Adaptation and Development of a New Method for Measuring Antibiotic and Dye Accumulation in *Acinetobacter baumannii*.

By

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Abstract

Acinetobacter spp. are nosocomial pathogens, commonly associated with opportunistic infections such as pneumonia, urinary tract and skin and soft tissue infections, particularly in intensive care units and in military casualties returning from Iraq and Afghanistan. Their propensity to acquire resistance determinants and over-express existing resistance genes allows them to avoid eradication by antibiotics and biocides. A common cause of multidrug resistance is innate and over-production of efflux pumps. This study aimed to establish a method to distinguish between isolates that demonstrate different levels of efflux and to apply the method to study defined efflux pump mutants. MICs of a range of antibiotics were determined against clinical isolates representative of resistant and susceptible isolates commonly found causing infection in Singapore hospitals and specific pump deletion mutants. A Hoechst 33342 accumulation assay was developed and used to compare efflux in the clinical isolates and pump deletion mutants and data were compared with those from ethidium bromide and norfloxacin accumulation assays. Measurement of accumulation of H33342, with supporting ethidium bromide accumulation data, showed a significant difference in the efflux activity of specific pump deletion mutants compared with the parental strain. Data obtained in this study support previous work carried out with other strains and isolates of *A. baumannii* and provide an insight into the contribution of RND systems AdeFGH and AdeIJK to MDR in clinical isolates commonly found in Singapore hospitals.

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Chapter 1. Introduction

1.1. *Acinetobacter* species

Acinetobacter are a Gram negative coccobacilli responsible for an increasing number of nosocomial infections in the UK and worldwide. *Acinetobacter* are currently defined as aerobic, non-fermenting, non-fastidious, non-motile, catalase positive and oxidase negative, with a DNA G+C content of 38% to 50% (Rossau, Van Landschoot et al. 1991). *Acinetobacter* are opportunistic pathogens and are a rising problem in immunocompromised patients within the hospital setting. The clinical success of this organism is down to a number of factors including its propensity to acquire antibiotic resistance determinants and to over-express existing intrinsic resistance genes, allowing it to avoid eradication by antibiotics and biocides. Its ability to survive desiccation means it is also able to persist in the hospital environment for extended periods of time (Wendt, Dietze et al. 1997; Jawad, Seifert et al. 1998).

The taxonomy of *Acinetobacter* has a long and complicated background and there are still difficulties with species identification. The bacterium was first identified in 1911 by the Dutch microbiologist, Beijerinck, who isolated a bacterium he named *Micrococcus calcoaceticus* from soil samples (Beijerink 1911). Since then, bacteria now known as *Acinetobacter* have been isolated many times and assigned to various genera. In 1971, the genus *Acinetobacter* was officially acknowledged by the Subcommittee on Nomenclature of *Moraxella* and Allied Bacteria (Lessel 1971). In 1986, the genera was divided into 12 DNA groups with formal species names (Bouvet and Grimont 1987) and over the past 26 years has been further categorised to give at least 33 individual genomic groups, 20 with formal species names (Table 1.1.) (Towner 2009).

Not all *Acinetobacter* species pose a threat to human health. Many species are non-pathogenic and can be isolated from the soil and often form part of the normal human skin flora (Baumann 1968; Seifert, Dijkshoorn et al. 1997). This has led to the common misconception that all *Acinetobacter* species are ubiquitous and that pathogenic species such as *A. baumannii* can be isolated from environmental sources. However, it is now generally accepted that this is not the case. Although *Acinetobacter* can be isolated from human skin, highly pathogenic species such as *A. baumannii* are rarely found colonising the skin of healthy humans (Berlau, Aucken et al. 1999). Clinically relevant strains of *Acinetobacter* are often found colonising hospital surfaces, hospital staff and medical instrumentation, although the natural habitat of these strains remains unknown.

1.1.1. *Acinetobacter baumannii - calcoaceticus* complex

Four of the identified *Acinetobacter* species are often grouped into the *Acinetobacter calcoaceticus – baumannii* (Acb) complex. This group comprises of *A. baumannii, A. calcoaceticus, A.* genomospecies (gsp) 3 and *A.* gsp 13 (recently renamed *as A. pittii* and *A. nosocomialis* respectively) (Gerner-Smidt 1992; Nemec, Krizova et al. 2011).

A. baumanni, A. pittii and *A. nosocomialis* are clinically relevant species, often implicated in infection, whereas *A. calcoaceticus* is largely non-pathogenic and is rarely identified in the hospital setting (Peleg, Seifert et al. 2008; Koh, Tan et al. 2011). Therefore, from a clinical perspective, identification of different species of *Acinetobacter*, especially those within the Acb complex, is extremely important. Species also has implications for treatment and infection control as many of the non-*A. baumannii* complex species are drug susceptible and infection control is not necessary (Chuang, Sheng et al. 2011). However, discrimination between species is

difficult, particularly between members of the *A. baummanii* complex, which cannot be differentiated phenotypically and are often misidentified (Gerner-Smidt, Tjernberg et al. 1991; Gerner-Smidt 1992). Semi-automated systems such as the API 20NE system are often unreliable and also cannot distinguish between closely related species (Bernards, van der Toorn et al. 1996). There are a number of methods currently used for the routine identification of *Acinetobacter* species in clinical laboratories; DNA-DNA hybridisation is the reference standard (Bouvet and Grimont 1986). However this process is lengthy and is impractical for a routine diagnostic laboratory. For this reason various methods have been adopted and verified for species identification. These include 16S rRNA gene restriction (ARDRA) (Vaneechoutte, Dijkshoorn et al. 1995), high resolution fingerprint analysis by amplified fragment length polymorphism (AFLP) (Janssen, Maquelin et al. 1997), ribotyping (Gerner-Smidt 1992), tRNA spacer analysis (Ehrenstein, Bernards et al. 1996), restriction analysis of 16S-23S rRNA intergenic spacer regions (Dolzani, Tonin et al. 1995; Chang, Wei et al. 2005) and sequence analysis of the *rpoB* gene (La Scola, Gundi et al. 2006). Nonetheless, apart from a small number of *Acinetobacter* reference laboratories, species identification is not always possible in most clinical laboratories.

1.1.2. Epidemiology

Acinetobacter is well-recognised for its ability to cause nosocomial outbreaks, and particular strains are able to cause epidemics in multiple hospitals within a city, in

Table 1.1. *Acinetobacter* spp. that have been given formal species names (Towner 2009).

***Acinetobacter* species**
A. calcoaceticus
A. baumannii
A. pittii
A. nosocomialis
A. haemolyticus
A. junii
A. johnsonii
A. lwoffii
A. radioresistens
A. ursingii
A. schindleri
A. parvus
A. baylyi
A. bouvetii
A. towneri
A. tandoii
A. tjernbergiae
A. gerneri
A. beijerinckii
A. gyllenbergii

various regions in a country and can even spread worldwide (van Dessel, Dijkshoorn et al. 2004; Coelho, Turton et al. 2006). *A. baumannii* European (EU) clones I and II were first identified as outbreak strains in North-western Europe in 1996 (Dijkshoorn, Aucken et al. 1996). A third clone was later identified as a European outbreak strain and named EU clone III (van Dessel, Dijkshoorn et al. 2004). These clones are widespread throughout Europe and contribute significantly to the spread of carbapenem-resistant *Acinetobacter* (Towner, Levi et al. 2008). Other individual lineages are more prevalent in certain areas of Europe, such as the AYE-VEB-1 clone found in France and Belgium (Naas, Bogaerts et al. 2006) and the OXA-40 (OXA-24) carbapenem resistant clone found in Spain and Portugal (Da Silva, Quinteira et al. 2004). The EU clones are in fact international lineages and not limited to Europe. Outbreaks have been identified in the USA and South Africa (van Dessel, Dijkshoorn et al. 2004; Petersen, Cannegieter et al. 2011). In Asia, carbapenem resistant European clones have been described in hospitals in China (Fu, Zhou et al. 2010) and Korea (Park, Lee et al. 2010). In Singapore, carbapenem resistance is also observed in clinical isolates, with outbreak isolates related to EU clones I and II identified in the hospital setting, and the majority of carbapenem resistance due to OXA-23 carrying clones (Park, Lee et al. 2010). In a study of UK hospitals between 2003 and 2006, sub-lineages of EU clone II dominated, with the South-east (SE) and OXA-23 clones being the most prevalent (Coelho, Turton et al. 2006).

1.2. *Acinetobacter* Infection

1.2.1. Hospital Acquired Infection

Acinetobacter species most commonly cause nosocomial infections, including ventilator-associated pneumonia, skin and soft-tissue infections, wound infections, surgical site infections, catheter-related and urinary tract infections, secondary meningitis and bloodstream infections (Forster and Daschner 1998). Studies have found that up to 18% of patients infected with *A. baumannii* develop bacteraemia, most often acquired in the intensive care unit (ICU) (Cisneros, Reyes et al. 1996). The assessment of the outcome of *Acinetobacter* infection is difficult and reported mortality rates range from 5% in general wards to 54% in the ICU (Poutanen, Louie et al. 1997; Siau, Yuen et al. 1999). Seifert *et al.* showed the crude mortality rate of *A. baumannii* bacteraemia to be as high as 44%. However, it is difficult to determine morbidity and mortality directly attributable to *Acinetobacter* as opposed to co-morbidity, which is very common in these patients. Death attributable to *A. baumannii* bacteraemia, at 19%, was assessed to be much lower than the crude mortality rate (Seifert, Strate et al. 1995). Several predisposing factors to infections with *Acinetobacter* have been identified. These include immunosuppression, unscheduled hospital admission, respiratory failure at admission, previous antimicrobial therapy, previous sepsis in ICU and invasive procedures; all of which have been recognised as risk factors for *Acinetobacter* infection (Garcia-Garmendia, Ortiz-Leyba et al. 2001). *Acinetobacter* can be cultured from different environmental sites within hospitals and it is thought that cross contamination between sites is a major mode of transmission in hospital outbreaks (van den Broek, Arends et al. 2006). Carriage of *Acinetobacter* on the hands of hospital staff and on medical instrumentation can contribute to the spread of the organism. The ability of certain *Acinetobacter* species to survive on dry surfaces for extended periods of time may also increase

transmissibility. It has been suggested that desiccation tolerance, along with multidrug resistance demonstrated by some strains, may explain why *Acinetobacter* is able to establish itself in the hospital environment and cause recurring nosocomial outbreaks (Jawad, Seifert et al. 1998).

1.2.2. Community Acquired Infection

Although most common in the hospital environment, community acquired *Acinetobacter* infection has been observed. Recently in Portugal, necrotising community acquired pneumonia due to *Acinetobacter lwoffii* contamination of a nebuliser in a previously healthy child was identified (Moreira Silva, Morais et al. 2011). This type of infection is usually associated with underlying conditions such as alcoholism, smoking, chronic obstructive pulmonary disease and diabetes mellitus and is a particular problem in tropical climates such as Southeast Asia and Australia, where skin carriage is more common due to environmental conditions (Anstey, Currie et al. 1992; Chu, Leung et al. 1999). In these areas *A. baumannii* can be a cause of severe community-acquired pneumonia, especially in young alcoholic patients (Chen, Hsueh et al. 2001).

1.2.3. Infection in Military and Disaster Zone Casualties

Acinetobacter species are also commonly isolated from deep wound and burn infections, and osteomyelitis in military and disaster zones. Reports from the Marmara earthquake in Turkey in 1999 described a high incidence of *Acinetobacter* strains as responsible for healthcare associated infection in trauma patients (Oncul, Keskin et al. 2002). *Acinetobacter* wound infections have been reported in military casualties returning from Iraq and Afghanistan, many of them exhibiting MDR (Murray, Roop et al. 2006; Johnson, Burns et al. 2007; Petersen, Riddle et al. 2007;

Scott, Deye et al. 2007). Due to the misconception that *A. baumannii* is ubiquitous and can be isolated from environmental sources it was initially considered that the organism was being introduced at the site of injury, or was due to skin colonisation at the time of injury. However, there is now evidence of the role of environmental contamination and transmission of organisms within health care facilities and it is likely that patients with a prolonged stay in US field hospitals provide a reservoir for this organism (Davis, Moran et al. 2005; Scott, Deye et al. 2007).

1.3. Treatment of *Acinetobacter* Infections

Due to the wide spectrum of intrinsic and acquired antibiotic resistance mechanisms present in this pathogen, treatment of *Acinetobacter* infections poses a major challenge. Clinical isolates displaying resistance to a number of classes of antibiotics are commonly observed and treatment of MDR isolates is now limited to a small number of antibiotics:

- **Sulbactam** is a β-lactamase inhibitor that binds penicillin binding protein (PBP) protein 2 (Urban, Go et al. 1995). Whilst its primary purpose is to limit the degradation of active β-lactams by β-lactamases, it also demonstrates intrinsic activity against *Acinetobacter* species when used alone (Levin 2002; Higgins, Wisplinghoff et al. 2004). However, sulbactam is most commonly used in combination with other antibiotics and an ampicillin-sulbactam combination provides an effective therapeutic option for the treatment of MDR *Acinetobacter* infections (Levin, Levy et al. 2003). Oliveira *et al.* showed that ampicillin-sulbactam may be more efficacious in treating carbapenem-resistant

Acinetobacter spp. than polymyxins (Oliveira, Prado et al. 2008) and it has been suggested that sulbactam should be the preferred treatment for infections with this pathogen (Levin 2002; Peleg 2007). Unfortunately, increasing clinical use has led to a rise in sulbactam resistance and minimum inhibitory concentrations (MICs) of >32 μg/ml have been observed in clinical isolates (Henwood, Gatward et al. 2002; Higgins, Wisplinghoff et al. 2004). Resistance to an ampicillin-sulbactam combination has also been seen; in a study conducted in Taiwan, 70% of clinical isolates were ampicillin-sulbactam resistant (Yang, Chang et al. 2010).

- **Polymyxins** are polycationic lipopeptide antimicrobials that show bactericidal activity against *Acinetobacter* spp. They include polymyxin B, polymyxin E and colistin. The polymyxins were discovered as chemotherapeutic agents in 1947 (Stansly, Shepherd et al. 1947), but use has been minimal due to concerns over neurotoxicity and nephrotoxicity (Falagas, Fragoulis et al. 2005; Falagas, Rafailidis et al. 2006). However, with MDR bacteria becoming more prevalent, the use of polymyxins has increased and in some cases is recommended for the treatment of carbapenem-resistant *Acinetobacter* (Kim, Peleg et al. 2009). Polymyxins show high success rates in the clinical setting. Kallel *et al.* showed a favourable outcome in 76% of patients treated with colistin in the ICU (Kallel, Bahloul et al. 2006) and others have shown success with polymyxin treatment against MDR *Acinetobacter* (Holloway, Rouphael et al. 2006; Falagas, Rafailidis et al. 2010). Resistance to these drugs is rare, although increasing use means that the isolation of resistant strains is on the rise (Matthaiou, Michalopoulos et al. 2008) with reported resistance levels of up to 18% in *A.*

baumannii isolates in South Korea (Ko, Suh et al. 2007). Colistin and other polymyxins target the lipid A component of lipopolysaccharide (LPS) of Gram negative bacteria during initial binding of the outer membrane. Mutations in the lipid A biosynthesis genes *lpxA, lpxC,* and *lpxD* can result in loss of ability to produce lipid A and therefore LPS. This prevents the interaction of colistin with LPS in colistin resistant *A. baumannii* isolates (Moffatt, Harper et al. 2010). An alternative mechanism of resistance was identified by Beceiro *et al*. who observed mutations in *pmrB* and upregulation of *pmrAB* leading to modification of lipid A and consequently colistin resistance (Beceiro, Llobet et al. 2011).

- **Carbapenems** are one of the most valuable treatment options against MDR *Acinetobacter*. This class of β-lactams show good bactericidal activity against β-lactamase producing MDR *Acinetobacter* isolates (Fishbain and Peleg 2010). However, increasing resistance to imipenem and meropenem has been observed in the last decade (Karageorgopoulos and Falagas 2008). Resistance to carbapenems is most commonly due to production of class D β-lactamases e.g. OXA-51-like, and OXA-23-like enzymes (Turton, Ward et al. 2006; Corvec, Poirel et al. 2007), increased production of multi-drug efflux pumps, such as AdeABC (Magnet, Courvalin et al. 2001; Huang, Sun et al. 2008), and decreased permeability due to reduced expression of porins such as CarO (Ravasi, Limansky et al. 2011). Carbapenem resistant isolates are often resistant to other classes of antibiotics, leaving polymyxins and tigecycline as the only remaining treatment options. A 2007 study of antimicrobial susceptibility in isolates collected from around the world identified

susceptibility to imipenem ranging from 60.6% in Latin America to 88.6% in North America. Susceptibility was also high in Europe (85.9%), whereas susceptibility rates in Asia were moderate to low (69.2%) (Reinert, Low et al. 2007). Since then, various studies have highlighted the emergence of carbapenem resistant isolates in the clinical setting (e.g. Scott, Deye et al. 2007; Enoch, Summers et al. 2008; Lee, Fung et al. 2011; Kempf and Rolain 2012). However, susceptibility data based on a particular carbapenem antibiotic cannot be generalised to all drugs in this class. Differing imipenem and meropenem resistance levels have been observed in clinical isolates (Ikonomidis, Pournaras et al. 2006). Misinterpreted susceptibility results can also give rise to dire consequences; based on susceptibility to imipenem, a fatal case of *A. baumannii* pneumonia by a meropenem resistant isolate was treated with meropenem (Lesho, Wortmann et al. 2005).

- **Tigecycline** is a member of a relatively new class of antimicrobials, the glycyclines, and is a semi-synthetic modified minocycline (Neonakis, Spandidos et al. 2011). Good *in vitro* activity has been demonstrated against 595 clinical isolates of *Acinetobacter* spp. isolated throughout the UK (Henwood, Gatward et al. 2002) and global studies have shown MIC_{90} values of 1-2 μg/ml (Reinert, Low et al. 2007; Garrison, Mutters et al. 2009). There is a shortage of clinical studies investigating the activity of tigecycline, although a good clinical and microbiological response to tigecycline treatment of MDR *Acinetobacter* has been observed in some cases (Poulakou, Kontopidou et al. 2009). Vasilev *et al.* identified a cure rate of 82.4% for resistant *A. baumannii* infections in a multicentre study (Vasilev, Reshedko et al. 2008). However,

Gordon *et al.* observed microbiological clearance of the infection in only 68% of cases in a retrospective study of tigecycline treated *A. baumannii* infections in a UK hospital. This result suggests that tigecycline monotherapy may not always be appropriate (Gordon and Wareham 2009). Due to this lack of clinical evidence, breakpoint concentrations to define resistance have not yet been established by CLSI, EUCAST or BSAC for this antibiotic class. Treatment failure has also been observed with tigecycline therapy. An increase in MICs often developed during therapy, after only three weeks exposure to the drug (Reid, Grim et al. 2007). Evaluation of tigecycline treatment in unrelated studies of patients with MDR *A. baumannni* infections also identified single *A. baumannii* isolates that developed resistance during treatment (Schafer, Goff et al. 2007; Anthony, Fishman et al. 2008). Tigecycline diffuses rapidly into tissues resulting in low mean peak serum concentrations at recommended doses. As a consequence, therapeutic failure is possible, even with susceptible isolates, and so tigecycline is not recommended for bacteraemia infections (Fishbain and Peleg 2010).

1.3.1. Synergistic antibiotic combinations

Due to the increase in the occurrence of MDR *Acinetobacter* and the increasingly limited spectrum of antibiotics available for treatment, there have been numerous attempts to identify synergistic combinations of antibiotics to use in the treatment of this organism. However, many of these studies have been conducted *in vitro* or in animal models and there are few clinical studies to confirm their findings. Most work

focuses on combinations that increase the efficacy of last line drugs such as carbapenems, tigecycline and colistin. Sheng *et al.* used time kill studies to identify synergism between imipenem and colistin, tigecycline, amikacin and ampicillin-sulbactam against carbapenem resistant *Acinetobacter* species (Sheng, Wang et al. 2011). Synergy has also been observed with a combination of colistin and glycopeptide antibiotics vancomycin and teicoplanin, which are usually associated with the treatment of Gram-positive infections (Gordon, Png et al. 2010; Wareham, Gordon et al. 2011). Clinical data comes from a limited number of studies and these do not always support the findings of *in vitro* studies. In a cohort study, Falagas *et al.* found that cure of infection was not improved with colisitin-meropenem combination therapy compared with colistin monotherapy (Falagas, Rafailidis et al. 2006). Nonetheless, there are some clinical studies that identify a synergistic effect with antibiotic combinations; a carbapenem and ampicillin-sulbactam combination has been shown to give a more favourable outcome than carbapenem monotherapy in a retrospective study of MDR *A. baumannii* bacteraemia infections in Taiwan (Kuo, Lai et al. 2007). A combination of rifampicin with both colistin and imipenem has shown high cure rates against carbapenem resistant *A. baumannii* infections in critically ill patients (Motaouakkil, Charra et al. 2006; Saballs, Pujol et al. 2006).

1.4. Mechanisms of Antibiotic Resistance

Acinetobacter spp. possess a wide range of antibiotic resistance mechanisms (Table 1.2) (Peleg, Seifert et al. 2008; Roca, Espinal et al. 2012) , both innate and acquired, chromosomal and plasmid borne, allowing this bacterium to survive challenge by

many classes of antibiotics. The plasticity of the *Acinetobacter* genome allows it to adapt well to antibiotic pressure by capturing antibiotic resistance genes. An 86 kb genomic resistance island (*Aba*R1) has been identified in clinical isolates of MDR *Acinetobacter* spp., harbouring 45 genes conferring resistance to various classes of antibiotics including β-lactams, aminoglycosides, tetracycline and chloramphenicol (Fournier, Vallenet et al. 2006). This highlights the remarkable ability of this organism to acquire large mobile genetic elements, allowing it to rapidly adapt to its surroundings. The most widespread mechanisms of resistance in *Acinetobacter* are modification of the target, degradation or inactivation of the antibiotic and reduced permeability and active efflux of the agent.

Target modification occurs in various systems in *Acinetobacter*. Mutations in *gyrA* and *parC* are common, resulting in modified DNA gyrase or topisomerase and preventing fluoroquinolones from interacting with the DNA-gyrase complex (Hamouda and Amyes 2004). Phosphoethanolamine modification of lipid A was shown to lead to resistance to colistin in *Acinetobacter* by reducing the affinity of lipopolysaccharide (LPS) for the antibiotic (Beceiro, Llobet et al. 2011).

β-lactamase enzymes confer resistance to various β-lactam antibiotics in *Acinetobacter* spp. by inactivation of the drug. Chromosomally encoded AmpC cephalosporinases can be found in all strains of *A. baumannii* and increased expression due to an upstream insertion sequence (IS) element, IS*Aba*1, provides resistance to cephalosporins (Bou and Martinez-Beltran 2000; Hujer, Hujer et al. 2006; Ruiz, Marti et al. 2007) Extended spectrum β-lactamases (ESBLs) including

Table 1.2 . Resistance mechanisms previously identified in *Acinetobacter* species (Roca, Espinal et al. 2012).

Antimicrobial	Resistance mechanism	Protein
β-Lactams	Chromosomal cephalosporinase	AmpC
	Carbapenem-hydrolyzing class D β-lactamases	OXA-51-like, OXA-23-like, OXA-24/40-like, OXA-58-like, OXA-143-like
	Metallo-β-lactamases	IMP, VIM, SIM-1, NDM
	Other β-lactamases	TEM, SHV, SCO-1, CARB, PER, VEB, CTX-M, GES, KPC, OXA-2, 10, 20, 37
	Decreased permeability	CarO, 47 kDa OMP, 44 kDa OMP, 37 kDa OMP, 33–36 kDa OMP, 22–33 kDa OMP, HMP-AB, 43 kDa OMP
	Efflux pump	AdeABC, AdeIJK, AdeFGH, AdeDE, AdeXYZ
	Modified penicillin-binding proteins	PBP
Aminoglycosides	Aminoglycoside-modifying enzymes	Acetyltransferases, Nucleotidyltransferases, Phosphotransferases

Antimicrobial	Resistance mechanism	Protein
	Target binding site modification	16S rRNA methylases
	Efflux	AdeABC, AbeM, AdeDE
Quinolones	Target site mutations	GyrA/ParC
	Efflux pump	AdeABC, AdeIJK, AdeFGH, AdeDE, AbeM, AbeS
Chloramphenicol	Efflux pump	AdeABC, AdeIJK, AdeFGH, AdeDE, AdeXYZ, CmlA, CraA, AbeM, AbeS
Tetracyclines	Efflux pump	TetA, TetB, AdeDE, AdeXYZ
	Ribosomal protection	TetM
Tigecycline	Efflux pump	AdeABC, AdeIJK
Polymyxins	Lipid A modification	PmrCAB
	Loss of lipopolysaccharide	LpxABC
	Porin loss	CarO, OmpA38, OmpA32, OmpW

VEB (Carbonne, Naas et al. 2005), PER (Naas, Bogaerts et al. 2006), TEM (Endimiani, Luzzaro et al. 2007), SHV (Huang, Mao et al. 2004) and CTX-M (Nagano, Nagano et al. 2004) have all been described in *A. baumannii*, and can be found both plasmid and chromosomally encoded. Carbapenem hydrolysing enzymes are the most clinically relevant β-lactamases in *Acinetobacter* and both metallo-β-lactamases (MBLs) and serine oxacillinases (OXA) have been identified in *Acinetobacter* (Poirel and Nordmann 2006). The most widespread of these are the OXA-type enzymes, which can be encoded chromosomally or on a plasmid. *bla*OXA-51-like enzymes are naturally occurring in *A. baumannii* and encoded chromosomally. In the presence of an upstream promoter, IS*AbaI*, *bla*OXA-51-like genes provide intrinsic resistance to carbapenems (Turton, Ward et al. 2006; Turton, Woodford et al. 2006). *bla*OXA-51-like genes have a high prevalence worldwide and have been described in several studies (e.g. Héritier, Poirel et al. 2005; Coelho, Woodford et al. 2006; Hujer, Hujer et al. 2006; Turton, Woodford et al. 2006; Evans, Brown et al. 2007). *bla*OXA-23-like, *bla*OXA-24-like and *bla*OXA-58 like gene clusters have also been described as conferring carbapenem resistance in *Acinetobacter* (Donald, Scaife et al. 2000; Afzal-Shah, Woodford et al. 2001; Da Silva, Quinteira et al. 2004; Boo, Walsh et al. 2006; Coelho, Woodford et al. 2006; Corvec, Poirel et al. 2007).

Loss of outer membrane proteins (OMPs) has also been implicated in *Acinetobacter* resistance to antibiotics. *Acinetobacter* possess few outer membrane porins, and intrinsic low level resistance can be partly attributed to low permeability of the outer membrane (Sato and Nakae 1991). Altered expression of OMPs in response to antibiotic challenge (Yun, Choi et al. 2008) can further reduce permeability to

antimicrobials and lead to MDR. Expression of OmpA38, OmpA32, CarO and OmpW is reduced in the presence of sub-MIC levels of tetracycline, suggesting a role for OMPs in tetracycline resistance (Yun, Choi et al. 2008). Loss of OMPs has been implicated in carbapenem resistance and is seen in clinical isolates worldwide (Bou, Cerveró et al. 2000; Tomás, Beceiro et al. 2005; Hwa, Subramaniam et al. 2010).

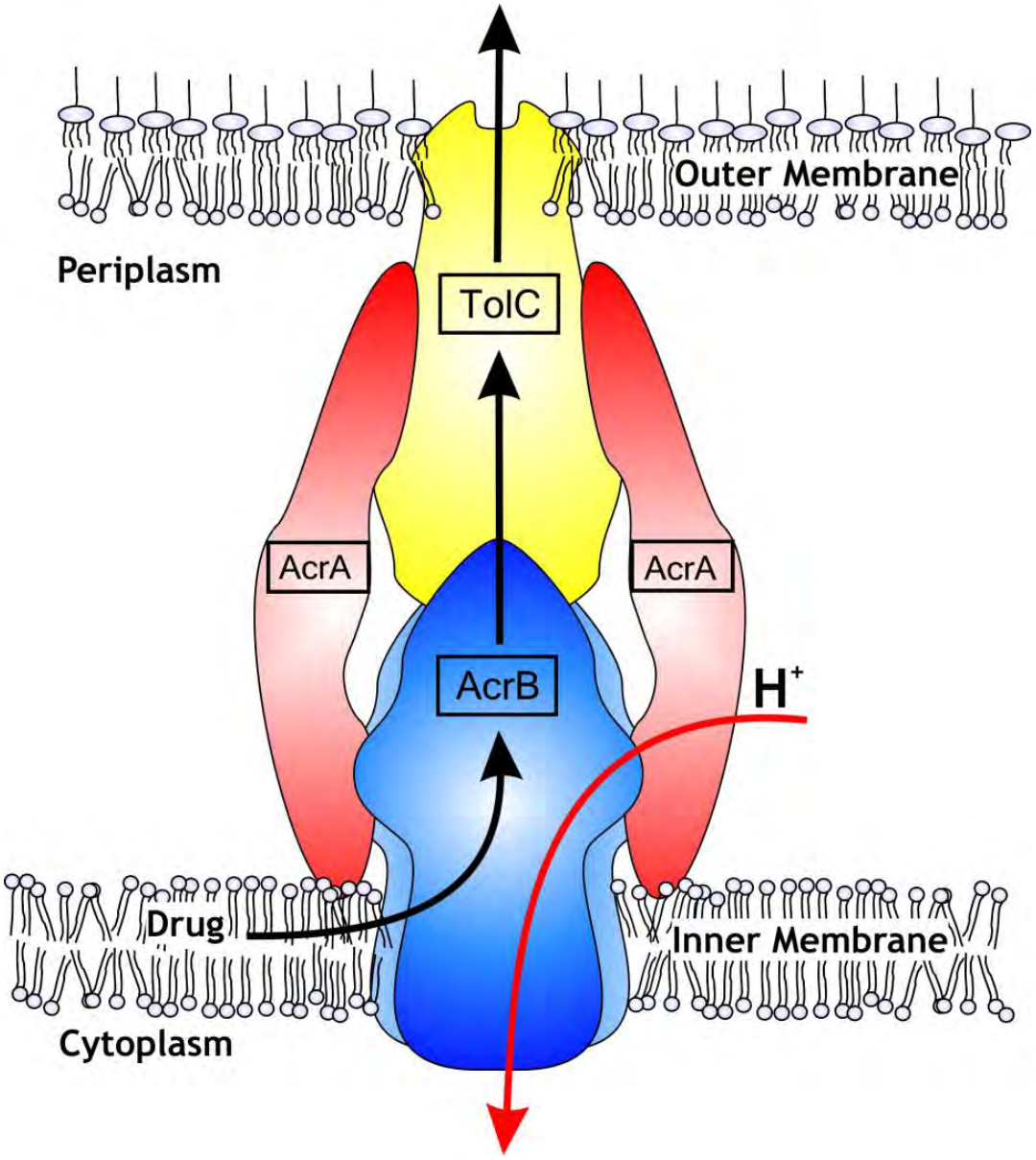
1.5. Efflux

Innate expression of efflux pumps in *Acinetobacter* allows a broad range of substrates to be removed from the cell, conferring resistance to various antibiotic classes. Increased expression of chromosomal efflux pumps and acquisition of additional efflux systems can then lead to MDR (Coyne, Courvalin et al. 2011).

