the last decades. However, little is known about the features that assist in colonisation and survival under adverse conditions encountered in the hospital and host niches. Therefore, the broad aim of this project was to examine a diverse range of mechanisms that play a role in *A. baumannii* persistence and resistance and have been presented in this thesis.

Efficient acquisition of iron is crucial for survival in many different environments, as this micronutrient is often available in limiting quantities. Therefore, the transcriptome of *A. baumannii* strain ATCC 17978, a widely used reference strain, was investigated under iron-replete and iron-limiting conditions. The iron acquisition mechanisms identified here were also examined using phenotypic assays. Furthermore, comparative genomic analyses were carried out to assess the distribution of the iron acquisition mechanisms in different fully sequenced *A. baumannii* isolates.

The clonal relationship of a broad collection of clinical *A. baumannii* isolates was analysed. This provided information about the prevalence of clonal lineages in Australia. Furthermore, various phenotypic characteristics, such as adherence and motility, were investigated and clone specific trends could be identified. An *A. baumannii* ATCC 17978 mutant strain displaying distinct adherence and motility phenotypes was isolated during the course of this study. Comprehensive analysis of the differences between the mutant and parental strain, by means of genomics, transcriptomics and phenotypic characterisation, aided in identification a various molecular mechanisms that play a role in *A. baumannii* adherence and motility.

The role of drug transporters in *A. baumannii* multidrug resistance was investigated by heterologous expression in *E. coli* hosts and by creating an *A. baumannii* directed insertion disruption mutant. A high-throughput cloning strategy was developed and proved successful for characterisation of various novel *A. baumannii* drug transporter proteins.

Overall, the wide range of experimental approaches and analyses utilised in this study generated a wealth of information on the *A. baumannii* persistence and resistance features. The results presented here have advanced the understandings about the factors that contribute to the success of *A. baumannii* as a human pathogen.