1.5.1. RND pumps

The resistance nodulation division (RND) family are the most common efflux systems in MDR *Acinetobacter* and a number of pumps of this type have been identified in species belonging to the Acb complex. RND pumps in Gram negative bacteria comprise of three components, forming a tripartite pump. The efflux protein is located in the inner membrane, the OMP channel spans the outer membrane and a periplasmic accessory protein links the two (Figure 1.1) (Piddock 2006). There is often a high level of homology between proteins in this family and RND pump proteins identified in *A. baumannii* show some similarity to the MexXY-OprM and

Figure 1.1. Schematic representation of an assembled tripartite RND efflux pump based on the AcrAB-TolC pump of *Salmonella* (Blair and Piddock 2009).



MexD previously characterised in *Pseudomonas aeruginosa* and MtrC from *Neisseria gonorrhoeae* (Magnet, Courvalin et al. 2001; Coyne, Courvalin et al. 2011). Most work carried out to date on the RND family of efflux pumps comes from the Courvalin team and is based on analysis of a single strain of *A. baumannii,* BM4454. This may not necessarily represent the paradigm for the species and further work is required to confirm these findings in other strains.

AdeABC is the most well characterised RND system in *Acinetobacter*. Found in both *A. baumannii* and other clinically relevant species (Magnet, Courvalin et al. 2001; Roca, Espinal et al. 2011), it is chromosomally encoded but has only been identified in clinical isolates and only confers MDR when overexpressed (Magnet, Courvalin et al. 2001). MIC studies with BM4454 mutants in which the efflux pump proteins are not produced, reveal that substrates for this pump include aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol and trimethoprim (Magnet, Courvalin et al. 2001; Marchand, Damier-Piolle et al. 2004). The role of AdeABC in efflux of carbapenems is still debated. Studies of clinical MDR isolates have shown a correlation between carbapenem resistance and overexpression of AdeABC (Héritier, Poirel et al. 2005; Huang, Sun et al. 2008). However, addition of efflux inhibitors carbonyl cyanide-*m*-chlorphenyl hydrazone (CCCP) did not affect carbapenem MICs in imipenem resistant clinical isolates from Greece (Pournaras, Markogiannakis et al. 2006). AdeABC is encoded as an operon, *adeABC*, and is regulated by the two-component system AdeRS. This system is encoded by *adeRS*, which is upstream and in the opposite direction to *adeABC* (Marchand, Damier-Piolle et al. 2004). AdeS is a sensor kinase and AdeR is its associated response regulator. Mutations in the AdeRS regulatory system are

associated with constitutive expression of AdeABC and MDR (Marchand, Damier-Piolle et al. 2004; Peleg, Adams et al. 2007). Overexpression of AdeABC has also been seen in a clinical isolate due to the insertion of IS*Aba1* into *adeS* (Ruzin, Keeney et al. 2007). However, MDR due to AdeABC overexpression has been seen in the absence of AdeRS mutations, suggesting another mechanism causing increased pump activity is also possible (Peleg, Adams et al. 2007). Inactivation of *adeB* causes loss of resistance to multiple antibiotics (Magnet, Courvalin et al. 2001), whereas inactivation of *adeC* does not have the same effect. This suggests that AdeC is not essential for MDR and that other OMPs may be recruited by AdeAB (Marchand, Damier-Piolle et al. 2004).

AdeIJK is found only in *A. baumannii* and has not been observed in any of the other members of the Acb complex. AdeJ shows 57% identity with AcrB from *Escherichia coli* and confers innate MDR (Damier-Piolle, Magnet et al. 2008; Lin, Ling et al. 2009). It exports β-lactams, fluoroquinolones, tetracyclines, tigecycline, lincosamides, rifampicin, chloramphenicol, co-trimoxazole, novobiocin and fusidic acid, but not aminoglycosides in BM4454 (Damier-Piolle, Magnet et al. 2008; Coyne, Guigon et al. 2010). AdeIJK can only be expressed at low levels in *A. baumannii* BM4454 and in *E. coli*, once expression reaches threshold levels the pump becomes toxic to the host (Damier-Piolle, Magnet et al. 2008; Coyne, Guigon et al. 2010). AdeIJK is encoded by the *adeIJK* operon. It is likely that the expression of this operon is regulated by a global mechanism as no local regulatory genes have yet been identified (Coyne, Guigon et al. 2010).

AdeFGH is encoded by the *adeFGH* operon but is not expressed in wild type *Acinetobacter* and so does not contribute to intrinsic resistance (Coyne, Rosenfeld et

al. 2010). Overexpression of the pump in BM4454 led to resistance to fluoroquinolones, chloramphenicol, trimethoprim, clindamycin, tetracyclines, tigecycline and sulfamethoxazole (Coyne, Rosenfeld et al. 2010). Spontaneous MDR mutants selected from BM4652 (BM4454Δ*adeABC*Δ*adeIJK*) on norfloxacin and chloramphenicol show mutations in a putative LysR-type transcriptional regulator, AdeL, located upstream of *adeFGH*. It is proposed that these mutations cause a constitutive phenotype, leading to increased expression of the AdeFGH efflux system (Coyne, Rosenfeld et al. 2010).

AdeDE was identified as a novel RND efflux system in *A. pittii* by Chau *et al.* (Chau, Chu et al. 2004). Encoded by *adeDE*, no OMP gene was found to be encoded as part of this operon. It is suggested that AdeDE recruits an OMP encoded elsewhere on the chromosome (Chau, Chu et al. 2004). Inactivation of AdeE in a clinical isolate reduced susceptibility to aminoglycosides, carbapenems, ceftazidime, fluoroquinolones, erythromycin, tetracycline, rifampin and chloramphenicol (Chau, Chu et al. 2004). Although initially described in *A. pittii*, AdeDE has also been observed in *A. nosocomialis* and *Acinetobacter gsp 17*. However, prevalence is still highest in *A. pittii*, with 70% of clinical isolates (n=83) studied containing the *adeDE* operon (Chu, Chau et al. 2006).

AdeXYZ is the other RND pump found in *Acinetobacter* spp. other than *A. baumannii*. It was identified in 90% of *A. pittii* isolates (n=83) tested in a study of blood culture isolates in China and was also been observed in *A. nosocomialis* and *A.* gsp. 17 (Chu, Chau et al. 2006). Although AdeXYZ has not been well characterised, the pump shows high homology with AdeIJK, suggesting a similar

function and possibly substrate range (Chu, Chau et al. 2006; Damier-Piolle, Magnet et al. 2008; Coyne, Courvalin et al. 2011).

1.5.2. Non-RND pumps

Acinetobacter spp. also possess a number of chromosomally encoded non-RND efflux systems: CraA contributes to intrinsic resistance to chloramphenicol (Roca, Marti et al. 2009), AmvA is a major facilitator superfamily (MFS) pump that exports erythromycin (Rajamohan, Srinivasan et al.), AbeM is a member of the multidrug and toxic compound extrusion (MATE) family and has a suggested role in resistance to a range of antibiotics and dyes (Su, Chen et al. 2005), and AbeS is a small multidrug resistance (SMR) efflux pump involved in chloramphenicol, fluoroquinolone, erythromycin, novobiocin and dye and detergent resistance (Srinivasan, Rajamohan et al. 2009).

Various acquired efflux systems have also been identified in *Acinetobacter* spp., carried either on plasmids, transposons or resistance islands (Vila, Marti et al. 2007). Most common are pumps of the MFS type that give resistance to tetracycline. The TetA pump, has been observed in 13.6% of tetracycline resistant strains (n=59) of *A. baumannii* and the TetB pump, conferring tetracycline and minocycline resistance, in 66% (n=59) (Marti, Fernandez-Cuenca et al. 2006). In a study of 32 clinical isolates of *A. baumannii* conducted by Mak *et al.*, 28 contained the *tetB* gene whilst *tetA* was not present in any isolate (Mak, Kim et al. 2009). The TetG and TetR MFS pumps have also been observed in *Acinetobacter* spp., as part of the acquired IS*AbaR1* resistance island (Fournier, Vallenet et al. 2006). The MFS pumps CmlA and FloR,

and the SMR pump QacE are also acquired on the IS*AbaR1* resistance island identified in *A. baumannii* AYE (Fournier, Vallenet et al. 2006).

1.6. Measurement of antibiotic accumulation by *Acinetobacter*

1.6.1. Background to this project

Acinetobacter is rapidly emerging as a highly significant pathogen in hospitals worldwide. Its propensity to acquire foreign resistance determinants and upregulate intrinsic mechanisms allows this organism to adapt and survive in the clinical setting. Key to its ability to resist challenge by a wide range of antibiotics is the overexpression of efflux pumps. The present study is part of a collaborative programme of research to characterise efflux pumps that confer clinically relevant MDR in order to guide antimicrobial therapy.

1.6.2. Hypotheses

- Previously uncharacterised putative efflux pumps revealed through comparative genomics contribute towards antimicrobial resistance in *Acinetobacter* species.
- Deletion of efflux pump genes, or inhibition of efflux pumps, in clinical isolates of *A. baumannii* will lead to reduced efflux and therefore reduced resistance to clinically relevant antibiotics.

1.6.3. Aims and Objectives

1. Adapt the method for measuring the accumulation of Hoechst 33342 by *Enterobacteriaceae* for use with *A. baumannii*.
2. Determine whether the levels of accumulation of Hoechst 33342 by *A. baumannii* are comparable with those of ethidium bromide and norfloxacin.
3. To use specific pump deletion mutants to
    a. determine the MICs of selected antimicrobial compounds to aid identification of efflux pumps that efflux clinically relevant antibiotics.
    b. measure the levels of efflux in pump deletion mutants and to identify compounds which inhibit efflux of Hoechst 33342, ethidium bromide and norfloxacin.

Chapter Two: Materials and Methods

2. Materials and Methods

2.1. Bacterial strains, growth, storage and identification

A. baumannii strain AYE was selected as a reference MDR strain for use in this study. AYE is epidemic in France and is associated with a mortality of 26% in infected patients (Poirel, Menuteau et al. 2003). This strain was chosen as a fully annotated genome sequence is available and has shown AYE to contain 52 antibiotic resistance genes, including an 86kb resistance island, harbouring 45 antibiotic resistance genes (Fournier, Vallenet et al. 2006). Bioinformatic analysis using AciBase revealed that AYE contained all previously described efflux pumps and the majority of predicted efflux pumps (L. Evans & L. J. V. Piddock, unpublished data).

Clinical isolates cultured from hospital infections in Singapore were provided by Kim Lee Chua. R1, R2 and R3 were representative of antibiotic resistant isolates, and S1, S2 and S3 were representative of antibiotic susceptible isolates (Table 2.1). Efflux pump deletion mutants were created in R2 and S1 by Kim Lee Chua (manuscript in preparation). These isolates were selected as representative of antibiotic resistant and susceptible clinical isolates in infections in Singapore hospitals. *adeFGH, adeIJK* and *A1S_1649* were deleted individually and in combination to give strains R2-Δ*adeFGH,* R2-Δ*adeIJK,* R2-Δ*adeFGH*Δ*adeIJK,* and R2Δ*adeFGH*ΔP7 (Table 2.1). 'Pump 7' (P7) is a putative RND family drug transporter identified in ATCC17978. a*deB* was deleted in S1 to give S1Δ*adeB*. AB211 and AB211Δ*adeB* were provided by Mike Hornsey (Queen Mary's School of Medicine and Dentistry, London). AB211 is a tigecycline resistant, post-therapy clinical isolate. AB211Δ*adeB* was created by Hornsey *et al*. by insertional inactivation of *adeB* in AB211.

Table 2.1. Strains used in this study

Code	Source	Reference
AYE	MDR clinical isolate (France)	Poirel *et al.*, 2003
R1	Resistant clinical isolate (Singapore)	Kim Lee Chua, unpublished
R2	Resistant clinical isolate (Singapore)	Kim Lee Chua, unpublished
R3	Resistant clinical isolate (Singapore)	Kim Lee Chua, unpublished
S1	Susceptible clinical isolate (Singapore)	Kim Lee Chua, unpublished
S2	Susceptible clinical isolate (Singapore)	Kim Lee Chua, unpublished
S3	Susceptible clinical isolate (Singapore)	Kim Lee Chua, unpublished
S1 Δ*adeB*	*adeB* pump deletion mutant in S1	Kim Lee Chua, unpublished
R2Δ*adeFGH*	*adeFGH* pump deletion mutant in R2	Kim Lee Chua, unpublished

R2Δ*adeIJK*	*adeIJK* pump deletion mutant in R2	Kim Lee Chua, unpublished
R2ΔadeFGHΔadeIJK	*adeFGH* and *adeFGH* double pump deletion mutant in R2	Kim Lee Chua, unpublished
R2Δ*adeFGH*ΔP7	*adeFGH* and P7 double pump deletion mutant in R2	Kim Lee Chua, unpublished
S1Δ*adeB*	*adeB* pump deletion mutant in S1	Kim Lee Chua, unpublished
AB211	Tigecycline resistant clinical isolate (UK)	Hornsey *et al.,* 2011
AB211Δ*adeB*	*adeB* pump deletion mutant in AB211	Hornsey *et al.,* 2011

All strains were routinely grown on Luria-Bertani (LB) agar (Sigma-Aldrich Ltd., UK), supplemented with appropriate antibiotics where necessary, and incubated overnight at 37°C. Overnight cultures were grown in LB broth (Sigma-Aldrich Ltd., UK), supplemented with appropriate antibiotics where necessary, and incubated overnight at 37°C with aeration (Table 2.2). Purity of cultures was confirmed by growth as opaque pink colonies on MacConkey agar. Colonies were confirmed as Gram-negative coccobacilli by Gram staining and microscopic observation. All strains and isolates were confirmed as *Acinetobacter* species by a *gyrB* PCR developed by Higgins *et al*. (Higgins, Wisplinghoff et al. 2007; Higgins, Lehmann et al. 2010). This protocol uses seven primers in a multiplex PCR to produce different sized amplimers, allowing differentiation between *A. baumannii, A. calcoaceticus, A. pittii* and *A. nosocomialis.* All primers were produced by Invitrogen (Invitrogen Ltd., UK; listed in Table 2.3). All primers were re-hydrated with UltraPure water (Invitrogen, UK) to a concentration of 100 µM; 25 µM working stock solutions of each primer were made. All primers were stored at -20°C. Polymerase chain reactions (PCR) were set up using 22 µl of 1.1 x ReddyMix PCR Master Mix (Thermo Scientific, UK, AB – 0794/B). A 25 µM stock of each of the seven primers was combined in equal volumes and 2 µl of this stock and 1 µl of whole cell lysate was added to the master mix. Whole cell lysates were prepared by adding a loopful of bacterial culture from an LB agar plate to 100 µl of sterile water and heating at 99ºC for ten minutes. The initial denaturation step was carried out at 94°C for two minutes. This was followed by 25 cycles of denaturation at 94°C for one minute, annealing at 60°C for 30 seconds and elongation at 72°C for one minute. This was followed by a final elongation stage at 72°C for ten minutes (Table 2.4, PCR 1). Products were separated by agarose gel

electrophoresis in 1% Tris-Boric acid-EDTA (TBE) at 100 volts. Gels were prepared by adding 1 g of electrophoresis grade agarose (Invitrogen Ltd, UK, 15510-027) to 50 ml of 1% TBE. The agarose was dissolved by heating in a 650 W microwave for approximately 90 seconds. Another 50 ml of TBE was then added before ethidium bromide was added to a final concentration of 0.1 µg/ml. The molten agarose was poured into a gel tray and allowed to set. Five microlitres of each sample was then loaded onto the gel along with a DNA ladder (Bioline).

2.2. Verification of efflux pump gene deletions

Primers designed by Kim Lee Chua (data unpublished) were used to confirm gene deletions in R2Δ*adeFGH,* R2Δ*adeIJK, R2ΔadeIJKΔadeFGH* and R2Δ*adeFGH*ΔP7 (Table 2.3). Primers were external to the deleted genes and designed to produce different sized amplimers with parental and mutant strains. Three PCRs were carried out with each pump deletion mutant and the parental strain, R2, to check for deletion of *adeIJK, adeFGH* and the pump 7 gene in each. The reaction contained 11 µl Reddymix Extensor PCR Mastermix (1.1) (Thermo Scientific, UK - AB-0794/B), 11 µl sterile water, 1 µl whole cell lysate and 1 µl forward primer and 1 µl reverse primer (25 µM) for each PCR (Table 2.4, PCR 2, 3, 4). The initial denaturation step was two minutes at 94ºC followed by 30 cycles of denaturation at 94ºC for ten seconds, annealing at 50ºC for 30 seconds and elongation at 68ºC for five minutes. The final denaturation step was for seven minutes at 68ºC. Products were separated by electrophoresis on a 1 % agarose gel as described previously (Section 2.1).

Table 2.2. Antibiotics, dyes and EPIs used in this study

Agent	Solvent	Supplier
Nalidixic acid	Distilled water	Sigma-Aldrich, UK
Ciprofloxacin	SDW and acetic acid	Sigma-Aldrich, UK
Kanamycin	Distilled water	Sigma-Aldrich, UK
Gentamicin	Distilled water	Sigma-Aldrich, UK
Ceftazidime	0.1 M sodium hydroxide	Sigma-Aldrich, UK
Imipenem	n/a	Biomerieux, UK
Meropenem	n/a	Biomerieux, UK
Piperacillin	Distilled water	Pfizer, UK
Ampicillin	1 M Sodium bicarbonate	Sigma-Aldrich, UK

Sulbactam	Distilled water	Pfizer, UK
Chloramphenicol	70 % methanol	Sigma-Aldrich, UK
Tigecycline	Distilled water	Pfizer, UK
Tetracycline	Distilled water	Sigma-Aldrich, UK
Erythromycin	100 % ethanol	Sigma-Aldrich, UK
Colistin	Distilled water	Sigma-Aldrich, UK
Trimethoprim	70 % ethanol	Sigma-Aldrich, UK
Hoechst	Distilled water	Sigma-Aldrich, UK
Ethidium bromide	Distilled water	Sigma-Aldrich, UK
Norfloxacin	Acetic acid and distilled water	Sigma-Aldrich, UK
CCCP	Dimethyl sulfoxide	Sigma-Aldrich, UK

PAβN	Distilled water	Sigma-Aldrich, UK
Verapamil	Distilled water	Sigma-Aldrich, UK

Table 2.3. Primers used in this study

Primer number	Description	Sequence	Reference
1079	*A. baumannii gyrB* forward	GTTCCTGATCCGAAATTCTCG	Higgins *et al.*, 2007
1080	*A. baumannii* and *A. nosocomialis gyrB* forward	CACGCCGTAAGAGTGCATTA	Higgins *et al.*, 2007
1081	*A. baumannii* and *A. nosocomialis gyrB* reverse	AACGGAGCTTGTCAGGGTA	Higgins *et al.*, 2007
1082	*A. calcoaceticus gyrB* forward	GACAACAGTTATAAGGTTTCAGGTG	Higgins *et al.*, 2010
1083	*A. calcoaceticus gyrB* reverse	CCGCTATCTGTATCCGCAGTA	Higgins *et al.*, 2010
1084	*A. pittii gyrB* forward	GATAACAGCTATAAAGTTTCAGGTGGT	Higgins *et al.*, 2010
1085	*A. pittii gyrB* reverse	CAAAAACGTACAGTTGTACCACTGC	Higgins *et al.*, 2010

1479	*adeB* forward check	GCAATCACCAAAGGAATTAC	Kim Lee Chua (unpublished)
1480	*adeB* reverse check	ATCTATTGGGCTGATATTAC	Kim Lee Chua (unpublished)
1481	*adeI* forward check	GCGATAAAGTCATTGTTGATG	Kim Lee Chua (unpublished)
1482	*adeK* reverse check	GTTCTGTGTAATCTGATATTGCTG	Kim Lee Chua (unpublished)
1483	*adeFGH* external check	ATTAAGTTTTGAGCGTATAAGCTTC	Kim Lee Chua (unpublished)
1484	*adeFGH* external check	AAGACGTAACTGGTAAAGAT	Kim Lee Chua (unpublished)

1104	Pump 7 external check	ACAGTCCGCAGCAGAGATTT	Kim Lee Chua (unpublished)
1105	Pump 7 external check	GCGGTTTTGCTCTGTTTAAT	Kim Lee Chua (unpublished)

Table 2.4. Parameters and primers used for PCRs

		PCR parameters					
PCR	**Primers**	**Initial Denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**	**Number of cycles**
1. *gyrB* multiplex Appendix 1	1079 1080 1081 1082 1083 1084 1085	94ºC (2 min)	94ºC (1 min)	60ºC (30 sec)	72ºC (1 min)	72ºC (10 min)	25

2. *adeABC* check Appendix 2	1479 1480	94ºC (2 min)	94ºC (10 sec)	54ºC (30 sec)	68ºC (5 min)	68ºC (7 min)	30
3. adeFGH check Appendix 3	1483 1484	94ºC (2 min)	94ºC (10 sec)	54ºC (30 sec)	68ºC (5 min)	68ºC (7 min)	30
4. adeIJK check Appendix 4	1481 1482	94ºC (2 min)	94ºC (10 sec)	54ºC (30 sec)	68ºC (5 min)	68ºC (7 min)	30
5. Pump 7 check Appendix 5	1105 1106	94ºC (2 min)	94ºC (10 sec)	54ºC (30 sec)	68ºC (5 min)	68ºC (7 min)	30

2.3. Phenotypic characterisation of clinical isolates and efflux pump deletion mutants

The phenotypes of susceptible clinical isolates S1, S2 and S3 were compared with those of resistant clinical isolates R1, R2 and R3. The effect of the deletion of efflux pump genes in mutants R2Δ*adeFGH,* R2Δ*adeIJK, R2ΔadeFGHΔadeIJK* and R2Δ*adeFGH*ΔP7 was determined by comparing the phenotype to that of the parental strain, R2. The effect of the deletion of efflux pump gene *adeB* was determined by comparing the phenotype of S1Δ*adeB* to that of the parental strain, S1.

2.3.1. Bacterial growth kinetics

Bacterial strains were grown with aeration in LB broth at 37°C overnight. Bacterial cultures were diluted 1:100 in sterile LB broth and 100 µl of this suspension was added to each well of a clear 96 well microtitre tray. Optical density (OD) at an absorbance of 600 nm was measured over 16 hours in the BMG FLUOstar Optima (BMG labtech, UK) at 37°C. Each experiment included three biological replicates and three technical replicates of each bacterial strain. The FLUOstar is sensitive to an OD 600 nm of between 0.0 and 4.0 and reproducibility is ±0.010 for the OD range of 0.0-2.0 (www.BMG-labetech.com).

2.3.2. Determination of the minimum inhibitory concentrations of antibiotics

The MICs of antibiotics and efflux inhibitors (EIs) (Table 2.2) were determined by the agar doubling dilution method according to the British Society for Antimicrobial Chemotherapy (BSAC) standard methodology (Andrews 2001). Stock solutions of 10,000 µg/ml of antibiotics were made up and appropriate amounts added to 20 ml of cooled, molten Iso-sensitest agar (Oxoid, UK) in sterile universals. This was dispensed aseptically into petri dishes and allowed to set. Overnight cultures of each bacterial strain to be tested were diluted 1:100 to give approximately 10^7 CFU/ml. Each agar plate was inoculated with 1 µl of diluted culture giving approximately 10^4 colony forming units (CFU) per spot. Plates were incubated overnight at 37ºC and read according to BSAC guidelines. The MIC was determined as the lowest concentration of antibiotic that inhibited visible growth (Andrews 2001). MICs of imipenem and meropenem were determined by E-test (Biomerieux, UK). Colonies grown overnight on LB agar were emulsified in sterile water to a concentration of approximately 10^8 CFU/ml. A sterile swab was soaked in the suspension and the surface of an iso-sensitest agar plate streaked in three directions. The plate was allowed to dry for approximately 15 minutes before E-test gradient strips were positioned on the surface. Plates were incubated overnight at 37ºC and read according to the manufacturer's instructions. Resistance was determined using BSAC recommended breakpoint concentrations for *A. baumannii*. MDR strains were defined as those resistant to three or more antimicrobial classes.

2.3.3. Development of a Hoechst 33342 accumulation assay to measure efflux in *A. baumannii*

The bisbenzamide dye Hoechst (H) 33342 is a fluorescent dye that is readily taken up by living cells and fluoresces with a high quantum yield upon binding to DNA and when in the hydrophobic environment of the lipid membrane. A H33342 accumulation assay has previously been used to measure efflux and identify MDR Gram-negative *Enterobacteriaceae* (Coldham, Webber et al. 2010). This assay was modified for use with *A. baumannii* and used to measure differences in efflux between clinical isolates R1-3 and S1-3, pump deletion mutant AB211Δ*adeB* and its parental strain AB211 and pump deletion mutants S1Δ*adeB,* R2Δ*adeFGH,* R2Δ*adeIJK, R2ΔadeFGHΔadeIJK* and R2Δ*adeFGH*ΔP7 and the parental strains, S1 and R2.

The assay was initially developed using isolates R1, R2, R3, S1, S2 and S3. First, viability of the clinical isolates at 1x, 2x and 4x H33342 concentrations used in the assay was determined. A stock solution of H33342 was made up and appropriate amounts added to 20 ml of cooled, molten Iso-sensitest agar (Oxoid, UK) in sterile universals. This was dispensed aseptically into petri dishes and allowed to set. Overnight cultures of each bacterial strain to be tested were diluted 1:100 to give approximately 10^7 CFU/ml. Each agar plate was inoculated with 1 µl of diluted culture giving approximately 10^4 CFU/spot. Plates were incubated overnight at 37º and plates were examined for inhibition of growth.

Clinical isolates R1, R2, R3, S1, S2 and S3, and reference strain AYE, were used to assess the suitability of the H33342 accumulation assay for determining differences in efflux between clinical isolates of *A. baumannii*. The ability of the assay to identify altered efflux in strains lacking efflux pumps was assessed with AB211 and efflux

pump deletion mutant AB211Δ*adeB* (Hornsey, Loman et al. 2011). Bacterial strains to be tested were grown with aeration in LB broth at 37°C overnight. A 4% inoculum (120 µl in 3 ml) of bacterial culture was added to fresh LB broth. This suspension was incubated with aeration at 37°C until the cells reached mid-logarithmic phase. Cells were harvested by centrifugation at 2200 x *g* in a Hereaus Megafuge 40R (Thermo Scientific, UK) for ten minutes at room temperature and resuspended in phosphate buffered saline (PBS) at room temperature. The OD at 600 nm was adjusted to either 0.1, 0.2, 0.3 or 0.5 and 180 µl of the cell suspension was dispensed into the wells of a black microtitre tray (Corning, Amsterdam). Assays with cell suspensions of varying optical density were carried out to assess the optimal number of bacterial cells to use in the assay. A 25 µM Hoechst H33342 stock was prepared to give a final concentration of 2.5 µM and loaded into the FLUOstar OPTIMA for injection after an initial reading. Fluorescence was measured over 117 minutes at excitation and emission wavelengths of 350 nm and 461 nm respectively in a FLUOstar OPTIMA. The level at which maximum fluorescence was reached and remained unchanged within the time period of the assay was taken as the steady state accumulation level. Student's *t*-tests were carried out to compare the steady state accumulation level of AB211Δ*adeB* with the parental strain, AB211; *P* values <0.05 were taken as significant. In order to quantitatively compare the efflux rate of the strains, the time taken for a 4 fold increase in fluorescence to occur after H33342 injection was calculated. Each assay was repeated three times with two biological replicates.

The assay was developed further for use with the efflux inhibitors (EIs) carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) and phenyl-arginine-β-naphthylamide

(PAβN). Concentrations of EIs non-inhibitory to the growth of *A. baumannii* clinical isolates, R1, R2, R3, S1, S2 and S3 were determined by MIC determination and growth kinetic assays. MICs were carried out by the agar doubling dilution method as previously described (Section 2.3.2). Growth kinetics in the presence of sub-MIC concentrations of EIs were then carried out to simulate conditions in the H33342 accumulation assay and ensure no inhibition of growth. Bacterial strains to be tested were grown with aeration in LB broth at 37°C overnight. A 4% inoculum (120 µl in 3 ml) of bacterial culture was added to fresh LB broth. This suspension was incubated with aeration at 37°C until the cells reached mig-logarithmic phase, which is equal to an OD at 600 nm of 0.6. Cells were harvested by centrifugation at 2200 x *g* in a Hereaus Megfuge 40R for ten minutes at room temperature and resuspended in 3 ml sterile LB broth. The OD at 600 nm of the suspension was measured and adjusted to 0.5 to standardise the number of cells in each culture and to simulate the conditions used in the H33342 accumulation assay. 176 µl of this suspension was added to each well of a clear 96 well microtitre tray, along with 4 µl of EI at the required concentration. OD at an absorbance of 600 nm was measured over 16 hours in a BMG FLUOstar OPTIMA (BMG, UK) at 37°C. Each experiment included three biological replicates and three technical replicates of each bacterial strain. Student's t-test were carried out to determine significant difference between growth with and without EI; *P* values <0.05 were taken as significant. Concentrations of EIs that did not restrict growth in the two hour time period of the H33342 accumulation assay were used in all experiments

2.3.4 Hoechst 33342 accumulation in efflux pump deletion mutants

The assay was carried out as described previously (Section 2.3.3). After resuspension in PBS, bacterial strains were adjusted to an OD at 600 nm of 0.5 and either 180 µl of the cell suspension, or 176 µl of cell suspension and 4 µl of EPI at the required concentration was dispensed into the wells of a black microtitre tray. Concentrations of 50 µM CCCP and 100 µM PAβN were found previously to be non-inhibitory to growth and were used in this and all subsequent assays. A 25 µM H33342 stock solution was prepared and loaded into the FLUOstar OPTIMA for injection after an initial reading; this gave a final concentration of 2.5 µM. Fluorescence was measured over 117 minutes at excitation and emission wavelengths of 350 nm and 461 nm, respectively, in a FLUOstar OPTIMA. The level at which maximum fluorescence was reached and remained unchanged within the time period of the assay was taken as the steady state accumulation level. As fluorescence is an arbitrary number and can vary between assays, fold change in fluorescence of mutants compared to the parent strain, and of strains with EI compared to without EI was calculated to enable comparison between individual runs. Student's *t*-tests were carried out to compare the steady state accumulation level of each mutant with the parental strain and of each strain with EI with the same strain without EI; *P* values <0.05 were taken as significant. Each assay was repeated at least three times with two biological replicates.

2.3.5 Ethidium bromide accumulation

Bacterial strains to be tested were grown with aeration in LB broth at 37°C overnight. A 4% inoculum (120 µl in 3 ml) of bacterial culture was added to fresh LB broth. This suspension was incubated with aeration at 37°C until the cells reached mid-logarithmic phase, which is equal to an OD at 600 nm of 0.6. Cells were harvested by centrifugation at 2200 x *g* in a Hereaus Megafuge 40R for 10 min at 4ºC and resuspended in 1M sodium phosphate buffer with 5 % glucose. The OD at 600 nm was adjusted to 0.5 and either 180 µl of the cell suspension, or 176 µl of cell suspension and 4 µl of EI at the required concentration was dispensed into the wells of a black microtitre tray. A 1 mM ethidium bromide stock was prepared and loaded into a FLUOstar OPTIMA for injection after an initial reading, to a final concentration of 0.1 mM. Fluorescence was measured over 117 minutes at excitation and emission wavelengths of 530 nm and 600 nm respectively in a FLUOstar OPTIMA. The level at which maximum fluorescence was reached and remained unchanged within the time period of the assay was taken as the steady state accumulation level. As fluorescence is an arbitrary number and can vary between assays, fold change in fluorescence of mutants compared to the parent strain, and of strains with EI compared to without EI was calculated to enable comparison between individual runs. Student's *t*-tests were carried out to compare the steady state accumulation level of each mutant with the parental strain; *P* values <0.05 were taken as significant. Each assay was repeated three times with two biological replicates.

2.3.6 Norfloxacin accumulation

Dry cell weights were determined for parental strain, R2, and reference strain, AYE. Bacterial strains were grown with aeration in Iso-sensitest broth at 37°C overnight. A 4% inoculum (10 ml in 250 ml) of bacterial culture was added to fresh Iso-sensitest broth. This suspension was incubated with aeration at 37°C. 20 ml samples were taken at an OD at 660 nm of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.65, 0.7, 0.75, 0.8 and 0.9 and placed in dry, pre-weighed universal tubes. Samples were centrifuged at 2200 x *g* in a Hereaus Megafuge 40R for 10 min at room temperature and the supernatant discarded. Tubes containing pellet were placed in a desiccator and weighed every 24 hours until weight reached a stable point (seven days). Cell weight per ml of culture was calculated and a standard curve of OD at 660 nm against dry cell weight was plotted.

Bacterial strains to be tested were grown with aeration in iso-sensitest broth at 37°C overnight. A 4 % inoculum (10 ml in 250 ml) of bacterial culture was added to fresh Iso-sensitest broth and incubated with aeration at 37°C until the cells reached mid-logarithmic phase, which is equal to an OD at 660 nm of 0.55. Cells were harvested by centrifugation at 4500 x *g* in a Hereaus Megafuge 40R for 20 minutes at 4ºC. Cells were resuspended and washed twice in 10 ml cold 50 mM phosphate buffer. Cells were resuspended to an OD at 660 nm of 20 in cold 50 mM phosphate buffer and incubated at 37ºC with stirring for ten minutes. At t=0, a 500 µl sample of each cell suspension was transferred to a tube containing 1 ml 50 mM phosphate buffer on ice and 10 µl of a 1000 µg/ml stock solution of norfloxacin was added to a final concentration of 10 µg/ml. To measure the effect of EIs on uptake, EIs were also added at t=0 if required. At t=300 seconds another 500 µl sample was transferred to

a microcapped centrifuge tube containing 1 ml 50 mM phosphate buffer on ice and all samples were centrifuged at 13,000 x *g* in a Hereaus Megafuge 40R for five minutes at 4ºC. Cell pellets were resuspended and washed twice in 1 ml cold 50 mM phosphate buffer to remove bound norfloxacin. Cell pellets were resuspended in 1 ml 0.1 M glycine (pH 3) and incubated overnight at room temperature in the dark. Samples were then centrifuged at room temperature for five minutes at 13,000 x *g.* The supernatant was transferred to a fresh tube and centrifuged as before. Samples were diluted 1:100 in 0.1 M glycine (pH 3) and fluorescence was measured in an LS 45 fluorescence spectrophotometer (Perkin Elmer) at an excitation and emission wavelength of 281 nm and 440 nm respectively. Standards of 0.001, 0.01 and 0.03 µg/ml norfloxacin in 0.1 M glycine were used to calculate concentration of norfloxacin in each sample, and from this dry cell weight curves were used to calculate ng of norfloxacin per mg of bacterial cells. Student's *t*-tests were carried out to compare concentrations of norfloxacin in each mutant with the parental strain, and of each strain with EI with the same strain without EI; *P* values <0.05 were taken as significant. Each assay was repeated three times with a single biological replicate.

Chapter Three: Measurement of efflux in *A. baumannii* clinical isolates and efflux pump deletion mutants

Chapter 3. Measurement of efflux in *A. baumannii* clinical isolates and efflux pump deletion mutants

3.1. Background

The bisbenzamide dye Hoechst (H) 33342 is a fluorescent dye that is readily taken up by living cells and fluoresces with a high quantum yield upon binding to DNA and when in the hydrophobic environment of the lipid membrane. This makes it an ideal fluorescent probe for the measurement of cellular accumulation.

3.2. Aims and Hypotheses

The aim was to assess the suitability of the H33342 accumulation method, previously used for the measurement of efflux in *Enterobacteriaceae* (Coldham, Webber et al. 2010), for *A. baumannii* and to use it to measure efflux in clinical isolates and pump deletion mutants. The hypothesis was that this assay could be used to compare levels of efflux in different clinical isolates of *A. baumannii* and to identify a difference in efflux between pump deletion mutants and parental strains.

3.3. Adaptation of the Hoechst 33342 accumulation method to measure dye accumulation in clinical isolates of *A. baumannii*

3.3.1. Phenotypic characterisation of strains used to develop H33342 assay

All strains were confirmed as *A. baumannii* using a *gyrB* multiplex PCR (Table 2.4, PCR number 1) (primer positions shown in Appendix 1); each isolate produced a 294 base pair (bp) amplicon and a 490 bp amplicon (Figure 3.1 and Table 3.1) characteristic of this species.

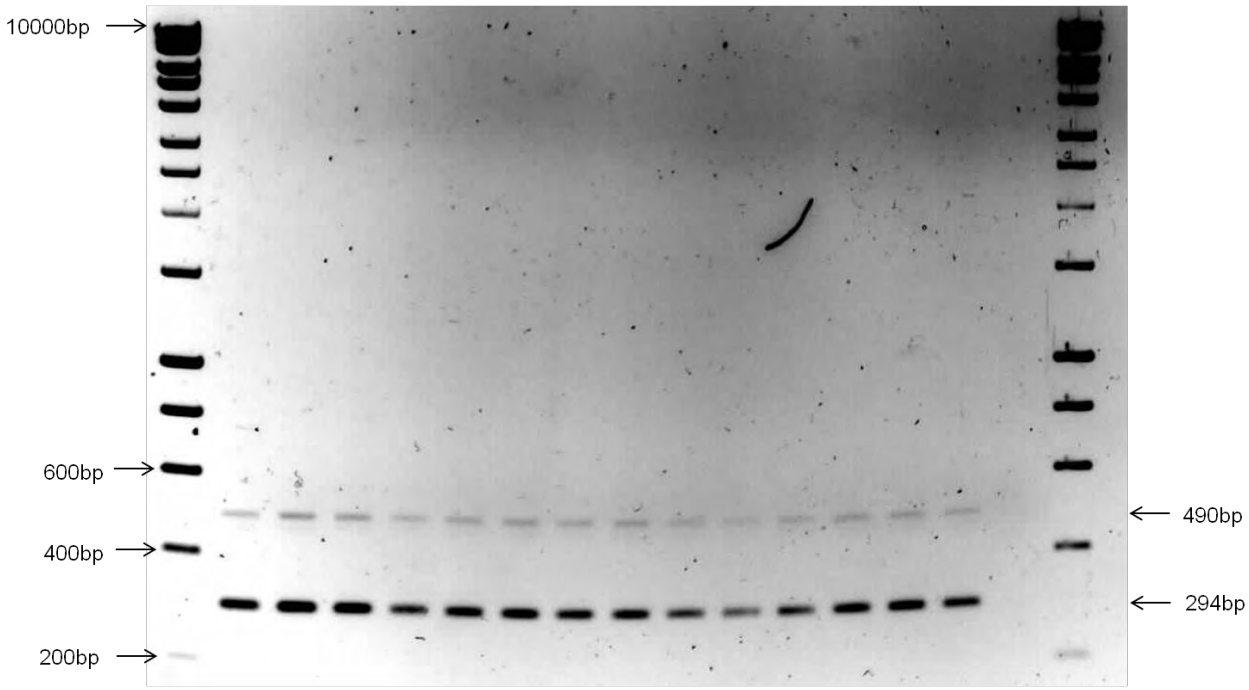
3.3.1.1. Bacterial growth kinetics

The generation time of antibiotic susceptible Singaporean clinical isolates was significantly lower than that of antibiotic resistant isolates (Figure 3.2 and Table 3.2). Generation time was lower than AYE in isolates S1, S2 and S3. Generation time was higher than AYE in isolates R1, R2 and R3. However, there was no significant difference in optical density at onset of stationary phase (500 min) between antibiotic susceptible isolates (S1, S2, S3) and antibiotic resistant isolates (R1, R2, R3). OD at 600 nm at onset of stationary phase was higher in isolates S1 and S2 than strain AYE. OD at 600 nm at onset of stationary phase was lower in isolate R3 than in strain AYE.

3.3.1.2. Antimicrobial susceptibility

AYE, R1, R2 and R3 were multidrug resistant (MDR) clinical isolates, resistant to three or more classes of antibiotics including β-lactams, fluoroquinolones, aminoglycosides and carbapenems (Table 3.1). Strain AYE and isolates R1 and R3 were susceptible to colistin (MIC ≤ 2). AYE, R2 and R3 were resistant to tigecycline. S1, S2 and S3 had resistance to fewer antibiotics. S1 and S3 were susceptible to ciprofloxacin, S2 and S3 were susceptible to colistin, S1 was susceptible to tigecycline, and S1, S2 and S3 were susceptible to carbapenems.

Figure 3.1. and Table 3.1. Confirmation of strains as *Acinetobacter baumannii* using a *gyrB* multiplex PCR.



Lane	Description	Predicted fragment size (bp)	Actual fragment size (bp)
1	AB211	294 + 490	294 + 490
2	AB211Δ*adeB*	294 + 490	294 + 490
3	R1	294 + 490	294 + 490
4	R2	294 + 490	294 + 490
5	R3	294 + 490	294 + 490
6	S1	294 + 490	294 + 490
7	S2	294 + 490	294 + 490
8	S3	294 + 490	294 + 490

9	AYE	294 + 490	294 + 490
10	S1Δ*adeB*	294 + 490	294 + 490
11	R2Δ*adeFGH*	294 + 490	294 + 490
12	R2Δ*adeIJK*	294 + 490	294 + 490
13	R2Δ*adeFGH*Δ*adeIJK*	294 + 490	294 + 490
14	R2Δ*adeFGH*ΔP7	294 + 490	294 + 490
15	Negative control	0	0

Figure 3.2. Growth kinetics of clinical isolates R1, R2, R3, S1, S2 and S3 at 37°C as determined by optical density.

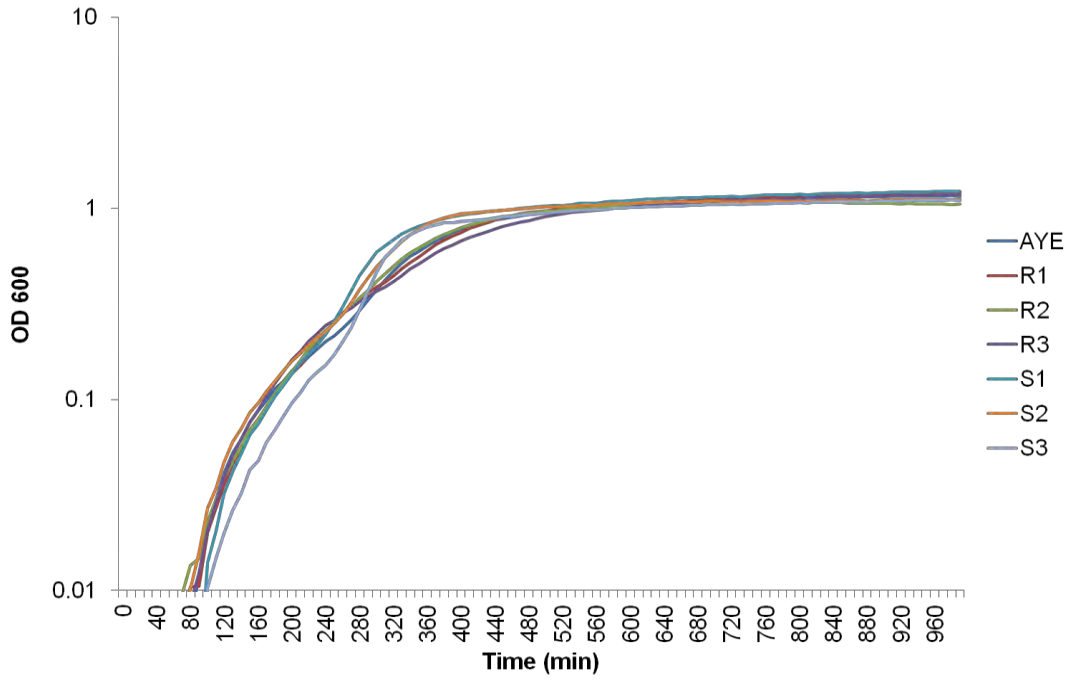


Table 3.2. Generation times and optical density at onset of stationary phase (540 min)

Strain	Generation Time (min)	OD 600 nm at onset of stationary phase (540 min)
AYE	78	0.99
S1	64	1.06
S2	71	1.04
S3	54	0.97
R1	95	1.01
R2	89	1.00
R3	119	0.96

3.3.1.3. Growth in the presence of efflux inhibitors and toxicity of H33342

To determine non-inhibitory concentrations of efflux inhibitors (EIs) for use in the H33342 accumulation assay, MICs of CCCP and PAβN against strain AYE and isolates S1, S2, S3, R1, R2 and R3 were determined (Table 3.3). MICs of both EIs were found to be lowest against S3 so this isolate was selected to determine growth kinetics in the presence of sub-MIC concentrations of EIs under the conditions of the H33342 accumulation assay. Concentrations of EIs that did not restrict growth in the two hour time period of the H33342 accumulation assay were used in all experiments. Concentrations of 50 μM CCCP and 100 μM PAβN had no effect on growth under conditions used in the H33342 assay (Figure 3.3). MICs of H33342 showed that all strains grew at concentrations up to 128 μg/ml (Table 3.3), much higher than the 2.5 μM concentration used in the accumulation assay.

3.3.2. Optimisation of the H33342 accumulation assay for measurement of efflux in clinical isolates and strain AYE

Previous work in this laboratory has shown that different levels of accumulation of H33342 can be distinguished for different strains of *Enterobacteriaceae* with a H33342 concentration of 2.5μM (Coldham, Webber et al. 2010). Therefore, this concentration was selected for use in experiments with *A. baumannii*. Assays were carried out using cell cultures adjusted to an OD at 600 nm of either 0.1, 0.2, 0.3 or 0.5. Cultures at an OD at 600 nm of 0.5 were found to produce measurable and

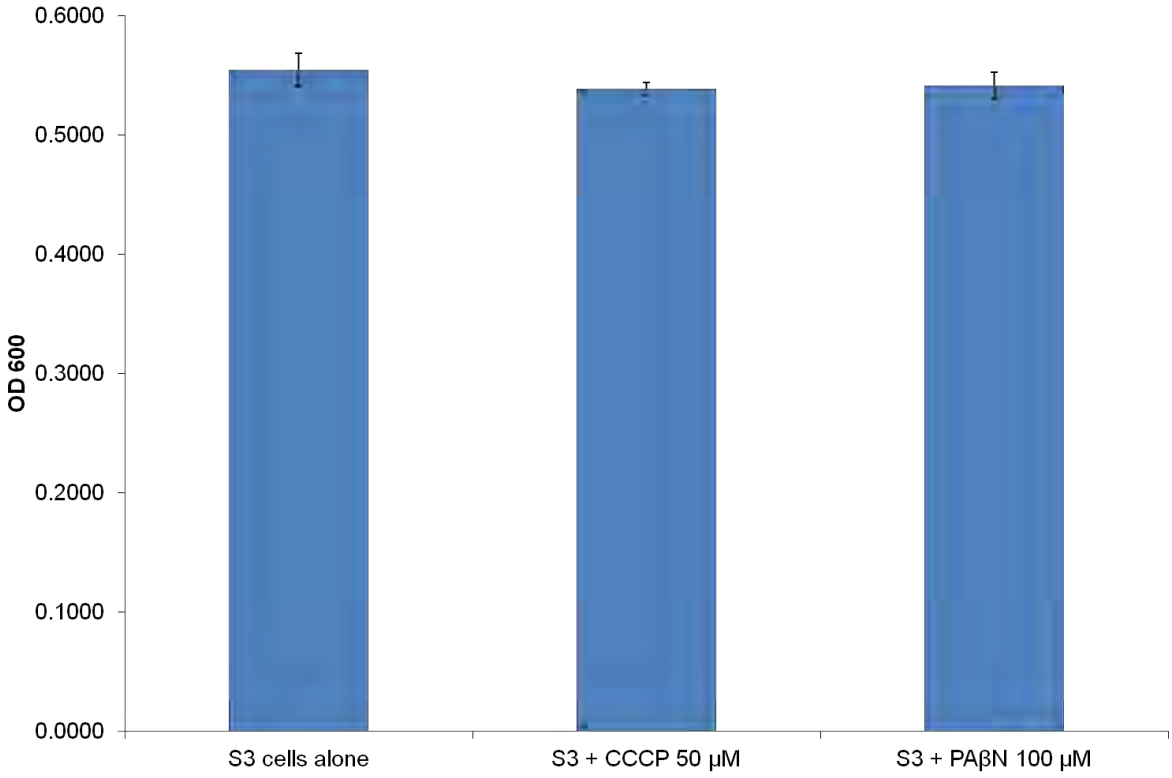
Table 3.3. MICs of a range of clinically relevant antibiotics and dyes against R1, R2, R3, S1, S2, S3 and AYE (µg/ml).

Strain	AYE	S1	S2	S3	R1	R2	R3
NAL	>1024	8	32	32	1024	>1024	1024
CIP	**128**	0.5	**16**	0.5	**8**	**256**	**128**
NOR	128	4	32	8	64	128	128
KAN	>1024	1	512	1	>1024	>1024	>1024
GEN	**1024**	1	2	**64**	**>1024**	**>1024**	**256**
TAZ	>1024	>1024	>1024	>1024	>1024	>1024	>1024
IMP[a]	0.38	0.094	0.094	0.094	**>32**	**>32**	**>32**
MER[a]	**>32**	0.38	0.5	0.25	**>32**	**>32**	**>32**
PIP	512	32	64	32	512	256	1024
AMP	512	32	512	512	512	1024	1024
AMP+SUL	32	8	64	64	32	64	64
CHL	512	128	128	128	526	128	256
TIG	**2**	0.12	**2**	**2**	0.5	**1**	**2**
TET	>1024	4	16	8	>1024	1024	>1024
ERY	512	8	16	8	512	512	1024
COL	2	8	1	2	2	4	2
TMP	>1024	16	8	32	32	128	128
ETBR	512	512	256	256	512	512	256
CCCP	256	256	256	128	256	256	256

PAβN	1024	1024	1024	512	1024	1024	1024
VER	>1024	>1024	>1024	>1024	>1024	>1024	>1024
H33342	512	512	256	256	256	256	512

NAL, nalidixic acid; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; TAZ, ceftazidime; TIG, tigecycline; IMP, imipenem; MER, meropenem; PIP, piperacillin, AMP, ampicillin; AMP+SUL, ampicillin + sulbactam; CHL, chlorampenicol; TET, tetracycline; ERY, erythromycin; COL, colistin; TMP, trimethoprim; VER, verapamil. [a], MIC values determined by E-test. Resistant values are shown in bold (as per BSAC recommended breakpoint concentrations, where available (Andrews 2001)).

Figure 3.3. Optical density of S3 at 2 hours after growth in the presence of sub-inhibitory concentrations of CCCP and PAβN.



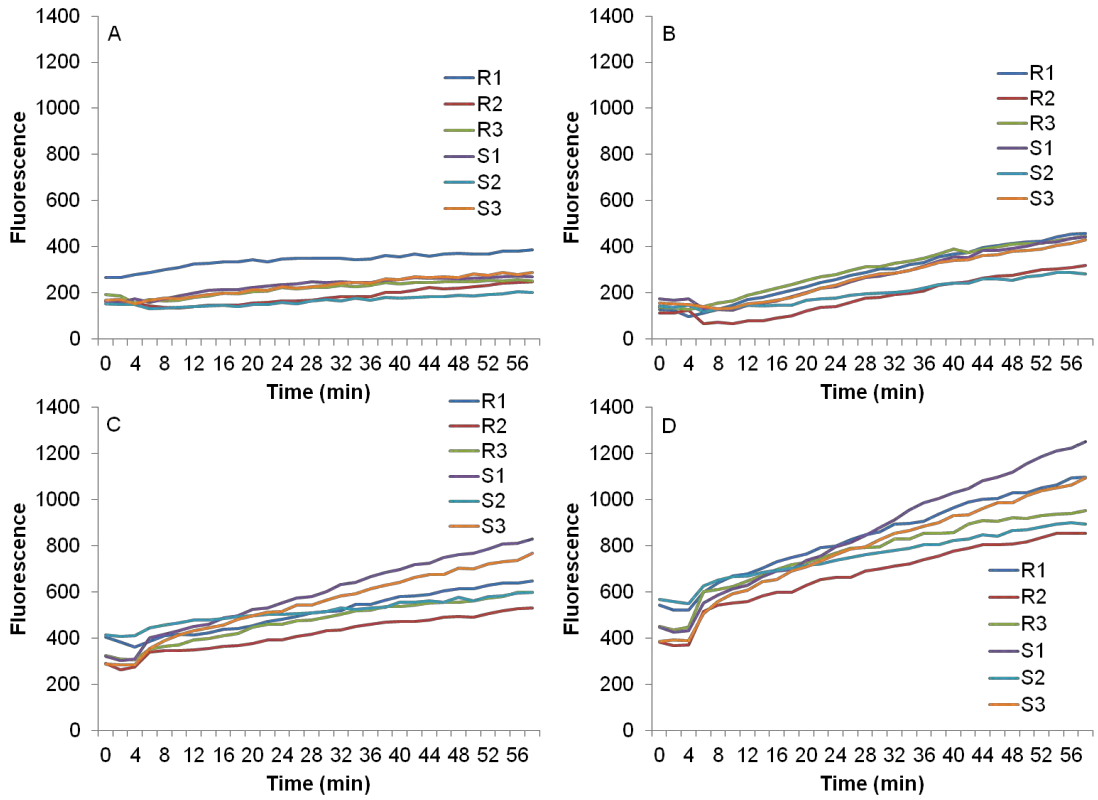
Data are displayed as a representative example of at least three experiments. The standard deviation represents variation between six biological replicates.

reproducible fluorescence (Figure 3.4). Heat-killed cells, which rapidly accumulate H33342, were included as a maximum fluorescence level control.

A H33342 accumulation assay with 2.5 μM H33342 and with cultures at an OD 600 nm of 0.5 was able to detect different levels of accumulation in the isolates. When data were compared with those for AYE differing levels of accumulation were seen for the clinical isolates S1- 3 and R1-2 (Figure 3.5). S1, S3, R1 and R2 showed significantly higher levels of accumulation of H33342 than AYE. Isolate S2 accumulated significantly lower levels of H33342 when compared with AYE. There was no significant difference in the level of H33342 accumulated in strain AYE and clinical isolate R3.

To assess the ability of CCCP and PAβN to inhibit pumps that efflux H33342, the H33342 accumulation assay was carried out in the presence of these EIs. CCCP is an uncoupler that can penetrate the membrane and disrupt the proton gradient (Lomovskaya and Watkins 2001). It therefore inhibits all efflux pumps that use the proton gradient, for example MFS, SMR and RND pumps. PAβN specifically blocks RND efflux pumps (Lomovskaya, Warren et al. 2001; Pagès, Masi et al. 2005). Addition of CCCP caused a significant increase ($p = \leq 0.05$) in accumulation of H33342 in AYE and all clinical isolates tested with fold changes of 1.42 or higher (Figure 3.6). S2 and R3 showed the most marked increase in the presence of CCCP, with a fold change of 2.00 and 2.15 respectively. Addition of PAβN also significantly increased accumulation ($p = \leq 0.05$) in all clinical isolates tested (Figure 3.7). The highest increase was seen in S3, with a fold change of 1.89.

Figure 3.4. Accumulation of 1400µg/ml H33342 in cell cultures of differing starting OD600



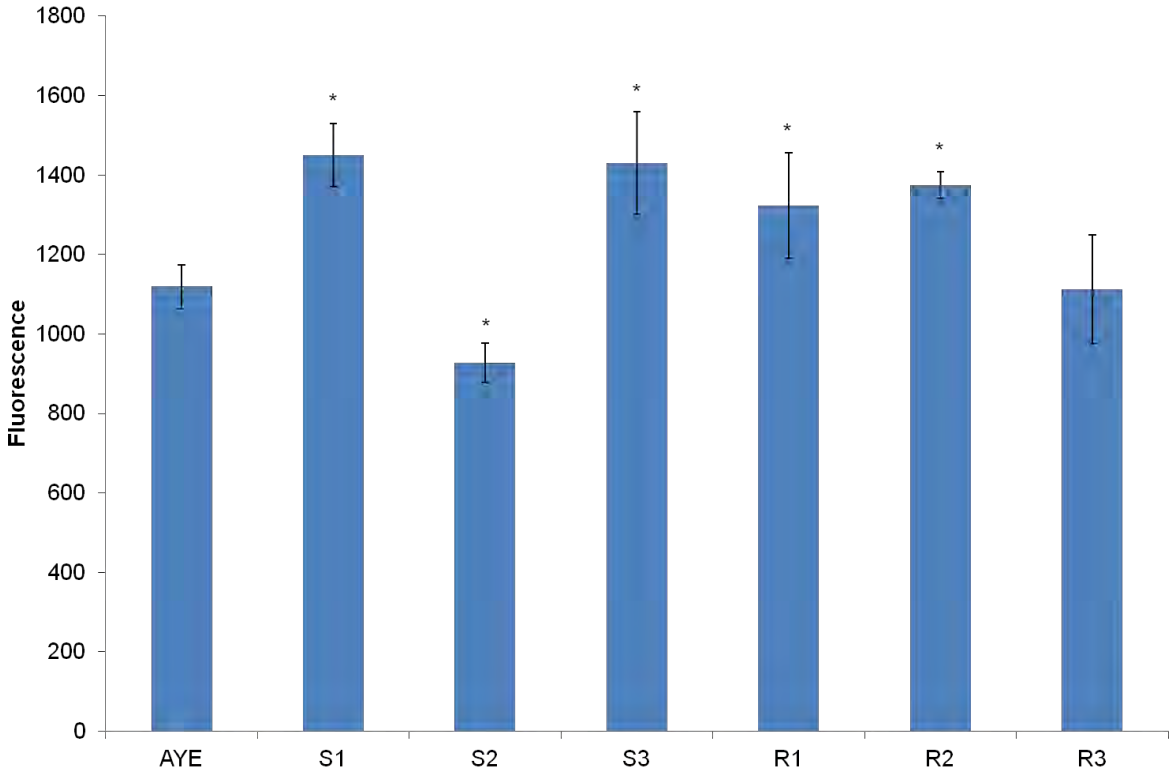
A, starting OD = 0.1

B, starting OD = 0.2

C, starting OD = 0.3

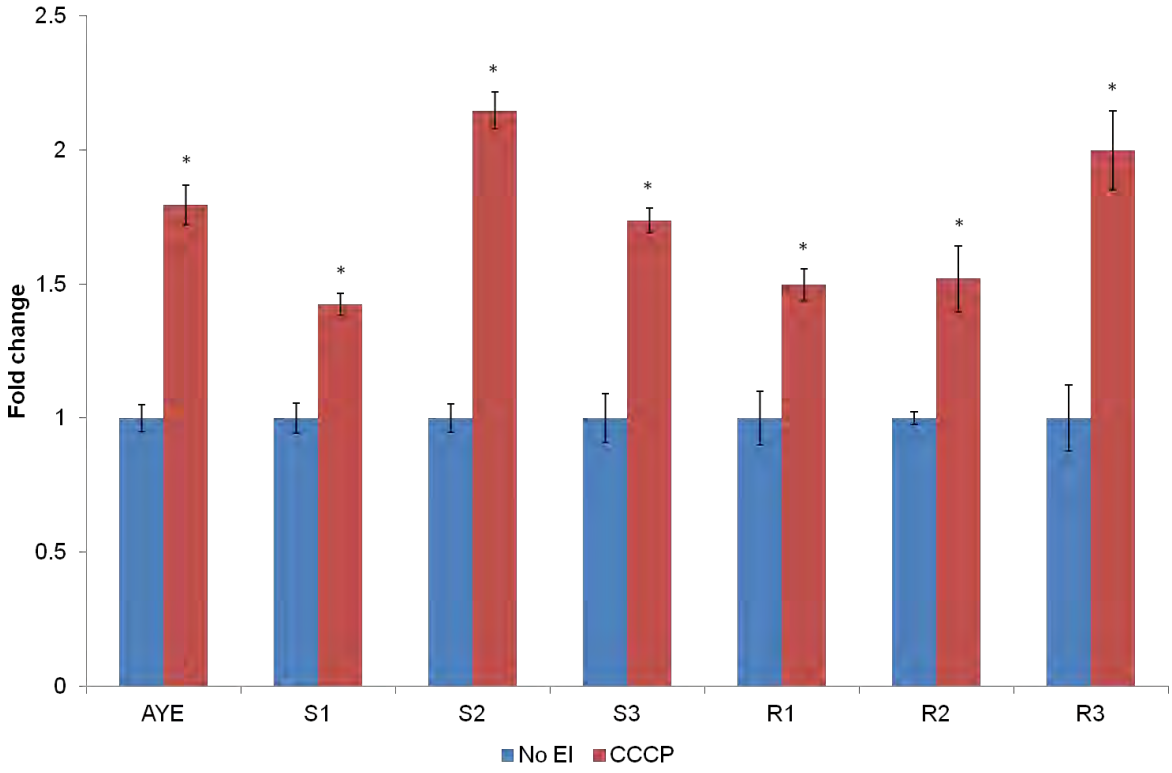
D, starting OD = 0.5

Figure 3.5 Steady state levels of accumulation of H33342 by strain AYE and clinical isolates S1, S2, S3, R1, R2 and R3 at 117 minutes exposure.



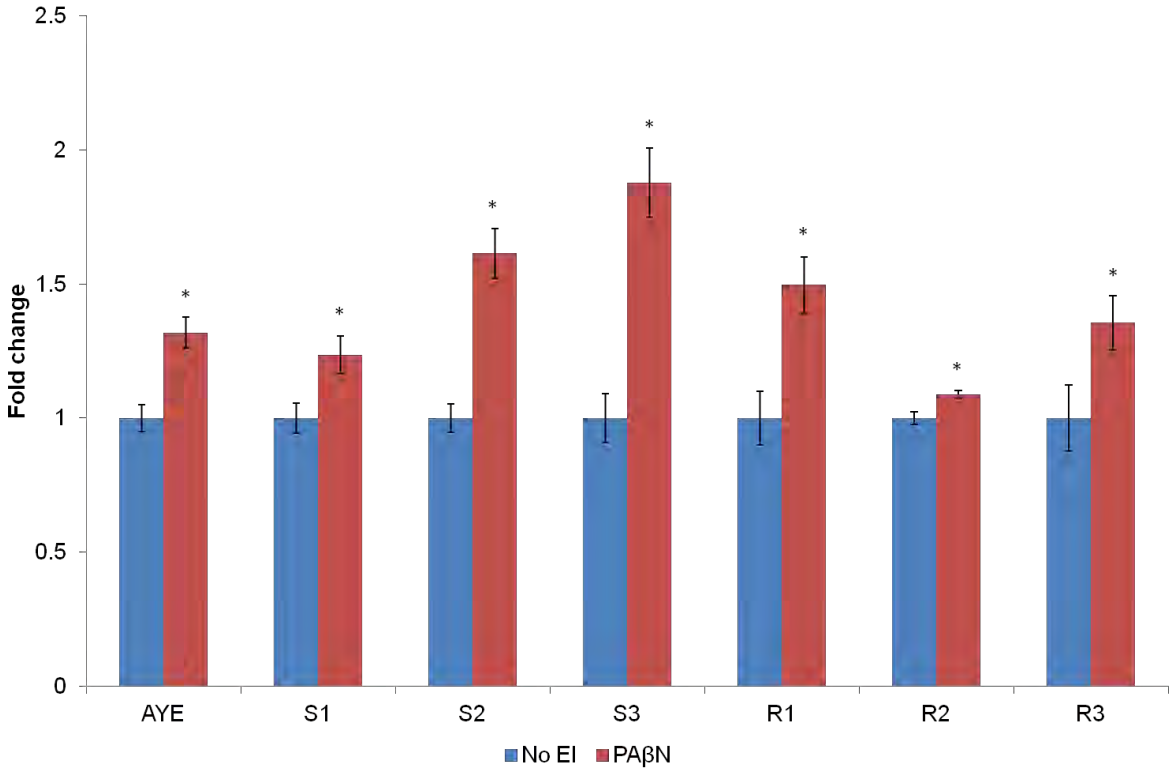
Data are displayed as a representative example of at least three separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values of the clinical isolates with those for AYE are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.6 Fold difference in levels of H33342 accumulated by AYE and clinical isolates S1, S2, S3, R1, R2, and R3 at steady state +/- CCCP



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of CCCP with those without CCCP are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.7 Fold difference in levels of H33342 accumulated by AYE and clinical isolates S1, S2, S3, R1, R2, and R3 at steady state +/- PAβN



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of PAβN with those without PAβN are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

3.3.3. Accumulation of H33342 by an *adeB* pump deletion mutant

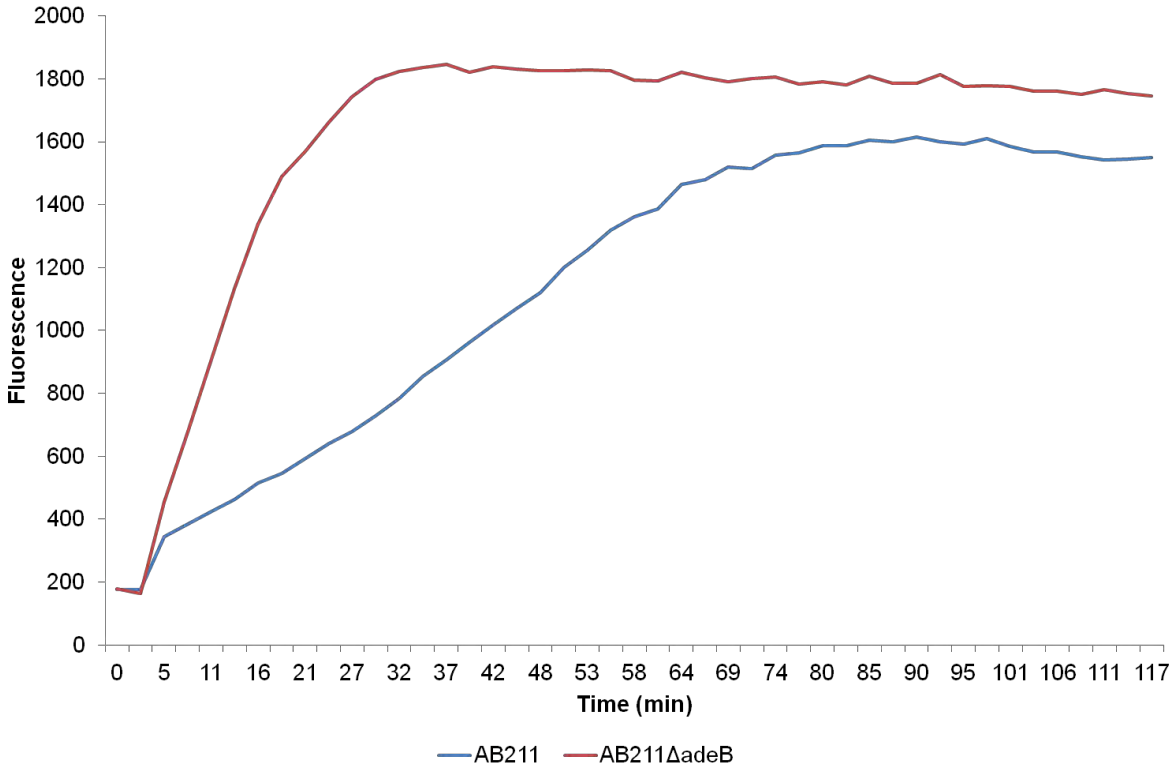
The ability of the H33342 accumulation assay to discriminate between pump deletion mutants and their parental strains was assessed using MDR strain AB211 and AB211Δ*adeB*. AB211*ΔadeB* has a deletion in the *adeB* gene, which encodes the inner membrane component of the AdeABC RND efflux pump. Previous studies showed that this efflux pump deletion mutant had reduced levels of resistance to tigecycline compared with the parental strain (Hornsey, Loman et al. 2011). AB211*ΔadeB* reached steady state accumulation level more rapidly (~32 min) than AB211 (~80 min) (Figure 3.8). Steady state levels of dye accumulation at 117 min were also significantly higher in the mutant (fluorescence = 1747) when compared with the parental strain (fluorescence = 1549), suggesting a reduced level of efflux.

3.3.4. Measurement of H33342 in specific efflux pump deletion mutants constructed for this project

3.3.4.1. Verification of gene deletions and phenotype of pump deletion mutants

Pump deletion mutants S1Δ*adeAB*, R2Δ*adeFGH,* R2Δ*adeIJK*, R2Δ*adeFGH*a*deIJK* and R2Δ*adeFGHΔP7* were constructed in the laboratory of collaborator Professor Kim Lee Chua as part of the MRC-A* funded research project. 'Pump 7' (P7) is a putative RND family drug transporter identified in ATCC 17978. All mutants were checked for deletion of the correct genes using 4 individual PCRs (Table 2.4, PCR numbers 2,3,4 and 5) (Primer positions shown in Appendix 2,3,4 and 5). Parental

Figure 3.8. Accumulation of H33342 in AB211 and pump deletion mutant AB211Δ*adeB*



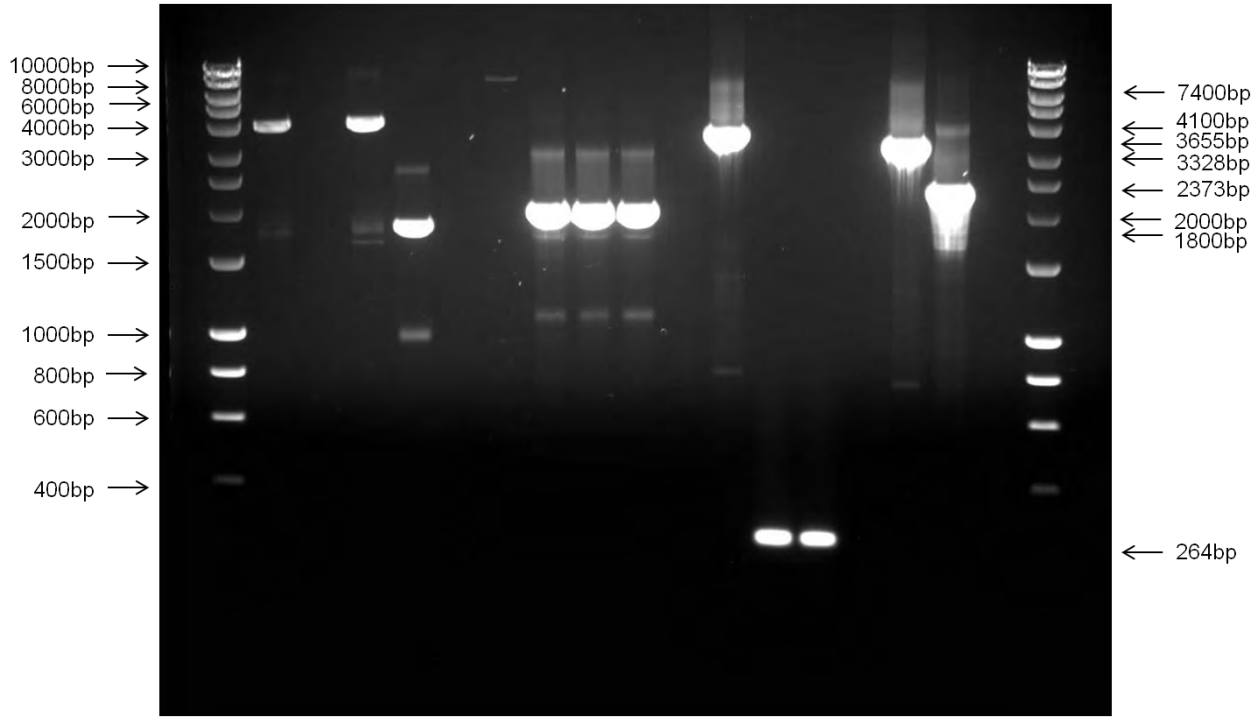
Data displayed are an exemplar of ≥ 3 separate experiments.

strains S1 and R2 were included as negative controls. Mutants were constructed in S1 and R2 because these strains were selected as representative of susceptible and resistant clinical isolates causing infection in Singaporean hospitals. Each mutant produced an amplimer of the size predicted for the mutation present (Figure 3.9 and Table 3.4)).

Comparison of the growth kinetics of each pump deletion mutant with the parental strain showed a reduction in the OD 600 nm at onset of stationary phase in S1Δ*adeAB*, R2Δ*adeIJK,* R2Δ*adeFGHadeIJK* and R2Δ*adeFGHΔP7* (Figure 3.10 and Table 3.5). OD 600 nm was significantly lower (p = ≤0.05) in S1Δ*adeAB* and R2Δ*adeFGHΔP7* compared with the parental strain, whereas R2Δ*adeIJK* and R2Δ*adeFGHadeIJK* had a higher OD 600 nm compared with the parental strain. The generation times of S1Δ*adeAB*, R2Δ*adeFGH,* R2Δ*adeFGHadeIJK* and R2Δ*adeFGHΔP7* were all significantly increased (p = ≤0.05) compared with the parental strain.

Deletion of a*deB* in Singaporean clinical isolate S1 had no effect on susceptibility to the antibiotics tested (Table 3.6). Deletion of *adeFGH* in Singaporean clinical isolate R2 resulted in a one dilution reduction in the MIC of tetracycline (Table 3.7). Deletion of this pump also reduced MICs of EIs CCCP and PAβN. Deletion of *adeIJK* in R2 conferred increased susceptibility to nalidixic acid, norfloxacin, chloramphenicol, tetracycline, tigecycline, trimethoprim, ethidium bromide, CCCP, PAβN and verapamil. The mutant R2 in which both *adeFGH* and *adeIJK* were deleted had the same susceptibility to antibiotics as the mutant in which *adeIJK* had been deleted.

Figure 3.9. and Table 3.4. Verification of gene deletions in pump deletion mutants (Table 2.4, PCR numbers 2,3,4 and 5).



Lane	PCR	Description	Predicted fragment size (bp)	Actual fragment size (bp)
1	*adeABC* check	AB211	4100	4100
2	*adeABC* check	AB211Δ*adeB*	0	0
3	*adeABC* check	S1	4100	4100
4	*adeABC* check	S1Δ*adeB*	1800	1800
5	*adeABC* check	Negative control	0	0
6	*adeFGH* check	R2	7416	7416
7	*adeFGH* check	R2Δ*adeFGH*	2000	2000
8	*adeFGH* check	R2Δ*adeFGH*Δ*adeIJK*	2000	2000

9	*adeFGH* check	R2Δ*adeFGH*ΔP7	2000	2000
10	*adeFGH* check	Negative control	0	0
11	*adeIJK* check	R2	3655	3655
12	*adeIJK* check	R2Δ*adeIJK*	264	264
13	*adeIJK* check	R2Δ*adeFGH*Δ*adeIJK*	264	264
14	*adeIJK* check	Negative control	0	0
15	P7 check	R2	3328	3328
16	P7 check	R2Δ*adeFGH*ΔP7	2373	2373
17	P7 check	Negative control	0	0

Figure 3.10. Growth kinetics of pump deletion mutants at 37°C as determined by optical density.

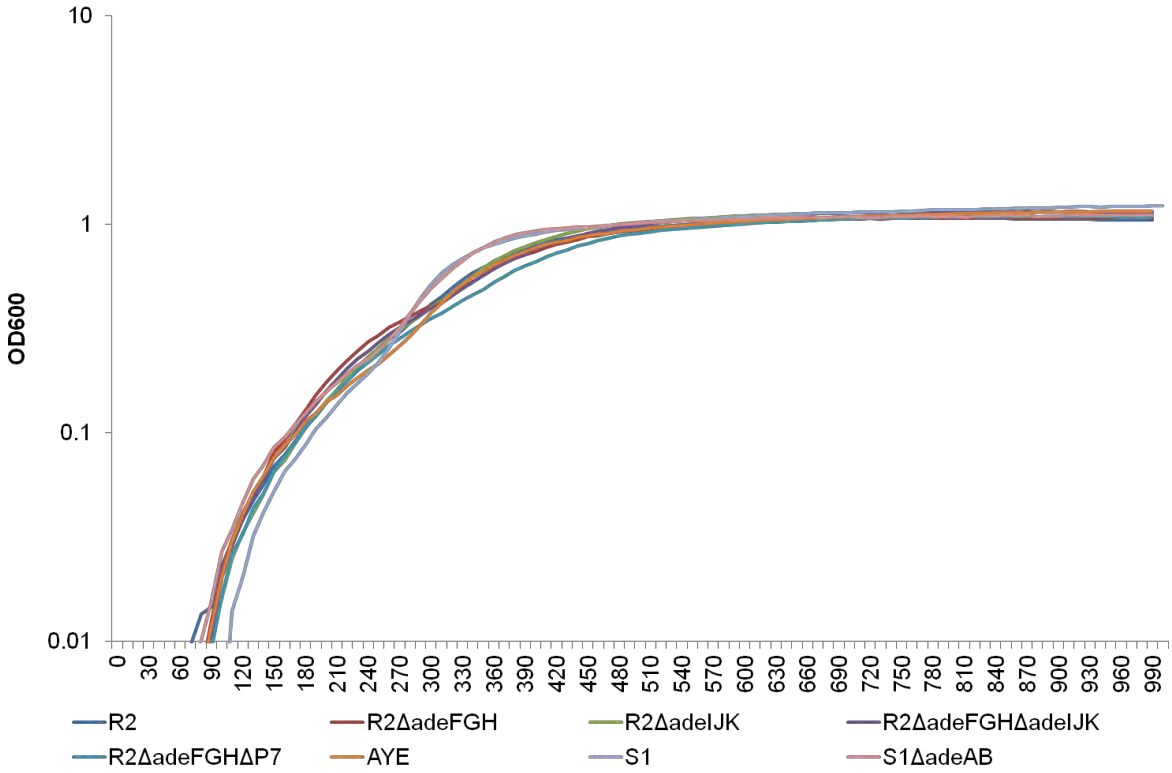


Table 3.5. Generation times and optical density at onset of stationary phase (540 min)

Strain	Generation Time (min)	OD 600 nm at onset of stationary phase (540 min)
AYE	78	0.99
S1	64	1.06
S1Δ*adeAB*	100	0.94
R2	89	1.00
R2Δ*adeFGH*	117	0.98

R2Δ*adeIJK*	91	1.06
R2Δ*adeFGH*Δ*adeIJK*	106	1.04
R2Δ*adeFGH*Δ*P7*	106	0.95

Table 3.6. MIC values of clinically relevant antibiotics for *A. baumannii* (µg/ml).

Strain	S1	S1Δ*adeB*
NAL	8	8
CIP	0.5	0.5
NOR	4	4
KAN	1	1
GEN	1	1
TAZ	>1024	>1024
IMPa	0.094	0.094
MERa	0.38	0.38
PIP	32	32
AMP	32	32
AMP+SUL	8	8
CHL	128	128
TIG	0.12	0.12
TET	4	4
ERY	8	8
COL	8	8
TMP	16	16
ETBR	512	512
CCCP	256	256
PAβN	1024	1024

VER	>1024	>1024
H33342	512	512

NAL, nalidixic acid; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; TAZ, ceftazidime; TIG, tigecycline; IMP, imipenem; MER, meropenem; PIP, piperacillin, AMP, ampicillin; AMP+SUL, ampicillin + sulbactam; CHL, chlorampenicol; TET, tetracycline; ERY, erythromycin; COL, colistin; TMP, trimethoprim; VER, verapamil. [a] , MIC values determined by E-test. Resistant values are shown in bold (as per BSAC recommended breakpoint concentrations, where available (Andrews 2001)).

Table 3.7. MIC values of clinically relevant antibiotics for *A. baumannii* (µg/ml).

Strain	R2	R2Δ*adeFGH*	R2Δ*adeIJK*	R2Δ*adeFGH* Δ*adeIJK*	R2Δ*adeFGH* Δ*P7*
NAL	>1024	>1024	**256**	**256**	>1024
CIP	256	256	256	256	256
NOR	128	128	**64**	**64**	128
KAN	>1024	>1024	>1024	>1024	>1024
GEN	>1024	>1024	>1024	>1024	>1024
TAZ	>1024	>1024	>1024	>1024	>1024
IMP[a]	>32	>32	>32	>32	>32
MER[a]	>32	>32	>32	>32	>32
PIP	256	256	256	256	256
AMP	1024	1024	1024	1024	1024
AMP+SUL	64	64	64	64	64
CHL	128	128	**64**	**64**	128
TIG	1	1	**0.25**	**0.25**	1
TET	1024	**512**	**128**	**128**	**512**
ERY	512	512	512	512	512
COL	4	4	4	4	4
TMP	128	128	**16**	**16**	128
ETBR	512	512	**256**	**256**	512
CCCP	256	**32**	**8**	8	**32**

PAβN	1024	**512**	**512**	**512**	**512**
VER	>1024	>1024	**1024**	**1024**	>1024
H33342	256	256	256	256	256

NAL, nalidixic acid; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; TAZ, ceftazidime; TIG, tigecycline; IMP, imipenem; MER, meropenem; PIP, piperacillin, AMP, ampicillin; AMP+SUL, ampicillin + sulbactam; CHL, chlorampenicol; TET, tetracycline; ERY, erythromycin; COL, colistin; TMP, trimethoprim; VER, verapamil. [a], MIC values determined by E-test. Resistant values are shown in bold (as per BSAC recommended breakpoint concentrations, where available (Andrews 2001)).

There was a small reduction in the MICs of tetracycline, CCCP and PAβN in R2Δ*adeFGH*Δ*P7,* but these changes were no different to those seen with the single deletion of *adeFGH* and so this mutant was not used in further experiments.

3.3.4.2. Accumulation of H33342 by efflux pump deletion mutants

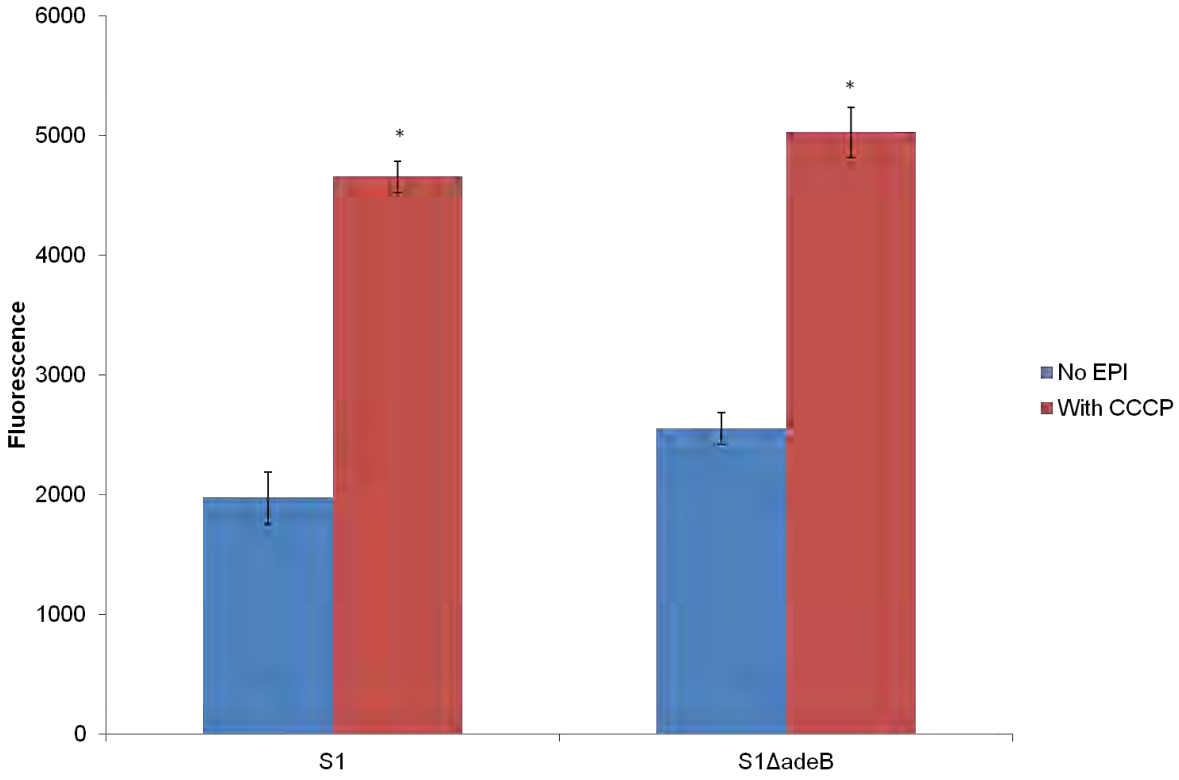
Accumulation of H33342 over time was studied in susceptible clinical isolate S1, S1Δ*adeB,* and three efflux pump deletion mutants from MDR clinical isolate R2, R2Δ*adeFGH,* R2Δ*adeIJK*, R2Δ*adeFGH*Δ*adeIJK*. Steady state levels of accumulation were taken as a measure of the level of efflux in individual strains. Strains with higher levels of steady state accumulation also displayed a higher rate of accumulation and reached steady state more rapidly.

Compared with the parental strain, there was no change in the level of H33342 accumulated at steady state in S1Δ*adeB* (Figure 3.11). Addition of CCCP caused a significant increase in the level of H33342 accumulated at steady state in both S1 and S1Δ*adeB,* with no significant difference between the parent and mutant.

Compared with the parental strain, when *adeFGH* was deleted in isolate R2, there was a significant 0.69 fold change in the level of H33342 accumulated (Figure 3.12). Accumulation was significantly increased in R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK* compared with the parental strain, with a fold change of 1.18 and 1.16, respectively.

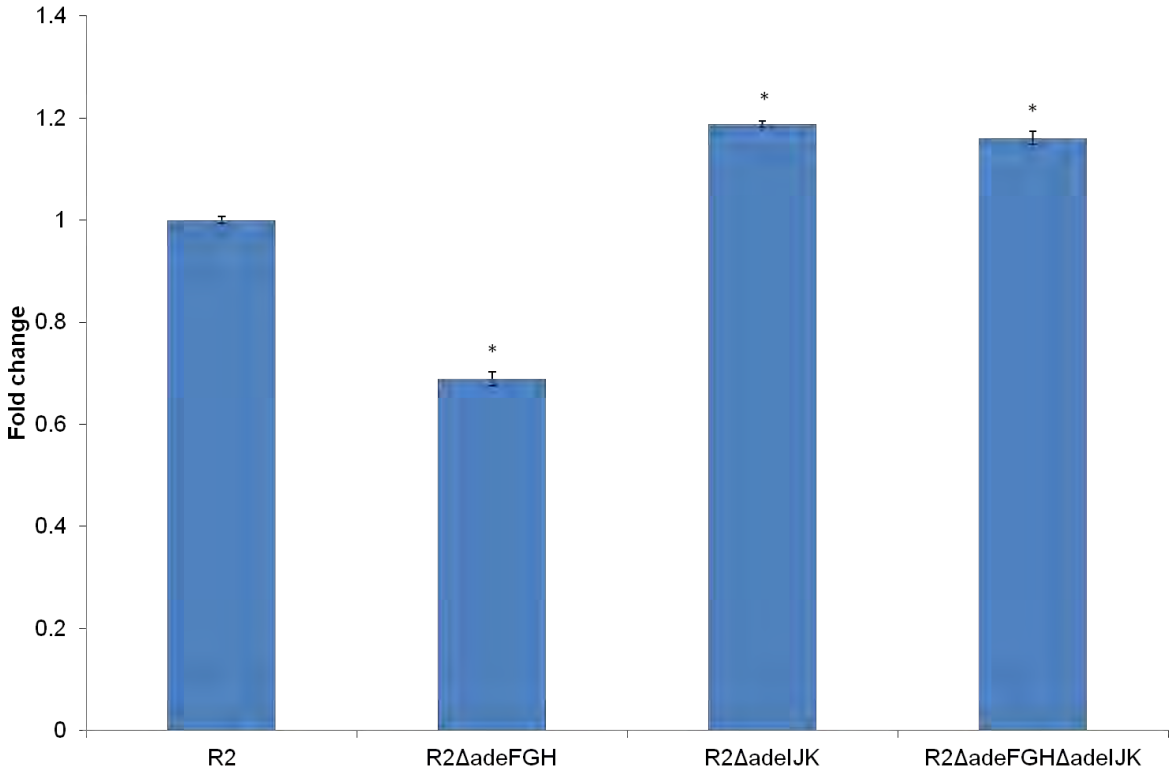
Addition of CCCP caused a significant increase in the level of H33342 accumulated at steady state (Figure 3.13). This increase was most pronounced in R2Δ*adeFGH*, with a 1.45 fold increase observed. The clinical isolate, R2, showed a smaller 1.31

Figure 3.11. Fluorescence of H33342 accumulated by S1 and pump deletion mutant +/- CCCP at steady state.



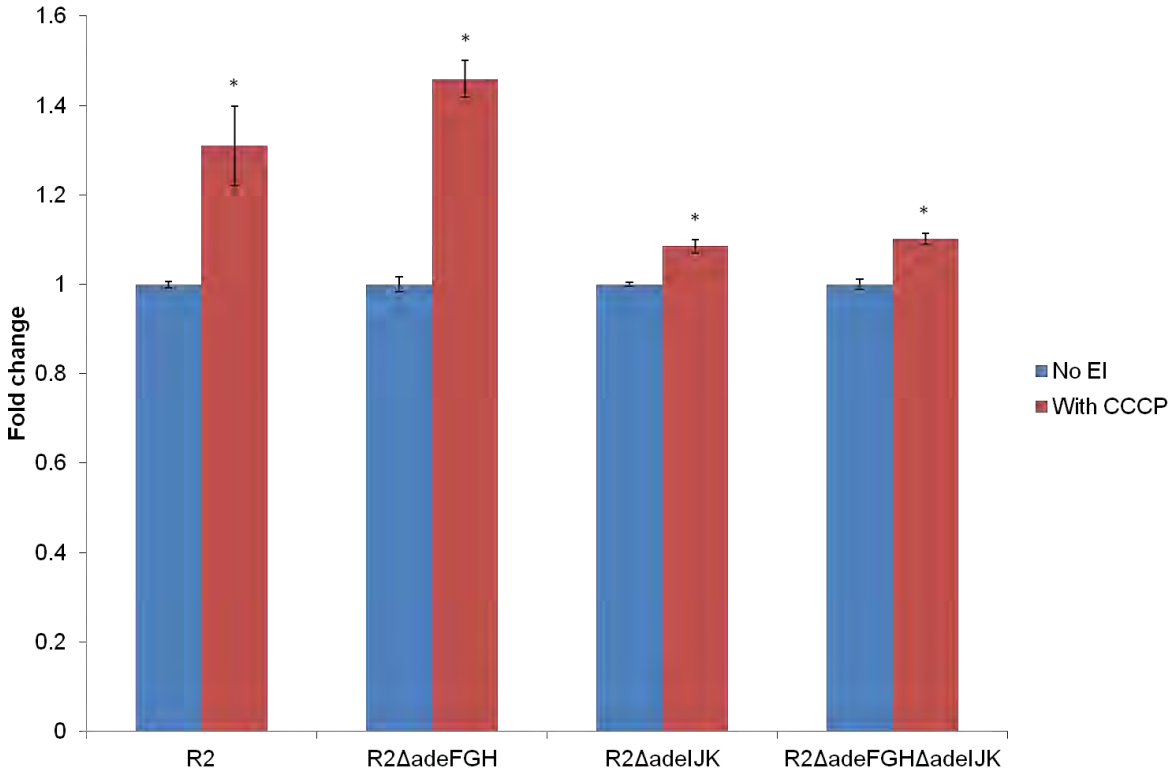
Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of CCCP with those without CCCP are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.12. Fold difference in levels of H33342 accumulated by R2 and pump deletion mutants at steady state.



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values of pump deletion mutants with those for the parental strain are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.13. Fold difference in levels of H33342 accumulated by R2 and pump deletion mutants at steady state +/- CCCP



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of CCCP with those without CCCP are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

fold increase. R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK* showed the smallest fold changes of 1.09 and 1.10 respectively.

Addition of PAβN also caused a significant increase in accumulation of H33342 in all strains (Figure 3.14). This increase was greatest in R2Δ*adeFGH*, with a 1.47 fold increase observed. Increase in H33342 accumulation was lower in the parental strain R2 and in R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK* mutants with respective fold changes of 1.12, 1.19 and 1.24.

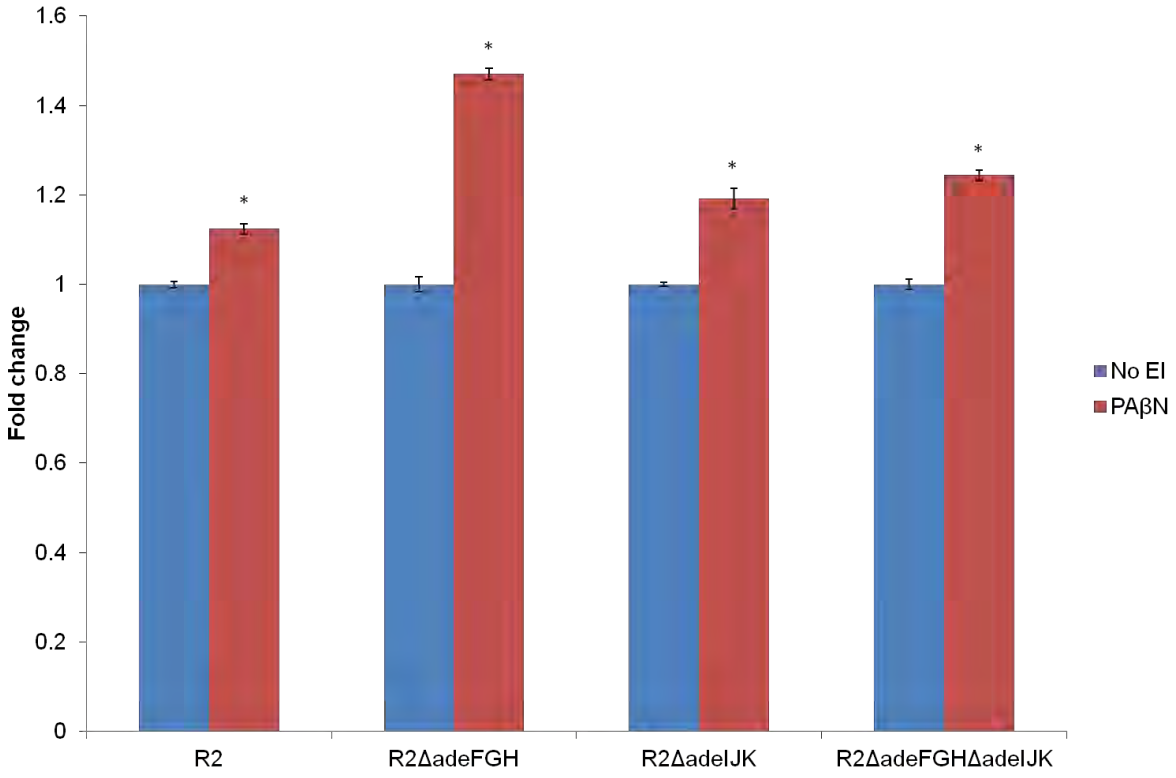
3.4. Accumulation of ethidium bromide

To compare with data obtained with H33342, accumulation of ethidium bromide was measured in strain AYE, clinical isolates and pump deletion mutants. Ethidium bromide is an alternative dye to H33342 and may be a substrate for different efflux pumps.

3.4.1. Accumulation of ethidium bromide in clinical isolates and strain AYE

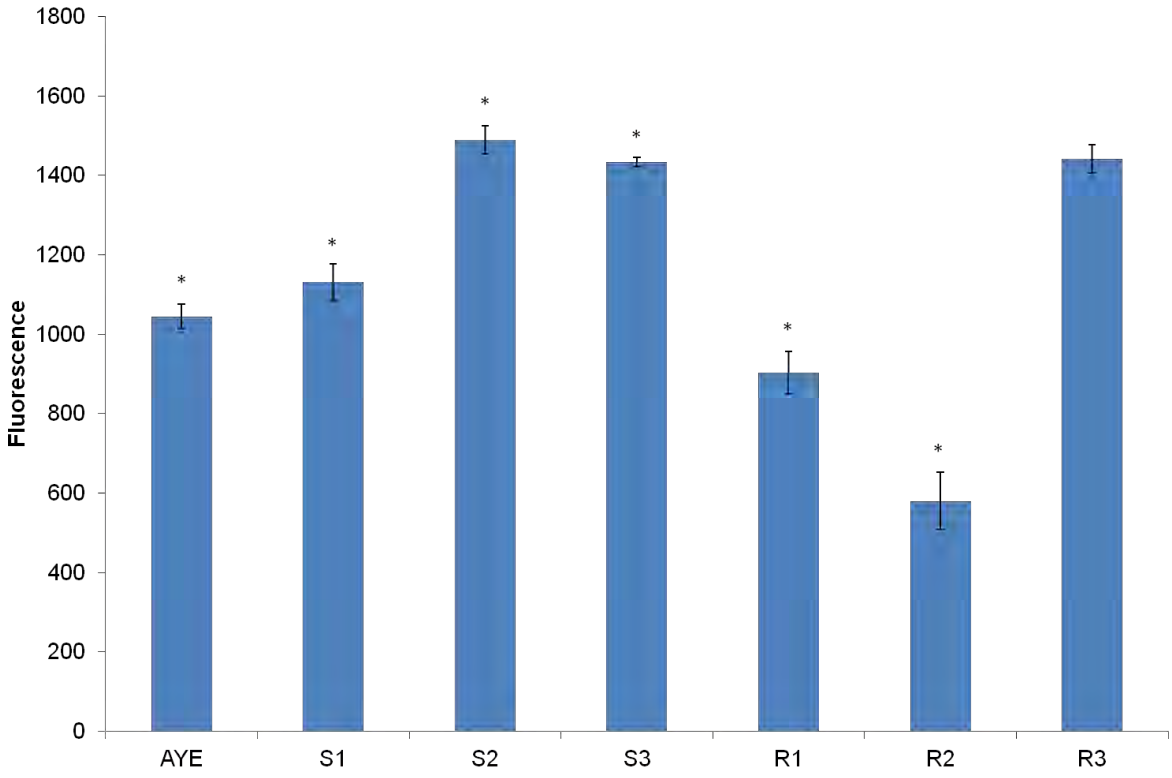
Singaporean clinical isolates S1-3 and R1-3 all showed a significant difference in accumulation of ethidium bromide compared with reference strain AYE (Figure 3.15). R1 and R2 accumulated less than the three antibiotic susceptible isolates. Isolates R3 and AYE accumulated more ethidium bromide than R1 and R2, similar to that seen for isolates S1-S3.

Figure 3.14. Fold difference in levels of H33342 accumulated by R2 and pump deletion mutants at steady state.+/- PAβN



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of PAβN with those without PAβN are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.15. Steady state levels of accumulation of ethidium bromide by strain AYE and clinical isolates S1, S2, S3, R1, R2 and R3 at 117 minutes exposure



Data are displayed as a representative example of at least three separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values of the clinical isolates with those for AYE are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Addition of CCCP caused a significant increase ($p = \leq 0.05$) in the level of ethidium bromide accumulated at steady state for isolates AYE, R1 and R2 with a 1.54, 1.60 and 2.59 fold increase, respectively (Figure 3.16). Addition of CCCP had a smaller effect on the level of ethidium bromide accumulated in S1 and S3 with a 1.10 and 1.04 fold increase observed. CCCP had no effect on the level of ethidium bromide accumulated in S2 and R3.

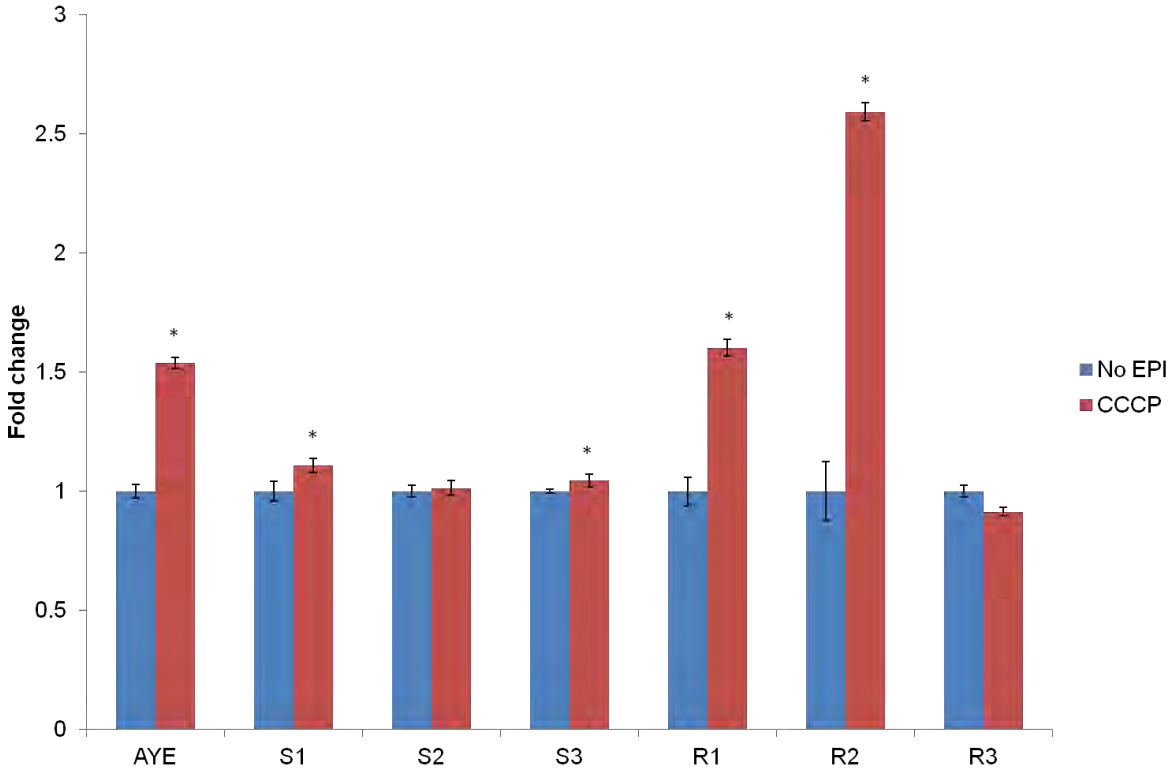
Addition of PAβN also significantly increased ($p = \leq 0.05$) the level of ethidium bromide accumulated in AYE, R1 and R2, with a 1.9 fold increase in strain AYE and a 1.4 fold increase in both R1 and R2 (Figure 3.17). PAβN had no effect on accumulation of ethidium bromide by isolates S1, S2, S3 and R3.

3.4.2. Accumulation of ethidium bromide by efflux pump deletion mutants

To compare with data obtained with H33342, accumulation of ethidium bromide was also studied in efflux pump deletion mutants from MDR clinical isolate R2, R2Δ*adeFGH,* R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK*. As found with H33342, R2Δ*adeFGH* accumulated significantly less ethidium bromide than R2, with a 0.81 fold change (Figure 3.18). R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK* accumulated 1.85 and 1.93 fold more ethidium bromide than R2 ($p = \leq 0.05$).

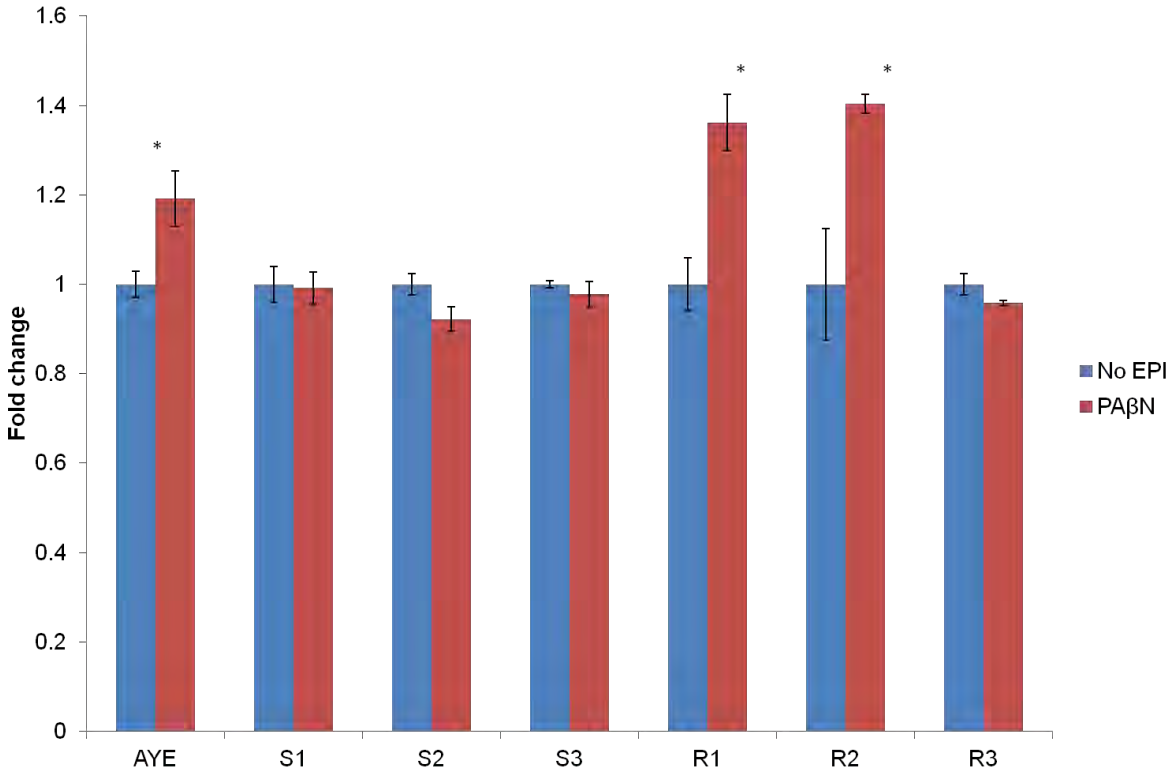
Addition of CCCP significantly increased levels of ethidium bromide accumulated in all strains at steady state (Figure 3.19). The highest increase was seen in R2Δ*adeFGH* with a 2.60 fold change. Addition of CCCP produced a 2.07 fold change

Figure 3.16. Fold difference in levels of ethidium bromide accumulated by AYE and clinical isolates S1, S2, S3, R1, R2, and R3 at steady state +/- CCCP



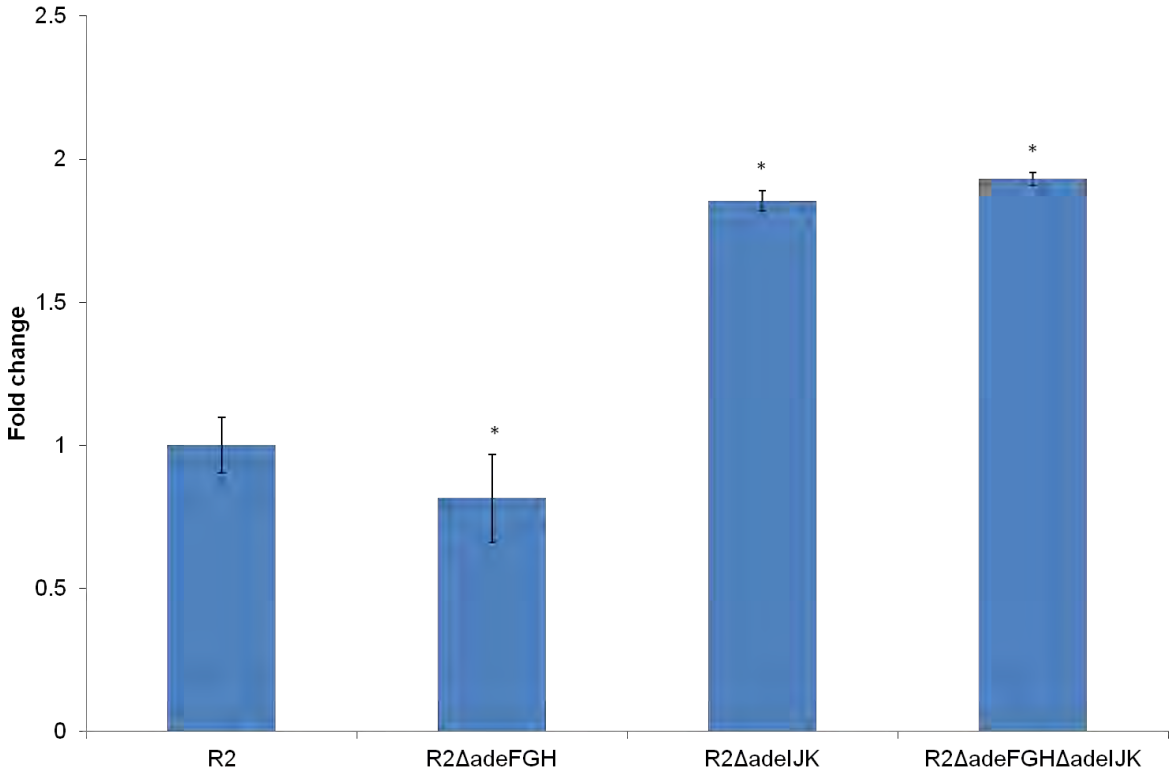
Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of CCCP with those without CCCP are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.17. Fold difference in levels of ethidium bromide accumulated by AYE and clinical isolates S1, S2, S3, R1, R2, and R3 at steady state +/- PAβN



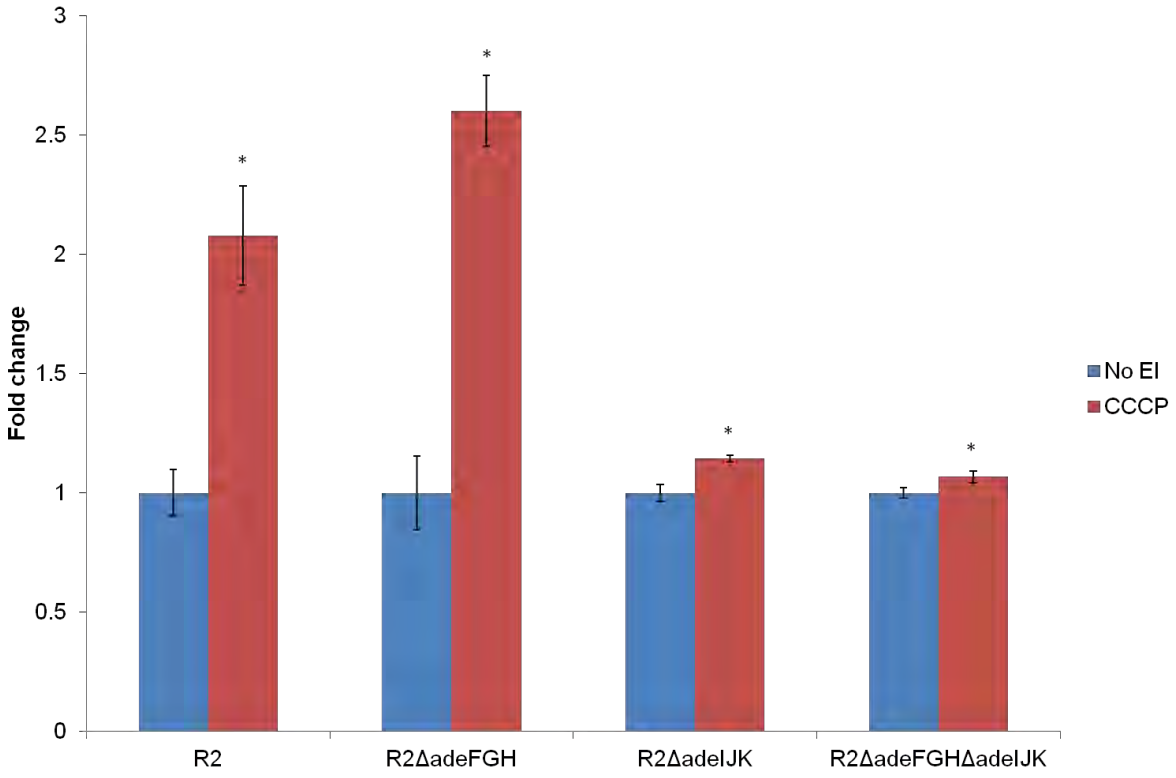
Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of PAβN with those without PAβN are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.18. Fold difference in levels of ethidium bromide accumulated by R2 and pump deletion mutants at steady state



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values of pump deletion mutants with those for the parental strain are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.19. Fold difference in levels of ethidium bromide accumulated by R2 and pump deletion mutants at steady state +/- CCCP



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of CCCP with those without CCCP are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

in the level of ethidium bromide accumulated in the parental strain, R2, and increases of 1.15 and 1.07 fold in mutants R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK.* This replicated the pattern seen with addition of CCCP in the H33342 accumulation assay.

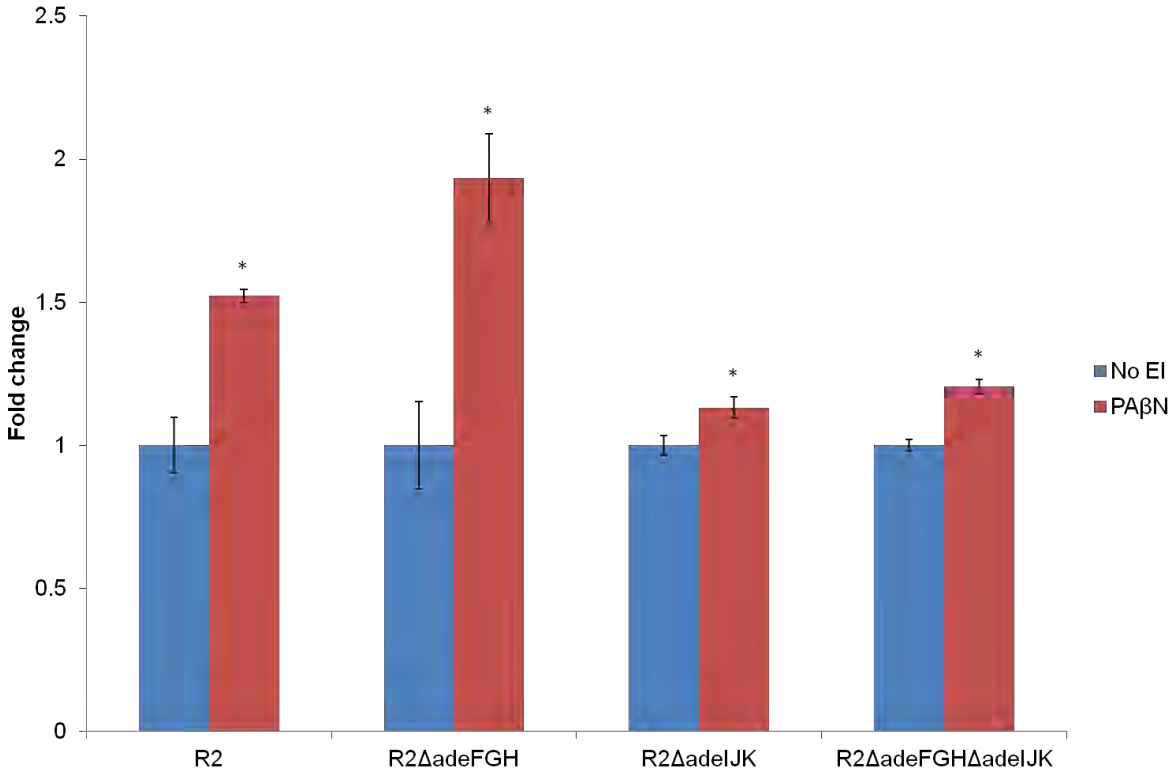
Addition of PAβN also increased the level of ethidium bromide accumulated by 1.52 fold in R2 (Figure 3.20). Addition of PAβN increased the level of ethidium bromide accumulated by R2Δ*adeFGH,* with a 1.93 fold change. Modest increases of 1.13 and 1.21 fold were seen for mutants R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK,* respectively.

3.5. Uptake of norfloxacin by efflux pump deletion mutants

To determine whether deletion of *adeFGH* and *adeIJK* affected the concentration of fluoroquinolone antibiotic accumulated, and whether data for H33342 and/or ethidium bromide was similar, norfloxacin accumulation was determined. Norfloxacin is a fluoroquinolone antibiotic which exhibits natural fluorescence. Fluoroquinolones have been shown to be substrates of RND pumps AdeABC, AdeFGH and AdeIJK (Coyne, Courvalin et al. 2011).

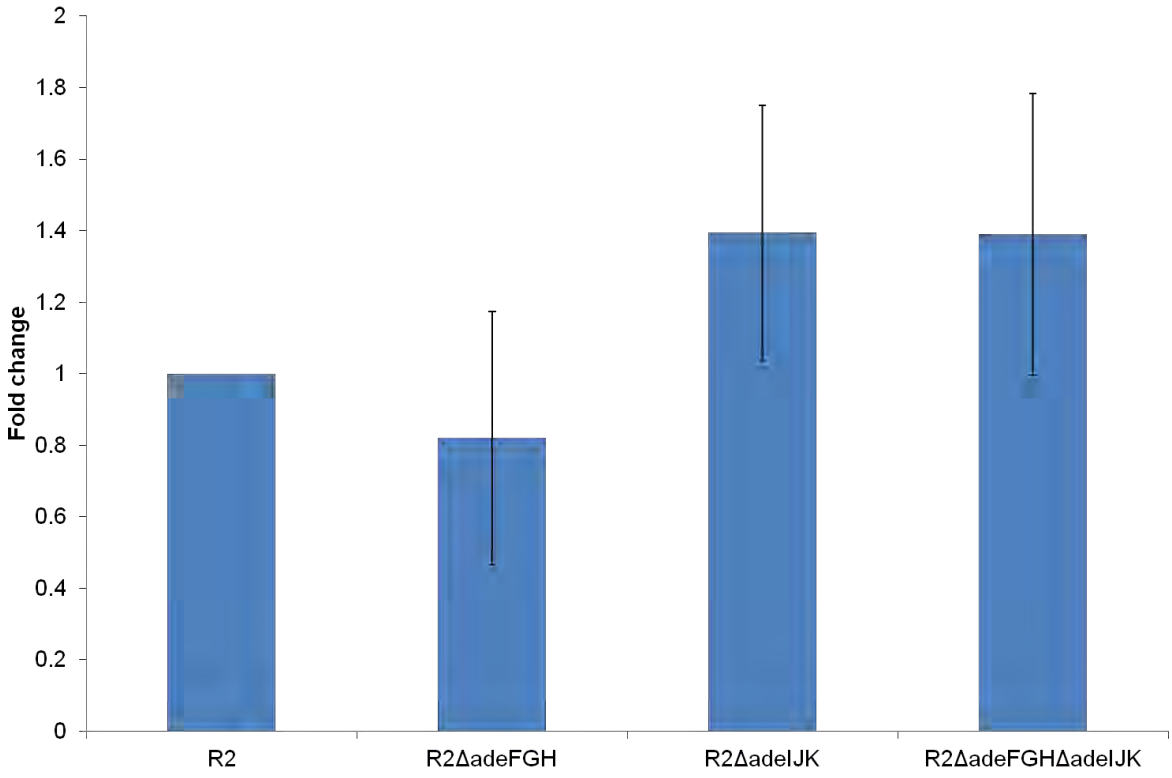
The pattern observed mirrored that seen in H33342 and ethidium bromide accumulation assays (3.21). As found with H33342, R2Δ*adeFGH* accumulated 0.82 fold less norfloxacin than R2, (Figure 3.21). R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK* both accumulated 1.39 fold more norfloxacin than R2. However, none of these changes were significant due to the large error between assays.

Figure 3.20. Fold difference in levels of ethidium bromide accumulated by R2 and pump deletion mutants at steady state +/- PAβN



Data are displayed as a representative example of at least 3 separate experiments. The standard deviation represents variation between three biological replicates. Significant differences in values in the presence of PAβN with those without PAβN are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.21. Fold difference in levels of norfloxacin accumulated by R2 and pump deletion mutants at steady state



Data are displayed as the mean of 3 separate experiments with a single biological replicate. The standard deviation represents variation between three separate experiments. Significant differences in values of pump deletion mutants with those for the parental strain are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

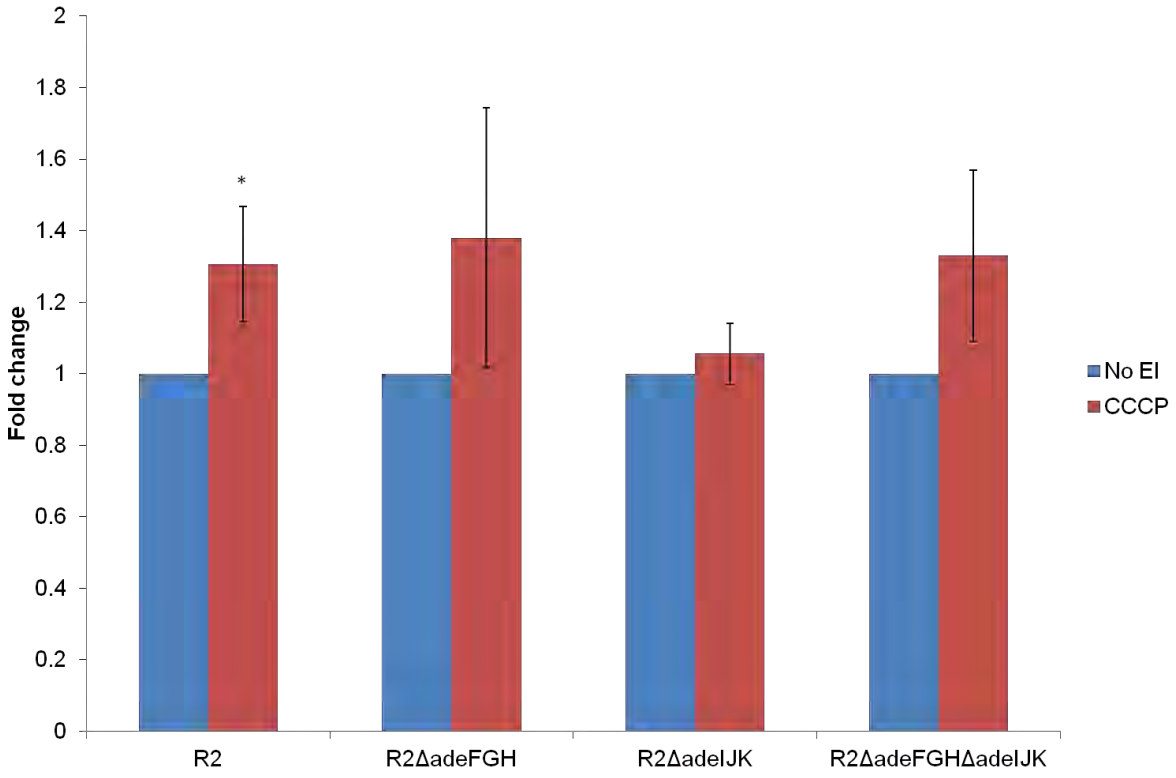
Addition of CCCP increased the level of norfloxacin accumulated in clinical isolate R2 by 1.31 fold (Figure 3.22). PAβN had no significant effect on the level of norfloxacin accumulated in any strains (Figure 3.23).

3.6. Comparison of data obtained with H33342 with that for ethidium bromide and norfloxacin

Levels of H33342 accumulated by strain AYE and Singaporean clinical isolates were different to levels of ethidium bromide accumulated (Table 3.8). Of the clinical isolates, S1, S3 and R2 accumulated the most H33342, whereas S2, S3 and R3 accumulated the most ethidium bromide. Addition of EIs had a different effect on the accumulation of H33342 and ethidium bromide. Addition of CCCP significantly increased the levels of H33342 accumulated by S2, S3 and R3. However, addition of CCCP had no effect on the levels of ethidium bromide accumulated by these isolates. Similarly, PAβN increased the levels of H33342 accumulated by S1, S2, S3 and R3, but had no effect on ethidium bromide accumulation.

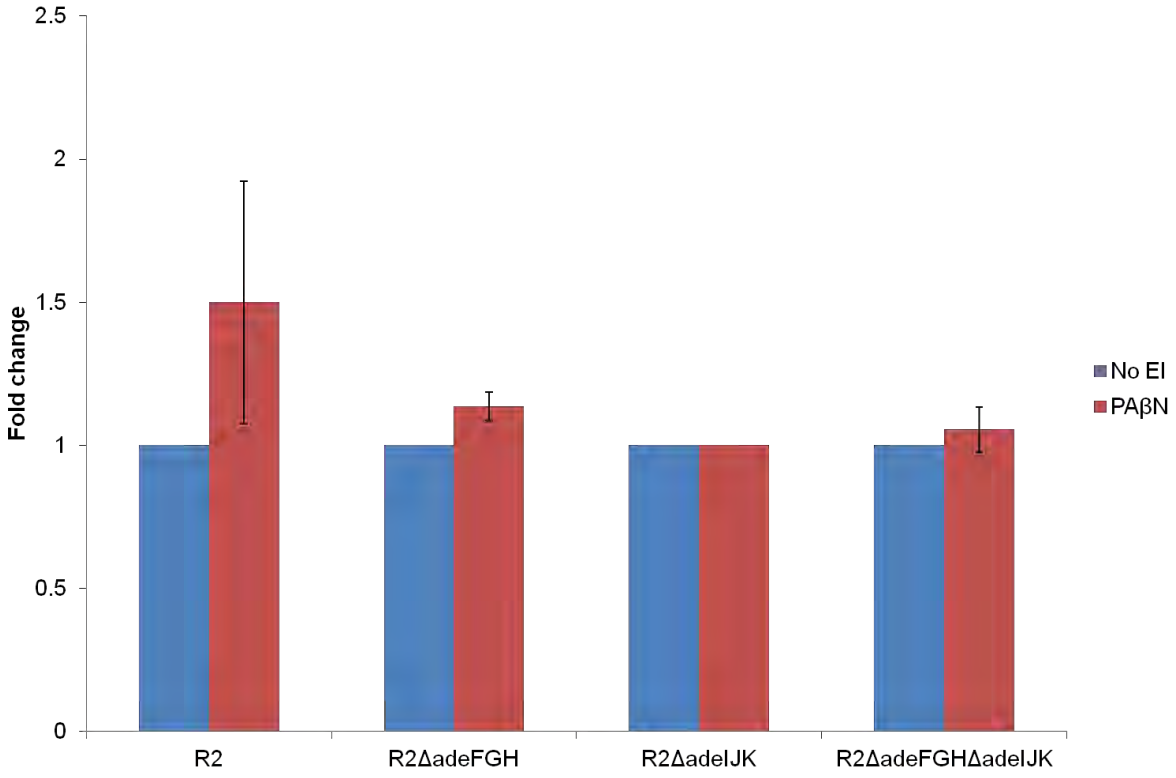
Levels of H44432 accumulated by efflux pump deletion mutants were similar to levels of ethidium bromide and norfloxacin accumulated (Table 3.9). There was a significant decrease in steady state levels of both H33342 and ethidium bromide accumulated in R2Δ*adeFGH*. There was an increase in steady state levels of both H33342 and ethidium bromide accumulated in R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK*.Addition of EIs also produced the same affect on accumulation of H33342 and ethidium bromide, with the highest increase in accumulation in R2 and R2Δ*adeFGH*. Measurement of

Figure 3.22. Fold difference in levels of norfloxacin accumulated by R2 and pump deletion mutants at steady state +/- CCCP



Data are displayed as the mean of 3 separate experiments with a single biological replicate. The standard deviation represents variation between three separate experiments. Significant differences in values in the presence of CCCP with those without CCCP are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Figure 3.23. Fold difference in levels of norfloxacin accumulated by R2 and pump deletion mutants at steady state +/- PAβN



Data are displayed as the mean of 3 separate experiments with a single biological replicate. The standard deviation represents variation between three separate experiments. Significant differences in values in the presence of PAβN with those without PAβN are indicated with a *, which indicates values returning a p value of ≤0.05 from a Student's t-test.

Table 3.8. Fold change in level of H33342, ethidium bromide and norfloxacin accumulated in strain AYE and clinical isolates

		AYE	S1	S2	S3	R1	R2	R3
H33342	Fold change compared to AYE	1	1.30	0.83	1.28	1.18	1.23	0.99
	Fold change with CCCP	1.80	1.42	2.15	1.74	1.50	1.52	2.00
	Fold change with PAβN	1.32	1.23	1.62	1.88	1.50	1.09	1.36
Ethidium bromide	Fold change compared to AYE	1	1.08	1.42	1.37	0.86	0.56	1.38
	Fold change with CCCP	1.54	1.11	1.01	1.04	1.60	2.59	0.91
	Fold change with PAβN	1.19	0.99	0.92	0.98	1.36	1.40	0.96

Table 3.9. Fold change in level of H33342, ethidium bromide and norfloxacin accumulated in efflux pump deletion mutants

		S1	S1Δ*adeAB*	R2	R2Δ*adeFGH*	R2Δ*adeIJK*	R2Δ*adeFGH*Δ*adeIJK*
H33342	Fold change compared to parental strain	1	1.29	1	0.69	1.19	1.16
	Fold change with CCCP	2.36	1.97	1.31	1.46	1.09	1.10
	Fold change with PAβN	N/A	N/A	1.12	1.47	1.19	1.24
Ethidium bromide	Fold change compared to parental strain	N/A	N/A	1	0.81	1.85	1.93
	Fold change with CCCP	N/A	N/A	2.08	2.60	1.15	1.07
	Fold change with PAβN	N/A	N/A	1.52	1.93	1.31	1.21

Norfloxacin	Fold change compared to parental strain	N/A	1	0.82	1.39	1.39
	Fold change with CCCP	N/A	1.31	1.38	1.06	1.33
	Fold change with PAβN	N/A	1.5	1.14	1.00	1.06

norfloxacin also produced similar results, although these changes were not significant.

Chapter 4: Discussion

Chapter 4. Discussion

Increased efflux as a result of over-expression of efflux pumps is a common mechanism of MDR in *A. baumannii* and resistance to a wide range of antibiotics can arise from a single mutation in regulatory genes such as *adeS* (Marchand, Damier-Piolle et al. 2004). An increase in the MIC of more than one class of antibiotics is often assumed to be the result of over-expression of efflux pumps, but levels of efflux are not usually measured.

H33342 displays low toxicity and low mutagenicity when used at a concentration of 10 μM (Durand and Olive 1982), making it an ideal fluorescent probe for the measurement of cellular accumulation. H33342 has previously been used to characterise membrane transport activity in mammalian cells (Lalande, Ling et al. 1981) and *Lactococcus lactis* (van den Berg van Saparoea, Lubelski et al. 2005), and to investigate the contribution of the ABC efflux pump VcaM to MDR in *Vibrio cholerae* and assess efflux activity in *E. coli* and *S. enterica* (Huda, Lee et al. 2003). Therefore, measurement of the accumulation of Hoechst 33342 could provide a sensitive and specific test to quickly and simply measure efflux in *A. baumannii*.

In this study, an assay previously developed in this laboratory to measure H33342 accumulation in *Enterobacteriaceae* (Coldham, Webber et al. 2010), was adapted for use with *A. baumannii*. Data were compared with that obtained with established methods using ethidium bromide and norfloxacin. The suitability of the H33342 accumulation assay for determining differences in efflux between isolates of *A.*

baumannii was assessed with four MDR clinical isolates and three antibiotic susceptible clinical isolates.

Strain AYE is a previously characterised strain with high resistance to over three classes of antibiotic, typical of a MDR strain of *A. baumannii*. Accumulation of H33342 by the cell was seen by the dramatic increase in fluorescence with injection of the dye as it entered the cells and bound to DNA and the lipid membrane. Addition of CCCP, which dissipates the proton motive force required by a broad range of efflux pumps, caused a significant increase in H33342 accumulation, suggesting reduced efflux due to the inhibition of active efflux pumps. Addition of PAβN, which inhibits RND transporters such as *Pseudomonas aeruginosa* MexAB (Lomovskaya, Warren et al. 2001) and *E. coli* AcrB (Yu, Aires et al. 2005), also resulted in higher levels of H33342 accumulation i.e. reduced level of efflux. However, this reduction was not as great as that seen with CCCP, suggesting that although RND pumps contribute to efflux in AYE, there are other families of CCCP susceptible efflux pumps that also export H33342.

To allow comparison of efflux levels in Singaporean clinical isolates, the level of H33342 accumulated at steady state in each clinical isolate was compared to the level accumulated by AYE. The H33342 assay revealed differences in levels of accumulation (efflux) between AYE and five of the six clinical isolates tested. Isolate S2 accumulated less H33342 than AYE, whilst isolates S1, S3, R1 and R2 accumulated more H33342 than AYE. This did not correlate well with the MICs of antibiotics for these isolates. It was hypothesised that strains exhibiting higher MICs of antibiotics would display increased levels of efflux, thus accounting, in part, for

their MDR phenotype. However, *A. baumannii* has been shown to possess various different antibiotic resistance mechanisms (Coyne, Courvalin et al. 2011). Any number of these may be present in these clinical isolates and contribute to MDR, masking the effect of efflux on the phenotype when measured by MIC. Further analysis of the genomes to identify the presence of genes encoding antibiotic resistance mechanisms in these isolates is required.

Addition of CCCP caused a significant decrease in efflux of H33342 in all isolates, with the largest effect seen in R3 and S2. These same isolates also accumulated less H33342, suggesting a higher level of efflux. In the presence of PAβN, accumulation of H33342 was also increased significantly in these two isolates. However, the increase was not as large as that seen with CCCP, suggesting that the transporters responsible for the higher levels of efflux of H33342 in these isolates are not inhibited by PAβN. Efflux in S3 was greatly reduced in the presence of PAβN, suggesting that efflux in this isolate may be largely due to RND transporters. This demonstrates how studying the effect of specific EIs on the accumulation of H33342 allows further insight into the classes of efflux pump that contribute to efflux, and therefore MDR, in clinical isolates of *A. baumannii*.

Comparison of the accumulation of H33342 and ethidium bromide in the clinical isolates of *A. baumannii* revealed different patterns, suggesting that these two compounds may be substrates for different pumps. Low levels of accumulation of ethidium bromide in isolates R1 and R2 suggest higher levels of efflux and may provide an explanation for the MDR phenotype displayed by these isolates. It may be that ethidium bromide and antibiotics tested in this study are substrates for the same

efflux pumps and so the levels of efflux of ethidium bromide therefore reflect levels of antibiotic efflux. In the presence of CCCP, efflux of ethidium bromide was most affected in strain AYE and isolates R1 and R2. This was expected as these clinical isolates showed the highest level of efflux of this dye. The presence of CCCP gave no significant decrease in efflux in isolates R3 and S2, the isolates that showed the least ethidium bromide efflux. Interestingly, these two isolates were those that showed the highest levels of efflux of H33342 and displayed the largest decrease in efflux of this dye with the addition of CCCP (Table 3.7). This suggests that clinical isolates of *A. baumannii* produce efflux pumps with different substrate specificities and these may be at different levels. This emphasises the need to use more than one substrate to measure efflux when explaining the role of efflux in MDR. Addition of PAβN affected efflux of ethidium bromide in a similar way to CCCP. Isolates R1 and R2 that effluxed ethidium bromide at high levels also showed the greatest decrease in efflux with PAβN. However, the increase was not as high as that seen with CCCP, suggesting that although RND transporters contribute to efflux of ethidium bromide in these isolates, other families of efflux pumps may also efflux dyes.

The H33342 accumulation assay was also used to assess whether efflux played a role in MDR in Singaporean *A. baumannii*. Genes encoding efflux pumps previously shown to efflux clinically relevant antibiotics in other strains of *A. baumannii* (Coyne, Courvalin et al. 2011) were deleted in clinical isolates S1 and R2 to create pump deletion mutants S1Δ*adeAB*, R2Δ*adeFGH*, R2Δ*adeIJK* and R2Δ*adeFGH*Δ*adeIJK* (Kim Lee Chua, manuscript in preparation). H33342 accumulation was also measured in AB211 and AB211Δ*adeB* (Wareham, Gordon et al. 2011).

The level of H33342 accumulated by AB211Δ*adeB* was higher than that in the parental strain AB211, suggesting that as a result of the lack of AdeB there was reduced efflux of this dye in the mutant. Deletion of *adeB* in susceptible strain, S1, had no significant effect on the MICs of the antibiotics tested, or on accumulation of H33342, suggesting that the RND pump AdeABC plays no role in resistance to antibiotics or efflux of H33342 in this strain. Deletion of *adeB* has previously been shown to reduce MICs of aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol and trimethoprim in an MDR isolate BM4454 (Magnet, Courvalin et al. 2001). A three-fold increase in ethidium bromide accumulation was also observed in this mutant (Magnet, Courvalin et al. 2001). However, as S1 is susceptible to several antibiotics and over-expression of AdeABC is required for MDR (Magnet, Courvalin et al. 2001) it may be that S1 only produces low levels of AdeABC and so deletion of *adeB* has no effect on the phenotype.

Deletion of *adeFGH* in MDR Singaporean isolate R2 had no effect on MICs of any of the antibiotics tested. Whilst over-expression of this system can lead to MDR, Coyne *et al.* have also demonstrated that *adeFGH* is not expressed in all isolates and therefore does not contribute to intrinsic resistance (Coyne, Rosenfeld et al. 2010). A triple pump deletion mutant, BM4679 (Δ*adeABC*Δ*adeIJK*Δ*adeFGH*) displayed the same level of resistance as BM4652 (Δ*adeABC*Δ*adeIJK*), indicating no expression of AdeFGH in this strain (Coyne, Rosenfeld et al. 2010). A lack of expression of AdeFGH in R2 may explain why deletion of these genes had no effect on resistance. However, when compared with the parental strain R2, R2Δ*adeFGH* did show a

significant decrease in accumulation of both H33342 and ethidium bromide, inferring increased efflux in the mutant. This may be due to increased expression of another efflux system in order to compensate for the loss of AdeFGH. This could also explain the lack of change in MIC seen with deletion of *adeFGH.* Previous work in *Salmonella enterica* serovar Typhimurium has shown that deletion of RND efflux pump genes can lead to compensatory altered expression of other efflux pump genes. For example, deletion of *acrB* in SL1344 resulted in a 7.9 fold increase in the expression of *acrF* (Eaves, Ricci et al. 2004). In order to confirm the hypothesis that another efflux pump could compensate for loss of *adeFGH*, transcriptomics are necessary to analyse the expression of individual efflux pump genes. Addition of CCCP and PAβN also had a greater effect on accumulation of H33342 and ethidium bromide in R2Δ*adeFGH* than in mutants lacking *adeIJK*, suggesting that efflux activity was originally higher in the Δ*adeFGH* mutant.

Changes in MICs to particular types of antibiotic tested against R2Δ*adeIJK* compared with the parental strains were similar to changes seen previously in pump deletion mutants of strain BM4454 (Damier-Piolle, Magnet et al. 2008; Coyne, Rosenfeld et al. 2010). The MICs of antibiotics against the double mutant R2Δ*adeFGH*Δ*adeIJK* were the same as for the single mutant, R2Δ*adeIJK* and confirmed data obtained with the single deletion of *adeFGH*. Deletion of *adeIJK* resulted in a significant increase in the steady state accumulation levels of both H33342 and ethidium bromide. This infers reduced efflux in these mutants, as a consequence of the removal of the efflux pump AdeIJK. Addition of both CCCP and PAβN to Δ*adeIJK* and Δ*adeFGHadeIJK* mutants significantly increased the steady state accumulation

levels of H33342 and ethidium bromide, suggesting that, despite lacking AdeIJK, these mutants still have efflux activity, presumably as a result of other pump systems. However, the increase in accumulation observed in these mutants was not as high as that seen with the parental strains and the Δ*adeFGH* deletion mutant. These data suggest that efflux is reduced in strains lacking *adeIJK*.

Unlike results seen with clinical isolates, the levels of H33342 and of ethidium bromide accumulated at steady state by Singaporean clinical isolate R2 and its efflux pump deletion mutants were similar. This confirms that both H33342 and ethidium bromide are substrates for the RND pumps deleted in this study and are suitable for measuring changes in efflux in these mutants. Although norfloxacin uptake studies did not show any significant differences in efflux between the R2 parental strain and mutants, perhaps suggesting that this agent is not transported by AdeFGH or AdeIJK, each experiment clearly indicated increased accumulation of norfloxacin in R2Δ*adeIJK* and R2Δ*adeFGHadeIJK*. Coupled with increased susceptibility to norfloxacin, it is highly likely that this agent is a substrate for *adeIJK*. Further work to identify the reason for the variability seen in experiments is warranted.

However, different classes of efflux pumps with different substrate ranges can be found in *A. baumannii* and these may contribute to efflux to varying degrees in different isolates. In order to gain a full picture of how efflux affects MDR in clinical isolates, it is useful to use more than one dye to measure efflux activity of pumps with different substrate ranges. It should also be noted that alterations in outer membrane permeability may contribute to changes in dye accumulation. Reduced expression of OMPs, such as OmpA, CarO and OmpW, has been seen previously in MDR isolates

of *A. baumannii* (Yun, Choi et al. 2008) and may, at least in part, account for a reduced level of accumulation observed in some isolates.

In summary, the H33342 accumulation assay previously developed for assessment of cellular permeability in *Enterobacteriaceae* has been adapted to provide a cheap, rapid and sensitive test for the measurement of efflux in *A. baumannii.* Measurement of the accumulation of H33342 provided a means of characterising MDR strains of *A. baumannii* and assessing the role of efflux in antibiotic resistance. Further assessment of the role of efflux in MDR can be investigated with the addition of EIs to inhibit different classes of efflux pumps. However, it should be noted that H33342 may not be a substrate for all efflux pumps present in *A. baumannii* and use of additional compounds such as ethidium bromide may be useful in further understanding the levels of efflux in individual isolates. The H33342 assay can also be used to assess the contribution of individual efflux pumps by comparing pump deletion mutants with their parental strain. Here, accumulation of H33342 has been used, with supporting ethidium bromide accumulation data, to show a significant difference in the efflux activity of specific pump deletion mutants compared with the parental strain. Data obtained in this study support previous work carried out with other strains and isolates of *A. baumannii* and provide an insight into the contribution of RND systems AdeFGH and AdeIJK to MDR in clinical isolates commonly found in Singapore hospitals.

Appendices

Appendix 1. The *gyrB* gene with primer locations indicated

tttaattgaacgattaagccaacttggtagccaggtttttatgacaactttagatcatgcat
cggtaaaaaaacatttacatgatctgtctatttcatatcaattattcagtgttgaatccggt
caagttagtcttgctgcaccataatttttgatgttacccatctttgaataaacctatattt
gctagggagaaaccATGAGTTCAGAGTCTCAATCAGCCTCTCAAACAGAACAAACCAATGAA
AAGGCTTATGATTCCTCTAGTATTAAAGTATTACGTGGATTAGATGCAGTTCGTAAACGTCC
GGGTATGTATATTGGGGATACAGACGACGGTACCGGTTTACACCATATGGTGTTTGAGGTTG
TCGATAATGCAATCGACGAAGCTTTAGCTGGCCACTGCGATGAAATTATTGTCACGATTCAC
GAGGATGAATCGGTCAGTGTTTCAGATAATGGCCGCGGTATTCCAACCGATATTCACCCTGA
AGAAGGGGTTTCTGCTGCAGAAGTAATTTTAACGATTCTGCATGCAGGCGGTAAGTTTGACG
ATAATAGCTATAAAGTTTCAGGTGGCTTACACGGCGTAGGTGTTTCGGTTGTTAACGCACTT
TCAAGTAAATTGCATCTAACAATTTACCGTGCTGGTCAAATCCATGAGCAAGAATATCATCA
TGGCGATCCGCAATATCCATTGCGTGTGATTGGTGAAACGGATAATACCGGAACAACTGTAC
GTTTTTGGCCAAGTGCAGAAACATTCAGTCAAACTATTTTTAATGTTGAAATTCTAGCACGC
CGTTTACGTGAGCTTTCTTTCTTGAATGCTGGTGTACGTATCGTTTTACGTGATGAACGTAT
TAACCTTGAGCATGTGTATGACTATGAAGGTGGTTTATCTGAGTTTGTAAAATACATCAACG
AAGGTAAAAACCATCTCAACGAAATCTTCCATTTCACAGCTGATGCTGACAACGGCATTGCT
GTAGAAGTAGCATTGCAATGGAACGATAGTTACCAAGAAAATGTTCGCTGTTTCACAAACAA
CATTCCACAAAAAGATGGTGGTACGCACTTAGCAGGTTTCCGCGCAGCTTTAACACGTGGCT
TAAACCAGTATCTTGAAAATGAAAATATTCTCAAGAAAGAAAAAGTGAATGTGACTGGTGAT
GATGCGCGTGAAGGTTTAACAGCCATTATTTCTGTTAAGGTTCCTGATCCGAAATTCTCGTC
TCAGACAAAAGAAAAATTGGTATCAAGTGAGGTAAAACCAGCGGTAGAGCAAGCAATGAACA
AAGAGTTCTCTGCTTACTTACTTGAGAATCCACAAGCTGCAAAATCAATTGCAGGCAAGATT
ATTGATGCTGCACGCGCACGTGATGCTGCACGTAAAGCACGTGAAATGACACGCCGTAAGAG
TGCATTAGATATTGCAGGTTTGCCTGGTAAATTGGCTGACTGTCAGGAAAAAGACCCAGCGC
TGTCTGAATTGTATCTTGTCGAGGGTGACTCTGCGGGTGGTAGTGCCAAACAAGGCCGTAAC
CGTAAAATGCAGGCTATTCTACCTTTAAAAGGTAAGATCCTGAACGTTGAGCGTGCGCGCTT
TGACAAAATGATCTCTAGTCAGGAAGTGGGTACATTAATTACAGCACTTGGCTGTGGTATTG
GCCGTGAAGAATATAACCCTGACAAGCTCCGTTATCATAAAATTATTATTATGACCGATGCC
GATGTCGATGGTTCGCACATTCGTACATTGTTATTAACATTCTTCTTCCGTCAAATGCCGGA
GTTGGTGGAACGTGGTCATATTTATATTGCACAGCCACCTTTGTATAAACTCAAAAAGGGTA
AGCAAGAGCAATATATTAAAGATAACGACGCATTAGAAACTTACTTGATTTCAAATGCGATT
GATGAGCTTGCTTTACATATTAGTGCTGATGCGCCTGCAATTACAGGTGAAGCGCTGGCAAA
AGTAATTCAAGACTATCAAGTTTCACAAAAGAGCTTACAACGCTTAACGTTACGTTATCCAG
CAAGCTTACTGGATGCATTACTCGAAGTAGATGCATTTAAAGCAGATCAAAACCATGATCAA
GCTTATGTCCAGCAATGGGCGTATCAAGTACGTGAAGCTGTACAAAGACTACAGCCAAGTTT
GCGCCCTGAAATTACGCTTGAAACATTTGAACGCGAGAATGCTCAAGGCGAGAAATCTGCGC
ATTACTGGCCACGTGTAACTGTATATGTTCATAACTTGCCACATGCTTACTTACTTGACGCA
GGTTTATTGAATTCTGCGGAATATGCTCGTTTATTGAAGAACTCGAAGAGTTGGTTCAAGTT
AATCGAAGATGGCGCATACTTACAAAAAGGTGATCGTCGTATTCAAGTCGCTAATTTCCATC
AGGTATGGCAGCACATTCTGCAAGATTCACGCCGTGGAATGATGATCCAGCGCTATAAAGGT
CTGGGCGAGATGAATGCTGAGCAGCTTTGGGAAACCACAATGGACCCTGAAAACCGTAATAT
GTTACAGGTGACTATTGATGATGCAATTGAAGCTGATCGCATGTTCTCATGCTTAATGGGCG
ATGATGTTGAACCACGTCGTGCCTTTATTGAAGAAAATGCATTAAATGCGGACATTGATGCT
TAAttgatagcaagattctaaatcagtctagggagagtagatgatgagtcaaaaaattctag
caagtatgctgatttcatgtgcggctttagtgagtcattctgcatttgcagcagacttggaa

gccgacatgaaaactttagcgaagagcactaaagcctttgctgaagcaaaagatatcgataa
cgctaaacagcagcttg

The *gyrB* gene of *A. baumannii* AYE is shown in capital letters while the upstream and downstream flanking regions are shown in lower case. Blue highlighted regions indicate primers 1079 and 1081 which produce a 490 bp amplimer in *A. baumannii* and. The red highlighted region indicates primer 1080, which along with 1081 produces a 294 bp amplimer in *A. baumannii* and *A. nosocomialis*.

Appendix 2. The a*deAB* operon with primer locations indicated

agtcatcttctaccacaagaataactttatcttggcaatcaaaagaaaaagaatgatcaaac
atagaaaatctggctatagaaagtgcttcaactcatcatacgctaaattatccgtatttctc
cacacttactccacactttagtgattatccctacacactcatcaaaaataatacgaacatca
aaaactcactaggtTTGGACAGTATGCAAAAGCATCTTTTACTTCCTTTATTTTTATCTATT
GGGCTGATATTACAGGGGTGTGATTCAAAAAAAGTCGCTCAAGCTGAGCCACCACCGGCTAA
AGTCAGTGTATTAAGCATTCAACCGCAATCGGTAAATTTTAGTGAAAATCTTCCTGCACGAG
TACATGCGTTCCGTACGGCGGAAATCCGTCCGCAAGTCGGAGGTATCATTGAAAAGGTTCTA
TTTAAACAAGGTAGTGAAGTTAGAGCAGGGCAAGCCTTATATAAAATTAATTCCGAGACTTT
TGAGGCCGATGTAAATAGCAATAGAGCTTCTCTCAATAAAGCTGAAGCTGAGGTGGCAAGAC
TCAAAGTTCAGTTAGAACGTTATGAGCAGCTATTACCAAGTAATGCGATTAGTAAGCAAGAA
GTAAGTAATGCTCAAGCTCAGTATCGTCAGGCTCTAGCCGATGTCGCTCAAATGAAAGCATT
GCTGGCCAGACAAAACTTGTAAtctgcaatatgcaacagttcgagcgcctatttctgggcgt
attgggcaatcttttgtcactgaaggtgcattggtcggtcagggcgataccaatacgatggc
aactattcaacagattgataaagtctatgttgatgttaagcaatcagttagtgagtatgaac
gcctacaagctgcgctacaaagcggtgaattatcagtcagcaaatagtgacaaaaccgttcg
tattaccaatagccacgggcaaccttataacgtcacagcaaaaATGTTGTTTGAAGATATTA
ATGTTGACCCGGAAACGGGCGATGTCACATTCCGTATTGAAGTTAATAACACTGAACGAAAA
TTACTTCCGGGCATGTATGTGCGTGTCAATATTGATCGTGCTTCTATTCCTCAAGCGCTATT
GGTTCCTGCGCAAGCGATCCAACGTAATATCAGTGGCGAGCCTCAGGTATATGTCATCAATG
CCCAAGGTACAGCGGAAATTCGTCCTATCGAAATTGGACAGCAATATGAGCAGTTCTATATC
GCTAACAAAGGCTTGAAAGTCGGTGACAAAGTCGTTGTTGAAGGTATTGAACGTATTAAGCC
AAATCAAAAATTGGCATTGGCAGCATGGAAAGCACCAGCCGTGGCAAATCATGCTTCAAGTG
TAGAAACCAAAACTTCTATAACTGAGGGGGCGCAACCATGAtgtcacaatttttattcgtc
gtcccgtttttgcttgggttatcgcgatctttattattatatttggattgctgagtattcct
aaactgccaattgcacgttttccaagtgtagccccgccacagGTGAATATTAGTGCGACTTA
TCCTGGTGCTACAGCTAAAACCATCAACGATAGCGTTGTAACCTTAATTGAGCGCGAATTAT
CGGGTGTAAAAAATCTACTCTACTATAGTGCGACAACAGATACCTCCGGTACAGCAGAGATT
ACCGCTACATTTAAACCGGGCACAGATGTGGAAATGGCTCAGGTGGACGTTCAAAATAAAAT
CAAGGCTGTAGAAGCTCGCTTACCGCAAGTTGTACGTCAGCAAGGTTTACAGGTTGAGGCTT
CATCGTCCGGATTTTTAATGCTGGTCGGGATTAACTCTCCAAATAATCAATATTCCGAAGTT
GATTTGAGTGATTATTTGGTTCGAAATGTTGTAGAAGAGCTAAAACGTGTCGAAGGTGTAGG
GAAGGTTCAATCTTTCGGTGCCGAGAAAGCTATGCGTATTTGGGTCGACCCAAATAAGCTTG
TTTCTTACGGTTTATCGATTAGTGATGTGAATAATGCCATTCGTGAAAATAATGTCGAAATT
GCACCCGGCCGACTTGGTGATTTACCAGCTGAAAAAGGCCAGCTCATTACTATTCCGTTGTC
TGCTCAAGGGCAATTGTCTAGTCTCGAGCAATTTAAAAATATCAGCTTAAAAAGTAAAACTA
ACGGTAGCGTAATTAAGTTATCTGATGTTGCCAATGTAGAAATAGGTTCACAAGCATATAAC
TTTGCCATTTTGGAAAATGGTAAGCCTGCTACCGCGGCTGCAATTCAATTAAGCCCGGGTGC
TAACGCCGTGAAAACTGCCGAAGGTGTTCGAGCAAAAATTGAAGAATTGAAGCTAAATTTAC
CGGAAGGCATGGAATTTAGTATTCCTTACGACACAGCGCCGTTTGTCAAAATTTCAATTGAA
AAGGTAATTCATACATTACTTGAAGCCATGGTTCTGGTTTTTATTGTGATGTATCTATTTTT
ACATAATGTCCGCTATACGCTTATTCCAGCGATTGTGGCGCCTATTGCCTTACTCGGTACTT
TTACCGTGATGTTGCTTGCCGGCTTTTCAATTAACGTACTCACCATGTTTGGTATGGTACTT
GCCATCGGGATTATTGTCGACGATGCAATTGTTGTCGTTGAAAACGTCGAAAGGATTATGGC
GACTGAAGGATTAAATCCTAAAGATGCAACCTCTAAAGCAATGAAAGAGATTACCAGCCCGA
TTATTGGTATTACGCTGGTATTGGCGGCAGTATTTTTACCTATGGCATTTGCGAGTGGTTCT

GTAGGGGTAATCTATAAACAGTTTACCTTGACCATGTCGGTATCTATTTTATTTTCAGCGCT
ATTGGCACTCATTTTAACACCGGCACTTTGTGCCACAATTTTAAAGCCAATCGATGGGCATC
ATCAGAAGAAGGGCTTCTTTGCATGGTTTGACCGTAGTTTCGATAAAGTCACTAAAAAGTAT
GAATTGATGCTGCTTAAAATCATCAAACATACAGTTCCAATGATGGTGATCTTTTTAGTAAT
TACCGGTATTACCTTTGCCGGAATGAAATATTGGCCAACAGCATTTATGCCAGAGGAAGATC
AAGGCTGGTTCATGACTTCGTTCCAGCTACCTTCAGATGCAACCGCAGAGCGTACTCGGAAT
GTAGTCAATCAATTTGAAAATAATTTGAAAGATAATCCCGATGTAAAAAGTAATACCACCAT
TTTGGGATGGGGTTTTAGTGGCGCAGGACAAAACGTAGCTGTGGCTTTTACGACACTTAAAG
ATTTCAAAGAGCGGACTAGCTCTGCATCTAAGATGACAAGCGCCGTTAATACTTCTATGGCG
AACAGTACGGAAGGTGAGACCATGGCCGTATTACCACCCGCTATTGATGAGTTAGGTACTTT
TTCAGGTTTCAGTTTACGTTTACAAGACCGCGCTAACTTAGGTATGCCTGCTTTACTGGCTG
CTCAAGATGAACTTATGGCAATGGCAGCCAAGAATAAAAAGTTCTATATGGTTTGGAATGAA
GGGTTGCCACAAGGTGACAATATTTCTTTAAAAATTGACCGTGAAAAGCTTAGTGCACTTGG
TGTTAAGTTTTCTGATGTTTCAGACATCATCTCTACATCAATGGGTTCAATGTATATCAATG
ACTTCCCTAATCAAGGACGTATGCAACAAGTCATTGTACAAGTTGAGGCTAAATCACGTATG
CAATTGAAAGATATCTTGAATCTGAAAGTCATGGGTTCAAGCGGTCAATTAGTCTCGTTATC
AGAAGTTGTAACGCCACAATGGAATAAGGCACCGCAACAATATAATCGTTATAACGGACGAC
CATCTTTGAGTATTGCTGGTATTCCTAACTTCGATACGTCATCGGGTGAAGCAATGCGTGAA
ATGGAACAACTGATTGCGAAATTACCGAAAGGTATTGGCTACGAGTGGACAGGTATTTCCTT
ACAGGAAAAGCAGTCTGAATCACAAATGGCCTTTTTACTTGGTTTATCCATGTTAGTGGTTT
TCCTTGTCTTGGCTGCACTCTATGAAAGCTGGGCAATTCCACTTTCTGTGATGCTGGTTGTG
CCACTCGGTATTTTTGGAGCAATCATTGCCATTATGTCTAGAGGGTTAATGAATGATGTGTT
CTTCAAAATCGGGCTAATTACCATTATTGGTCTATCGGCAAAGAATGCAATTTTGATTGTTG
AATTTGCGAAAATGCTGAAAGAAGAAGGCATGAGTTTGATTGAAGCCACTGTTGCTGCAGCC
AAACTTCGCTTACGGCCAATCCTGATGACATCACTTGCATTTACCTGTGGTGTAATTCCTTT
GGTGATTGCCTCAGGTGCAAGTTCAGAAACTCAACATGCTTTAGGCACAGGGGTTTTTGGTG
GCATGATTTCAGCCACTATTCTGGCTATTTTCTTTGTTCCCGTGTTTTTTATCTTCATTCTG
GGTGCAGTAGAAAAGCTATTTTCCTCTAAGAAAAAAATCTCATCTTAAgttcaatgcatcag
gggaaatactcaatttcctctgatgcatttttactgatctgaatgagagaatattactaact
aataaatgtcagccatttatagtctttggggttttaaatttatataaaaagtcagactagcc
tatatttttttacagctctaaatatttttttcaaataataacctgttaaagatttttctgctg
aaagcaaaccgctaacagttcctgaaaataccaattcaccacctttgtcacccgccaaaggg
ccaatatcaacaatccagtcggcttgacttatgatgtctaagttatgttcaatcacaatgac
tgtgttgccttttctcaactaagttatttaataaactaattagttttttcagtatcagaagggt
gtagtcctgtactc

The genes annotated as *adeA* and *adeB* in *A. baumannii* ATCC17978 (primer design based on this strain) are shown in capital letters while the upstream and downstream flanking regions are shown in lower case. Blue highlighted regions indicate primers 1479 and 1480, which are used to check for the deletion of this region.

Appendix 3. The a*deFGH* operon with primer locations indicated

cttgaagcttctccttaaaacacaaataaggtgataaaaacttgagtatttcgaacttactc
atctgctgaaataacagcaatttctataccataagttaaggatttatgaagatgtctaagca
atgatctgtattgacggattaaatgttgaaaaatcaatcaaattttaagattaagttttgag
cgtataagcttcagcaatattatgctgctcgaccaaagtacgaattgtatattcatgaccag
tttctttatcacaggcaaattcacatttctggtcgaaaggtaaagctgtaccgccaagtaat
ggacagcctgcaaaaagttcagcgacccaatcaacaaacacttttactttaagcgataaatg
acgattttgaagataaactgctgaaatcggcatcggtgctggcgtccactgaggcaatacct
cttttaacaaacctgattctaaatggttggtgagcatataacgtgggccttgaattatacca
aaaccttgcaaagccaagtcgatataagcatcaccgtcatttaccgaaacacgtccacgtac
tgacacacttttaattaaatcatcaaccacaaagtcccaatcgaagttgcgtccggtacggc
ttgaaaagaaatgaatcgctttatgatttttttgcaaatcttcaattgaggtaggttcgcca
tatttttctaaataaatcggtgaagcagcggttgcacactggaaagttccgatacgacgcgc
aattaagcttgaatcttttaattcacccacccgaattgcacaatcaacagcttctccaacca
aatctacaggtcggtcgttcagaccaattactaaatcaatatcaggatagcgtgcatgaaaa
tcgcggagccttggaattaaaattaaacgtccaatcgatacaggcacatcaatacgaagctg
acctcttggcccccgctctgcatcatgaaaagaagattcaatatcggcaacatccgctaaaa
tacgggctgtccgatcataatatacggcgccatccggtgtgagactaatttttcgtgttgtc
cgattaagcaatcgaacttgtaaatgcttctctaaagcctgaatggttgtagtcacagaagc
acgcggtaaacccaaactatcagctgctaaactgaaactatttgtttcaacaactttgttga
atactctcatggcatgaaatagatccacacgcacactcctattaatgcattttctagtgcaa
acacatcaattgttataaaaatacgaatagtgttatcaaatttaatatatttattcgtgatt
gacaaacttttatgctccgtccattccataaaccctgtttctcccatacggaccataactaa
aagtctgaaaacaggtatccaaataactaggagcacctcatgtcattttcccgcaaacagtt
tgcactgtctgccatctttgttgctattttagcaaccggtggcagttttATGTTGTTACATG
AAAATGCCGATGCAAAAGCTGCACCAACCGCTGCCCAACAAGCTGCTACTGTTGATGTAGCC
CCAGTAGTAAGCAAAACCATTACCGATTGGCAAGAATATTCCGGTCGTTTAGAAGCAATTGA
TCAAGTTGATATTCGGCCTCAAGTTTCAGGAAAACTCATTGCCGTACATTTCAAAGATGGAA
GCCTCGTTAAAAAAGGTGATTTACTTTTCACAATCGACCCTCGTCCTTTTGAAGCAGAACTG
AACCGTGCAAAAGCCCAGCTTGCTTCAGCTGAAGCACAGGTAACATATACCGCCAGCAATCT
TTCGCGTATTCAACGTCTCATTCAGAGTAATGCTGTTTCTCGTCAAGAACTGGATTTAGCCG
AAAATGATGCACGTTCAGCGAATGCTAACCTACAAGCCGCTAGAGCTGCTGTCCAATCTGCA
CGTTTAAATCTGGAATACACCCGTATTACTGCACCAGTCAGCGGCCGGATTTCACGAGCTGA
AGTGACCGTGGGTAATGTCGTCTCTGCAGGTAATGGCGCACAGGTTTTAACAAGTTTAGTGT
CTGTATCACGCCTATACGCATCTTTCGATGTTGATGAACAAACTTACCTGAAATATATCAGT
AATCAGCGTAATTCAGCACAAGTACCTGTCTATATGGGACTTGCCAATGAAACAGGCTTTAC
TCGCGAAGGTACAATCAACTCAATCGATAACAACCTGAATACAACCTCAGGAACAATCCGTG
TTCGTGCAACTTTTGACAATCCAAACGGTGTTTTATTACCGGGCCTATATGCACGAATTCGT
TTAGGTGGAGGTCAACCTCGCCCAGCGATTCTGATTAGTCCAACCGCGGTTGGCGTCGACCA
AGATAAACGTTTTGTCGTAGTAGTTGATGCGAAAAATCAAACTGCTTATCGCGAAGTAAAAC
TCGGTGCCCAACAAGATGGCTTGCAAATCGTAAATAGCGGATTACAAGCGGGTGATCGTATT
GTAGTGAATGGTTTACAGCGAATTCGTCCGGGTGACCCTGTTACACCGCATCTCGTTCCTAT
GCCAAATTCACAAATCACTGCTAACGCTACTCCTCAACCTCAGCCAACAGATAAAACATCAA
CTCCGGCAAAAGGTTAAtcacatatgaatatttctaagtttttattgatcggccgatcttt
gctggtgttttatcagtattgattttactcgccggtctgctttcggtattccaattaccgat
ttctgaatatcccgaggttgttccaccatctgtggtggtacgcgcccaatatccgggtgcaa

acccaaaagtgattgctgaaacggttgcatctccgctcgaagagtcaatcaacggcgtcgaa
gacATGCTGTATATGCAATCTCAAGCAAACAGCGACGGTAACCTAACCATTACGGTGAACTT
TAAGCTCGGTATCGACCCAGATAAAGCCCAACAATTGGTTCAAAACCGTGTGTCTCAGGCCA
TGCCCCGTTTACCTGAAGATGTACAGCGCTTAGGTGTAACCACACTAAAAAGCTCACCTACT
CTAACTATGGTAGTGCATCTGACCTCACCAGATAATCGCTATGACATGACCTACTTACGTAA
CTATGCGGTGCTCAACGTGAAAGACCGTTTAGCGCGTTTACAAGGGGTTGGTGAAGTCGGAT
TATTTGGTTCTGGTGACTACGCGATGCGTGTATGGCTTGACCCGCAAAAAGTAGCACAGCGT
AACCTTACAGCCACTGAAATTGTGAATGCAATCCGTGAACAAAACATTCAGGTTGCAGCGGG
TACAATCGGTGCATCACCAAGTAATTCACCTTTACAGCTTTCGGTCAATGCTCAAGGTCGTT
TAACCACTGAGCAAGAATTTGCCGATATCATTTTAAAAACTGCTCCCGACGGTGCAGTCACC
CGATTGGGTGATGTTGCACGTGTTGAACTCGCAGCTTCACAATATGGCTTACGTTCATTGCT
TGATAATAAACAAGCTGTCGCAATTCCAATTTTCCAAGCTCCGGGTGCGAATGCTTTACAAG
TTTCCGATCAAGTGCGTAGCACAATGAAGGAGCTTTCAAAAGATTTTCCATCTTCAATTAAA
TACGACATTGTTTATGACCCGACTCAGTTCGTACGGGCCAGTATTAAAGCCGTCGTTCATAC
CTTACTTGAAGCAATTGCACTGGTTGTTGTGGTTGTTATTTTATTCTTACAAACATGGCGCG
CCTCAATCATTCCATTGCTTGCCGTACCGGTTTCAATTATTGGTACATTCGCGCTCATGCTC
GCTTTTGGTTACTCAATCAATGCGCTATCACTGTTCGGGATGGTGCTTGCCATCGGCATTGT
CGTCGATGACGCGATTGTGGTCGTCGAAAATGTCGAGAGGAATATTGAAGCAGGCTTAAACC
CAAGGGAGGCGACTTACCGTGCCATGCGAGAAGTCAGTGGACCGATTATTGCCATTGCTTTA
ACACTTGTTGCAGTATTCGTACCTCTTGCCTTTATGACAGGCTTAACAGGGCAATTCTATAA
ACAATTTGCCATGACCATTGCCATTTCAACGGTTATTTCGGCTTTTAACTCACTCACCCTAT
CTCCAGCTTTGGCAGCGCTGTTACTTAAAGGACACGATGCTAAACCTGATGCCTTAACACGT
ATTATGAATCGTGTATTCGGTCGTTTCTTTGCACTGTTTAACCGTGTGTTTTCACGTGCTTC
AGACCGTTATAGTCAAGGCGTCAGCCGTGTCATTTCCCATAAAGCTTCGGCAATGGGTGTCT
ATGCAGCACTTCTAGGTTTAACCGTTGGTATTTCCTATATTGTTCCGGGCGGTTTCGTTCCT
GCGCAGGACAAACAATATTTAATTAGCTTTGCGCAGCTACCAAACGGCGCATCATTAGATCG
TACCGAAGCAGTCATTCGTAAAATGAGTGACACTGCACTTAAACAGCCTGGTGTAGAAAGTG
CTGTTGCCTTCCCTGGCCTCTCAATTAATGGTTTCACCAATAGCTCTAGCGCTGGTATTGTC
TTTGTGACTTTAAAGCCATTTGATGAACGTAAGGCAAAAGACTTATCTGCAAATGCAATTGC
AGGTGCGCTCAACCAGAAATATTCAGCTATTCAAGATGCCTATATCGCGGTTTTCCCACCAC
CACCAGTGATGGGCTTAGGTACTATGGGCGGCTTTAAACTGCAACTTGAAGACCGAGGTGCC
TTAGGCTATTCAGCCTTGAACGATGCTGCTCAAAACTTTATGAAGGCAGCACAATCAGCCCC
TGAACTTGGTCCAATGTTCTCAAGTTATCAAATTAACGTGCCTCAACTCAATGTAGATCTGG
ACCGTGTAAAAGCTAAACAGCAAGGCGTTGCTGTGACAGATGTTTTCAACACTATGCAGATT
TATTTAGGTTCTCAGTACGTTAACGACTTTAACCGTTTTGGACGTGTTTATCAGGTCCGTGC
ACAAGCCGATGCGCCTTTCCGTGCTAACCCTGAAGATATTTTGCAGCTTAAAACCCGTAATA
GTGCCGGACAAATGGTGCCATTATCTTCATTGGTGAATGTAACTCAAACCTATGGTCCTGAA
ATGGTCGTTCGTTATAACGGTTACACATCAGCAGATATTAACGGCGGCCCTGCCCCAGGTTA
TTCATCTAGCCAAGCAGAAGCTGCGGTTGAACGTATTGCTGCACAAACTCTACCGCGTGGTA
TCAAGTTTGAATGGACAGATTTAACTTACCAAAAAATCTTGGCTGGTAATGCTGGACTTTGG
GTATTCCCTATTAGCGTATTACTCGTGTTCTTAGTATTAGCTGCTCAGTATGAAAGCTTAAC
CCTACCATTAGCAGTTATCTTAATTGTACCAATGGGAATCTTAGCGGCTCTGACAGGTGTCT
GGTTGACAGCTGGAGATAACAACATCTTTACCCAAATCGGTCTAATGGTACTGGTGGGGCTA
GCCTGTAAAAATGCTATTTTGATTGTCGAATTTGCGCGGGAACTTGAAATGCAAGGTGCGAC
TGCCTTTAAAGCAGCCGTTGAAGCAAGTCGTCTACGTTTACGCCCAATTTTAATGACCTCTA
TTGCATTTATTATGGGTGTAGTGCCACTGGTTACTTCAACTGGCGCAGGTTCTGAAATGCGA
CATGCGATGGGTGTTGCCGTATTCTTCGGTATGATCGGTGTAACATTCTTTGGTTTATTCCT

CACCCCGGCCTTTTACGTTCTGATTCGTACCCTCAACAGCAAACATAAACTACATTCTGCGG
CAGTTCATGAAGCGCCGTTAGCTAGCCCACACGATCATTAAggaggacatttggtgattaca
tcaaaacaaaactggttgttgtcctcactcatgggaagcctgctccttgcaggctgctcatt
ggccccagaatatcaacctgcaaaagttataGTGCCAGTCAAATTCAAAGAATCTGACCCCA
AACTTGAAGATAATAACTGGAAGATTGCCCAACCTGCCGATCAACAAACTCGTGGTGAATGG
TGGCGCATTTACAATGATACTCAACTGAATGAACTTGAACAGCAAGCTATCGCAGGCAACCA
GAACCTAAAAGCGGTGGCAGCAAATATTCAGGCTTCACGTGCACTACGTTCGGCAGCTCAAG
CTGAACGCTTACCAAGTATTGATGCCGGATTTGGGCCAACCCGCCAAAAGCCGTCTCCAGCT
TCACTCGGTTTAGATGACAATGCACATACTTCGGCTCAAACCTTATGGCGAGCTCAAGCCAA
TGTTTCATATGAGCTCGATTTATTTGGTCGTGTAGCAAGTAGTGTCAACGCAGCAACAGCGG
ATCTACAGCAACAAGAGGCACTATATCAGTCGGCACTTTTAGCTCTACAAGCGGATGTAGCT
CAAGGTTATTTTCTGATACGTCAACTTGATACCGAACAGGCAATTTATAACCGTACAATCAA
ATTATTAGGTGAAACACGAGATTTAATGCAGCTTCGTTTTAAAAACGGACTGGTCAGTGAAT
TAGATGTTTCTCGTGCACAAACCGAACTTGCTACCGCACAAACCACTGCCCTAAATATTGCT
CGTAACAGAGCCAGTGCAGAACATGCGCTTGCAGTCTTATTAGGAAAACCACCAGCAGACTT
TAACTTGGCTGTTCAACCTTTAACTGCAAATAGTATCCGTCTCCCTGCCGGTTTGCCGTCAA
CTTTACTTGAAAGACGACCCGATATTGCGGCTGCAGAGCGTGCAATGGCAGCAGATAATGCA
CGTATTGGGATTGCTCGTGCAGCATTTTTCCCAAAACTCAGTCTTACAGGAGCTTTAGGTTA
TGAATCTTCAAGTTTAAGCGAGTTGGGTAAATGGTCGAGTCGGACTTTTTTACTAGGACCTG
TCGCTGGTACTATTTTGTCGTTACCTTTATTTGATGGTGGACAACGTAAAGCAGGCGTTGCT
CAAGCAAGAGCGGCTTATGAGGAAAGCGTCGCCAACTATAGACAAACTGTACTAAACGCATT
TCGCGAAGTTGAAAATGGTTTATCTGATCAAAGAATTCTCGATCAGCAAATTCAGGCTCAAA
ACCAAGCACTCTCCTCTTCTCGTCATGCCAATCAACTTTCTCATTTACGTTATCGAGAAGGT
GCTATTAGCTATCTTGATGTCATTGATTCTGACCGCACTATTTTGCAACAAGAACAATTAGC
AGCTCAGCTGAAAGGCAGCCAAATCATTGCAAGCATCAATTTAATCCGTGCTTTAGGGGGCG
GTTGGAGTAGTTAAtaaaaaagcgccttacggcgtctttttttatatacacaaattatttttg
catatacagggaattttgcacaaacagcttcaacttttgcttttacatcagcaataacttttt
tcgtcacctttgctatcaatcacgtcagcgatccaaccagcaagttcacgaacttcagcttc
accaaaaccacgagttgttactgccggagtaccgatacggataccagaagtcacaaatggag
aacgtgggtcatttggaactgagtttttgttcacagtaatgtgagcagcacctaaccaagcg
tctgcatctttaccagttacgtcttgtttgattaaagataacaagaataagtggttgtctgt
accaccagaaacaacatcataaccacgagcaatgaatacttcagccattgcttgagcatttt
tcacaacttgttgttggtaagctttaaagtcgtcagacattgcttctttgaagcagattgct
ttagcagcaatcgcatgcattaatggaccaccttggttaccagggaatacagctgattgaag
ttttttctcgatttcttcattcgctttcgcaaggattaaaccagaacgtggaccacgaagtg
ttttgtgagtcgtcgttgttgttacgtcagcaatttgaactgggtttggatatacaccagca
gcaacaagacctgcaacgtgagccatatcaacaaaaaggtaagcaccaactttgtccgcgat
gtcacggaaacgttgccaatctacaacacggctataagcagagaaaccagcaacgatcatac
gtggcttgtgttccaatgctaaacgttcaacttcttcgta

The genes annotated as *adeF, adeG* and *adeH* in *A. baumannii* ATCC17978 (primer design based on this strain) are shown in capital letters while the upstream and downstream flanking regions are shown in lower case. Blue highlighted regions

indicate primers 1481 and 1482, which are used to check for the deletion of this region.

Appendix 4. The a*delIJK* operon with primer locations indicated

attagcaacaagtatcgcgcttgttggttgtagcaaaggctccgatgagaaacagcaagctg
ctgctgctcagaaaATGCCGCCTGCAGAAGTAGGTGTTATTGTTGCTCAACCACAAAGTGTT
GAACAAAGCGTTGAGCTTTCAGGCCGTACTTCAGCATATCAAATTTCTGAAGTTCGTCCTCA
GACAAGTGGCGTGATTTTAAAACGCTTATTTGCTGAAGGAAGCTATGTTCGTGAAGGTCAGG
CGCTTTATGAGCTCGACTCTAGAACGAACCGTGCAACGTTAGAAAATGCAAAAGCATCACTC
CTACAACAACAGGCAAATCTAGCTTCACTACGTACCAAGTTAAATCGTTATAAACAACTTGT
TTCTAGTAATGCTGTGTCTAAACAGGAATATGATGACTTACTTGGTCAAGTCAATGTTGCAG
AAGCACAAGTTGCAGCAGCTAAGGCTCAAGTAACAAATGCAAATGTAGATCTTGGTTATTCT
ACAATTCGCTCTCCTATTTCTGGCCAATCTGGTCGTTCTTCAGTAACGGCTGGTGCTTTGGT
TACTGCAAACCAGACTGACCCGTTGGTAACGATTCAACAGTTAGATCCTATCTATGTTGATA
TTAATCAGTCTAGTGCTGAGTTATTGCGTTTACGTCAACAACTAAGCAAAGGCAGTTTAAAT
AACAGTAACAACACGAAAGTAAAATTAAAGCTTGAAGATGGTTCTACCTATCCAATCGAAGG
GCAACTTGCTTTCTCTGACGCTTCTGTAAACCAAGATACAGGAACAATTACATTACGTGCCG
TATTCTCTAACCCGAATCATTTATTGCTTCCGGGTATGTATACCACTGCGCAAATTGTTCAG
GGCGTTGTTCCAAATGCTTACCTGATTCCTCAAGCTGCCATTACTCGTTTACCTACAGGGCA
AGCTGTAGCGATGCTTGTTAATGCTAAAGGGGTTGTTGAGAGCCGTCCTGTTGAAACCTCTG
GTGTTCAAGGACAAAATTGGATTGTGACTAACGGCTTAAAAGCCGGCGATAAAGTCATTGTT
GATGGTGTTGCCAAAGTTAAAGAAGGGCAAGAAGTATCAGCAAAACCTTATCAAGCTCAACC
AGCAAACTCTCAAGGTGCAGCACCAAATGCTGCGAAACCGGCTCAATCAGGTAAACCTCAAG
CAGAACAGAAAGCAGCTTCAAATGCATAAggggtagattgaatggcacaatttttattcat
cgccccatatttgcgtgggtgattgcattagtcattatgttggcgggtattcttacgctaac
aaaaATGCCTATTGCACAATATCCAACGATTGCACCACCAACCGTAACGATTGCTGCGACTT
ATCCTGGTGCATCGGCTGAAACAGTTGAAAATACTGTAACCCAGATCATTGAACAACAAATG
AATGGTCTTGATGGCTTACGTTATATTTCATCTAACAGTGCTGGTAATGGTCAGGCATCTAT
TCAATTAAACTTTGAACAAGGTGTTGACCCTGATATTGCACAGGTTCAAGTTCAAAACAAAT
TGCAATCTGCAACTGCGCTTTTACCTGAAGATGTACAACGTCAAGGTGTAACAGTTACTAAA
TCTGGTGCGAGCTTCTTGCAAGTTATTGCATTCTATTCACCAGATAACAACCTGTCAGACTC
TGACATTAAAGACTACGTAAACTCGTCAATTAAAGAACCGCTTAGCCGTGTTGCCGGTGTTG
GTGAGGTACAGGTCTTCGGTGGCTCATACGCAATGCGTATCTGGCTTGATCCAGCTAAATTA
ACAAGCTACCAACTTACTCCTAGTGATATTGCAACTGCCTTACAAGCGCAGAACTCGCAAGT
TGCTGTAGGTCAGTTAGGTGGTGCTCCGGCTGTACAAGGTCAAGTTCTTAACGCAACAGTAA
ATGCACAAAGCTTATTGCAGACTCCTGAACAGTTTAAAAATATCTTCTTAAAGAACACAGCA
TCAGGTGCTGAGGTTCGATTAAAAGATGTTGCTCGCGTAGAATTAGGTTCGGATAACTATCA
ATTTGACTCGAAGTTTAACGGTAAACCGGCAGCTGGTCTTGCAATTAAAATTGCAACAGGTG
CTAACGCACTCGACACAGCCGAAGCAGTTGAACAACGTTTATCTGAACTACGTAAGAACTAT
CCAACAGGTCTTGCAGATAAACTGGCTTATGACACGACTCCATTTATCCGTCTTTCAATTGA
AAGTGTAGTACACACATTAATTGAAGCCGTGATTTTGGTATTCATTGTCATGTTCCTATTCT
TACAAAACTGGCGTGCAACGATTATTCCAACGCTTGCAGTTCCAGTAGTTGTATTAGGTACA
TTTGCGGTCATTAATATCTTTGGCTTCTCAATTAACACCTTAACCATGTTCGCTATGGTATT
GGCAATCGGTCTTCTGGTCGACGACGCCATTGTTGTAGTCGAAAACGTTGAACGTGTGATGA
GTGAAGACCATACCGATCCGGTTACCGCCACTTCTCGCTCAATGCAGCAGATTTCTGGTGCG
TTAGTAGGTATTACCAGCGTATTGACAGCGGTATTCGTACCAATGGCTTTCTTTGGTGGTAC
AACAGGTGTAATTTACCGCCAGTTCTCGATTACCCTTGTAACTGCAATGGTTCTGTCGTTAA
TTGTAGCGTTGACGTTCACACCGGCACTTTGTGCAACTATCTTGAAACAGCATGATCCTAAT
AAAGAACCAAGCAATAATATCTTTGCGCGTTTCTTTAGAAGCTTTAACAATGGTTTTGACCG

CATGTCGCATAGCTACCAAAATGGTGTTAGCCGCATGCTTAAAGGCAAAATCTTCTCTGGCG
TGCTCTATGCTGTTGTAGTTGCCCTTTTAGTCTTCTTGTTCCAAAAACTCCCGTCTTCATTC
TTACCAGAAGAAGATCAGGGTGTGGTCATGACACTTGTACAATTACCACCAAATGCAACGCT
TGACCGTACCGGTAAAGTGATTGACACCATGACTAACTTCTTTATGAATGAAAAAGACACCG
TGGAATCTATTTTCACTGTTTCTGGTTTCTCATTCACAGGTGTTGGTCAAAACGCTGGTATT
GGCTTCGTTAAGTTGAAAGACTGGAGCAAACGTACGACACCAGAAACTCAAATTGGTTCATT
GATTCAGCGTGGTATGGCATTAAATATGATCATTAAAGATGCATCATATGTTATGCCGTTAC
AGCTTCCAGCAATGCCTGAACTTGGTGTAACTGCCGGATTTAACTTGCAGCTTAAAGATTCA
AGTGGTCAAGGCCATGAGAAACTGATCGCAGCTCGTAACACGATTTTAGGTTTGGCATCACA
AGATAAACGTCTTGTAGGTGTGCGTCCAAATGGTCAGGAAGATACTCCTCAATATCAAATTA
ATGTAGATCAGGCTCAAGCTGGTGCTATGGGCGTTAGTATTGCCGAAATCAACAATACAATG
CGTATTGCATGGGGTGGCTCATACATTAACGATTTCGTTGACCGTGGTCGTGTGAAAAAAGT
TTATGTTCAAGGTGATGCGGGCAGCCGTATGATGCCTGAAGACTTAAACAAATGGTATGTAC
GTAATAACAAAGGTGAGATGGTTCCATTCTCGGCGTTTGCTACAGGCGAATGGACGTATGGT
TCTCCACGTCTCGAACGTTATAACGGTGTGTCATCAGTTAACATTCAAGGTACACCTGCACC
TGGCGTGAGCTCTGGTGATGCCATGAAAGCAATGGAAAAAATTATTGGTAAGTTGCCTTCTA
TGGGCTTACAAGGTTTCGACTATGAATGGACAGGCTTATCACTTGAAGAACGTGAGTCTGGT
GCTCAAGCGCCGTTCTTATACGCACTTTCATTGTTAATCGTATTCCTTTGCTTGGCTGCACT
ATATGAAAGCTGGTCAATTCCGTTCTCGGTTTTACTTGTGGTACCACTTGGTGTCATTGGTG
CAATCGTATTGACCTACTTGGGCATGATTATTAAAGGAGATCCAAATCTCTCAAATAACATT
TACTTCCAAGTAGCGATTATTGCGGTTATCGGTCTTTCTGCAAAAAATGCGATCTTGATTGT
TGAATTCGCAAAAGAATTGCAGGAAAAAGGTGAAGATCTACTTGATGCAACCTTACATGCTG
CAAAAATGCGTTTACGTCCAATTATCATGACCACCCTTGCCTTCGGTTTCGGTGTACTTCCA
CTTGCACTTTCAACAGGTGCCGGTGCAGGAAGTCAGCACTCTGTAGGCTTTGGTGTACTTGG
TGGCGTACTCAGCGCGACGTTCTTAGGTATCTTCTTTATCCCTGTATTCTATGTGTGGATTC
GTAGTATCTTTAAGTACAAACCAAAAACCATAAACACTCAGGAGCATAAATCGTGAtgcaaa
aagtatggtctatttcaggtcgtagcattgcggtatctgcacttgcgcttgctttggcagct
tgtcaaagcATGCGCGGCCCAGAACCAGTCGTGAAAACCGATATACCACAAAGCTATGCATA
TAACAGCGCTTCTGGTACGTCTATTGCTGAACAGGGTTATAAACAGTTCTTTGCTGACCCGC
GTTTGCTTGAAGTGATTGATTTGGCTCTTGCCAATAACCGTGACTTACGTACAGCAACGCTC
AATATTGAACGTGCTCAACAGCAATATCAGATTACACAGAACAACCAGCTTCCAACAATCGG
AGCAAGTGGTAGTGCAATTCGTCAGGTTTCTCAAAGCCGTGATCCGAATAATCCCTACTCTA
CTTATCAAGTAGGTTTGGGTGTAACTGCTTATGAGCTAGATTTCTGGGGTCGTGTTCGTAGC
CTCAAAGATGCTGCTTTAGATAGTTATCTTGCAACACAAAGTGCTCGTGATTCGACTCAAAT
CAGTCTGATTAGCCAAGTTGCTCAAGCATGGTTAAATTATTCGTTTGCAACAGCAAACTTAA
GACTGGCAGAGCAAACGCTTAAAGCACAGTTAGATTCTTACAATCTCAACAAAAAACGTTTT
GATGTAGGTATTGACAGTGAAGTTCCATTACGTCAAGCACAGATTTCTGTAGAAACTGCGCG
TAATGATGTAGCGAACTACAAAACTCAAATTGCTCAAGCACAAAACTTGTTGAACTTGCTTG
TAGGCCAACCTGTTCCACAAAACTTGTTACCTACACAACCTGTAAAACGCATTGCTCAACAA
AATGTGTTTACTGCCGGTTTACCAAGTGACTTGTTAAATAACCGTCCGGATGTAAAAGCTGC
TGAATACAACTTAAGCGCTGCGGGTGCGAATATCGGTGCTGCAAAAGCACGTTTATTCCCAA
CCATTAGCTTAACGGGTTCGGCTGGTTATGCATCAACTGACTTAAGTGATCTATTTAAGTCT
GGTGGTTTTGTATGGTCAGTTGGTCCAAGCTTAGATTTACCAATCTTTGACTGGGGTACACG
CCGTGCCAATGTAAAAATTTCTGAAACTGATCAGAAAATTGCATTGTCTGATTATGAAAAAT
CAGTTCAGTCGGCGTTCCGTGAAGTTAATGACGCGCTTGCAACTCGTGCCAACATTGGTGAG
CGTTTAACAGCACAACAACGTCTAGTAGAAGCGACTAACCGCAACTACACACTTTCAAATGC
CCGCTTCCGTGCTGGTATTGATAGTTACTTGACTGTTCTTGATGCGCAGCGTTCTTCATATG

CAGCTGAACAAGGTTTGTTATTGCTTCAACAAGCAAACTTAAACAACCAAATCGAGTTATAC
AAAACTCTAGGTGGCGGTTTAAAAGCAAATACTTCAGATACAGTGGTACATCAACCATCTAG
TGCTGAACTTAAAAAGCAATAAgttttaaatcactttcttataaaagctcacttcggtgggc
ttttttattgcaactcatctgaatatttttttatcttgaaagctaaagaaatttagattgata

The genes annotated as *adeI, adeJ* and *adeK* in *A. baumannii* ATCC17978 (primer design based on this strain) are shown in capital letters while the upstream and downstream flanking regions are shown in lower case. Blue highlighted regions indicate primers 1483 and 1484, which are used to check for the deletion of this region.

Appendix 5. The pump 7 operon with primer locations indicated

tcaaaatcaggaacctgcctgcattcatcagaatatttacagtacagctcccagaagttatc
aggaaatgtagttttaattggttctggacagtccgcagcagagatttttttagatctatttg
atcgtcagtatatgaatggtcgaactacaccagactttcatctttattggttaacccgttct
gctggtttctttccaatggaaaatacaccattcggtttagaacattttagtccggattacat
gcattacttttaccatttaccatcaggtttaaaggcagagttaccagcgcaacaagcaaatt
tatataaaggtattagcagtaaaacgatacgtgatatttatgagcgtttatatcatcgtagt
atcggtggaatgcctacagatgttacgttggctggacatcatcaactgcaatatgcagagct
cttagaaaaccagaaaacaaagttgtgttttgaacaccgtcagcaaaataagaattatattc
tggaggctgattgtgttattgcagcaacgggttatcgttatccaaaccctaagtttctacaa
aatttacaatctttaatcatacgagatcaggccgaccattggcaggtgggttcaaattttaa
agtcaattatcaaggtgagggatctatatttattcaaaatatggaagtttatcaccacggcg
taggtacgcctgatttaggcttaggagcatatagggcagccacaattgcaaatcaattggtt
ggtgaagagttattcaaacttgatcattctcagtcgttccaacaatttggagtgaatgcatc
agcgcaagaagaagtttctgttcgcaatattgcagcctcaaaaataaccgagttttcaagaa
ctttaaataaatcagggccaatgcatcattctgctttgggagcgtatgcacgaagtaaatta
tctgagcatattgaataagctgaacagaggatatgtgtatggaacgttcttctgtgtccgcg
gctcaacctcatgagcaaagtttacaacgctacgtacttctgacggttATGCTTGGTACAGG
TACAGTTAGTTTAAATAACAGTAGTTTCAACCCTGCAATACCTCATTTAATGTCCTATTTTC
AGGTAGGTGAGGTATGGGCAAGTTGGGTCGTTGTTGCCTTTTTATTAGCAATGAGTATCAGT
TTGCCTTTAGCTGGATTCCTAAGCCAGCGTTTTGGTAAGCGGTCAATTTATTTAATTGCCTT
ATTGGGATTTGCTTTGGCTTCTACAGCAGGTGGTTTATTTAATCAGTTTGAATCGGTGTTGA
TTGCTAGAGCTCTGCAAGGCTTTTGTAGCGGATTAATGATTCCATTATCTCTTGGACTAATT
TTTTCAGTTACGCCAAGTGAACAACGGGGAAGTACAACAGGATTATGGGGTGCCATGATCAT
GCTAACCTTAGCAGTTGGGCCCATGCTCGGAGCACTGGTTTTGGTATGGCTTAATTGGAAGG
CCTTATTTTTTATCAATTTACCTGTTGCTTGTCTGGCATTAATTTTAGGCTATGTTTTTCTA
CCTAAAGAGCAAGGGGACAATAAGCAAGAGTTTGACTGGGCAGGATTCTTTTTTCTCGGTTC
AAGCATAGTTTTATTACTTGGGACACTGAGCCAAATCCATCAAATTCAGGACTTATTTCAAC
CTCTATATGGTGCGCTTTTAGTTCTAAGTGTGCTGCTTTTTATAAGGTTTATTTTTTTACAA
AAGAATAAGTCGATGCCATTAATCGAGCCAGCTTTGTTTGCTACGAAGGGTTTCCGCTACAG
CTTAGTCATTTGTGTGGCACAAACAGTAGGCTTATTTATTGGCATGTTGCTCATTCCACTAT
GGATACAGCACCTTCTAAAGTTAAGCCCGCTTTGGACTGGTTTTGCGCTAATGAGCAGTGCA
GTAGTTACCGGTATTTGTAGTCAACCTGCCGGAAAATATCTAGATCATTACGGTGCTGCAAA
AATCATGTCTCTTGGACTAATGATTACCGTGGCTTCTTTTCTTTTGCTTGCTTGGGCACCTA
TACAAAATGTCTGGTTCATTGTGTTTTGTATGATATTACATGGCTTAGGAATGGGACTTTCT
TATATGCCATCGACTACGGCAGGTTTGAACAGCTTACGCCAGCAGCAACAGCATCTAGTTAC
TCAAGCGGCCGCTCTAAATAATTTATTCCGACGTATTTTTGCAGCAGTGGCAGTCGTTATTG
CCGCGTTATATCTTCAGCTAAGACAACAATCATTGCCGTTAAATACTCAAGCAATATTTACA
TCATTTCACACTATGCAGGAAATCTTTGTGTGTTGTGCAATTCTTATACTTTGCACTTTGCC
TTTCGCATGGCGTTTTCCTGAACAGTACAGAATCGACCAATTAAAGAAATAGgattaataaa
aatgggacaattacatccagttttatcaaatacacatgtgattcaaaatactttacaggctt
ggcagcatccaaattcggatcaggcaaaatatgttgagcaacgtgttattaaacaattactt
caagctttaatttttgaagatattattcattcagaatatgatggaaaaaattttattataga
agtacaaaatagtcagggccaaaccattcgttatgtcgctgcagggcaaagacaatatagtt
acaaactagtacgtctcgcccgcaatcaggatgtatttcgtcaagatgaaaacggtcattat
cagattgctactttaaatttagttattgatgaaattcttagaaccataaccgatgcagcaaa

agttgaagattttatttttgaattaaaacgaacatttattcatgatttacagtctcaagcat
gttttgatcactatgctttgccggcaattcaatatccatatgatattttagaatcatattta
atggatggtcatccatatcatccgtgttacaagtcacgtgtaggttttagtttacaagacaa
tgtacgctatggtgtggagttcgcccaaccaatcgccttggtgtggttggctgtgcatcagg
acattgttgcaaaaaaacattcagaagatatagaaccagattcatttttttaaggagcaatt
gaattcacaagatcaagagctattcctgcaacatctctcagatcgtgatttgaaggttgatg
aatatatttggattccggttcatccttggcaatgggaaaaccatttaatttctatttttgct
gaagaaattctaaatggtaaaattgtttatttagggcaaagtcaggatcgctatttagccca
acaatctctacgcactatgactaacttgcagcatccggaaaagccttatatcaaactgtcga
tgagtctaactaatacatcaagctcacgtgttctggctaaacatacagtaatgaatgggcca
attattacagactggttgcaacgcttaattaaacagagcaaaaccgccaagagcttgattt
tgctgtattacgtgaagtgtatggtttaagcgtggattttactaaattgccaaaatcacatg
ctcaacaggcctatggcacaattggatgtctatggcgtgagagtgtgcatcaatatttacgt
gaag

The genes annotated as A1S_1649 (pump 7) in *A. baumannii* ATCC17978 (primer design based on this strain) are shown in capital letters while the upstream and downstream flanking regions are shown in lower case. Blue highlighted regions indicate primers 1104 and 1105, which are used to check for the deletion of this region.

References

References

Afzal-Shah, M., N. Woodford, et al. (2001). "Characterization of OXA-25, OXA-26, and OXA-27, molecular class D beta-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*." Antimicrob Agents Chemother **45**(2): 583-588.

Andrews, J. (2001). "Determination of Minimum Inhibitory Concentrations." Journal of Antimicrobial Chemotherapy Supplement **48**(Suppl. S1): 5-16.

Anstey, N. M., B. J. Currie, et al. (1992). "Community-acquired *Acinetobacter* pneumonia in the Northern Territory of Australia." Clin Infect Dis **14**(1): 83-91.

Anthony, K. B., N. O. Fishman, et al. (2008). "Clinical and Microbiological Outcomes of Serious Infections with Multidrug-Resistant Gram-Negative Organisms Treated with Tigecycline." Clinical Infectious Diseases **46**(4): 567-570.

Baumann, P. (1968). "Isolation of *Acinetobacter* from soil and water." J Bacteriol **96**(1): 39-42.

Beceiro, A., E. Llobet, et al. (2011). "Phosphoethanolamine Modification of Lipid A in Colistin-Resistant Variants of *Acinetobacter baumannii* Mediated by the *pmrAB* Two-Component Regulatory System." Antimicrobial Agents and Chemotherapy **55**(7): 3370-3379.

Beijerink, M. (1911). "Pigmenten als oxydatieproducten gevormd door bacterien." Vers. Konin. Akad. Wet. Ams. **19**: 1092-1103.

Berlau, J., H. Aucken, et al. (1999). "Distribution of *Acinetobacter* species on skin of healthy humans." Eur J Clin Microbiol Infect Dis **18**(3): 179-183.

Bernards, A. T., J. van der Toorn, et al. (1996). "Evaluation of the ability of a commercial system to identify *Acinetobacter* genomic species." Eur J Clin Microbiol Infect Dis **15**(4): 303-308.

Blair, J. M. A. and L. J. V. Piddock (2009). "Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update." Current Opinion in Microbiology **12**(5): 512-519.

Boo, T. W., F. Walsh, et al. (2006). "First report of OXA-23 carbapenemase in clinical isolates of *Acinetobacter* species in the Irish Republic." J Antimicrob Chemother **58**(5): 1101-1102.

Bou, G., G. Cerveró, et al. (2000). "Characterization of a Nosocomial Outbreak Caused by a Multiresistant *Acinetobacter baumannii* Strain with a Carbapenem-Hydrolyzing Enzyme: High-Level Carbapenem Resistance in *A. baumannii* Is Not Due Solely to the Presence of β-Lactamases." Journal of Clinical Microbiology **38**(9): 3299-3305.

Bou, G. and J. Martinez-Beltran (2000). "Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC beta-lactamase in *Acinetobacter baumannii*." Antimicrob Agents Chemother **44**(2): 428-432.

Bouvet, P. J. and P. A. Grimont (1987). "Identification and biotyping of clinical isolates of *Acinetobacter*." Ann Inst Pasteur Microbiol **138**(5): 569-578.

Bouvet, P. J. M. and P. A. D. Grimont (1986). "Taxonomy of the Genus *Acinetobacter* with the Recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and Emended Descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*." International Journal of Systematic Bacteriology **36**(2): 228-240.

Carbonne, A., T. Naas, et al. (2005). "Investigation of a nosocomial outbreak of extended-spectrum beta-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a hospital setting." J Hosp Infect **60**(1): 14-18.

Chang, H. C., Y. F. Wei, et al. (2005). "Species-level identification of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex by sequence analysis of the 16S-23S rRNA gene spacer region." J Clin Microbiol **43**(4): 1632-1639.

Chau, S. L., Y. W. Chu, et al. (2004). "Novel resistance-nodulation-cell division efflux system AdeDE in *Acinetobacter* genomic DNA group 3." Antimicrob Agents Chemother **48**(10): 4054-4055.

Chen, M. Z., P. R. Hsueh, et al. (2001). "Severe community-acquired pneumonia due to *Acinetobacter baumannii*." Chest **120**(4): 1072-1077.

Chu, Y. W., S. L. Chau, et al. (2006). "Presence of active efflux systems AdeABC, AdeDE and AdeXYZ in different *Acinetobacter* genomic DNA groups." J Med Microbiol **55**(Pt 4): 477-478.

Chu, Y. W., C. M. Leung, et al. (1999). "Skin carriage of *Acinetobacters* in Hong Kong." J Clin Microbiol **37**(9): 2962-2967.

Chuang, Y. C., W. H. Sheng, et al. (2011). "Influence of genospecies of *Acinetobacter baumannii* complex on clinical outcomes of patients with acinetobacter bacteremia." Clin Infect Dis **52**(3): 352-360.

Cisneros, J. M., M. J. Reyes, et al. (1996). "Bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical findings, and prognostic features." Clin Infect Dis **22**(6): 1026-1032.

Coelho, J., N. Woodford, et al. (2006). "Occurrence of OXA-58-like carbapenemases in *Acinetobacter* spp. collected over 10 years in three continents." Antimicrob Agents Chemother **50**(2): 756-758.

Coelho, J. M., J. F. Turton, et al. (2006). "Occurrence of carbapenem-resistant *Acinetobacter baumannii* clones at multiple hospitals in London and Southeast England." J Clin Microbiol **44**(10): 3623-3627.

Coldham, N. G., M. Webber, et al. (2010). "A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*." J Antimicrob Chemother **65**(8): 1655-1663.

Coldham, N. G., M. Webber, et al. (2010). "A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*." Journal of Antimicrobial Chemotherapy **65**(8): 1655-1663.

Corvec, S., L. Poirel, et al. (2007). "Genetics and expression of the carbapenem-hydrolyzing oxacillinase gene blaOXA-23 in *Acinetobacter baumannii*." Antimicrob Agents Chemother **51**(4): 1530-1533.

Coyne, S., P. Courvalin, et al. (2011). "Efflux-mediated antibiotic resistance in *Acinetobacter* spp." Antimicrob Agents Chemother **55**(3): 947-953.

Coyne, S., G. Guigon, et al. (2010). "Screening and Quantification of the Expression of Antibiotic Resistance Genes in *Acinetobacter baumannii* with a Microarray." Antimicrobial Agents and Chemotherapy **54**(1): 333-340.

Coyne, S., N. Rosenfeld, et al. (2010). "Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*." Antimicrob Agents Chemother **54**(10): 4389-4393.

Da Silva, G. J., S. Quinteira, et al. (2004). "Long-term dissemination of an OXA-40 carbapenemase-producing *Acinetobacter baumannii* clone in the Iberian Peninsula." J Antimicrob Chemother **54**(1): 255-258.

Da Silva, G. J., S. Quinteira, et al. (2004). "Long-term dissemination of an OXA-40 carbapenemase-producing *Acinetobacter baumannii* clone in the Iberian Peninsula." Journal of Antimicrobial Chemotherapy **54**(1): 255-258.

Damier-Piolle, L., S. Magnet, et al. (2008). "AdeIJK, a Resistance-Nodulation-Cell Division Pump Effluxing Multiple Antibiotics in *Acinetobacter baumannii*." Antimicrobial Agents and Chemotherapy **52**(2): 557-562.

Davis, K. A., K. A. Moran, et al. (2005). "Multidrug-resistant *Acinetobacter* extremity infections in soldiers." Emerg Infect Dis **11**(8): 1218-1224.

Dijkshoorn, L., H. Aucken, et al. (1996). "Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods." Journal of Clinical Microbiology **34**(6): 1519-1525.

Dolzani, L., E. Tonin, et al. (1995). "Identification of *Acinetobacter* isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic-spacer sequences." J Clin Microbiol **33**(5): 1108-1113.

Donald, H. M., W. Scaife, et al. (2000). "Sequence analysis of ARI-1, a novel OXA beta-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92." Antimicrob Agents Chemother **44**(1): 196-199.

Durand, R. E. and P. L. Olive (1982). "Cytotoxicity, Mutagenicity and DNA damage by Hoechst 33342." Journal of Histochemistry & Cytochemistry **30**(2): 111-116.

Eaves, D. J., V. Ricci, et al. (2004). "Expression of *acrB, acrF, acrD, marA,* and *soxS* in *Salmonella enterica* serovar Typhimurium: role in multiple antibiotic resistance." Antimicrobial Agents and Chemotherapy **48**(4): 1145-1150.

Ehrenstein, B., A. T. Bernards, et al. (1996). "*Acinetobacter* species identification by using tRNA spacer fingerprinting." J Clin Microbiol **34**(10): 2414-2420.

Endimiani, A., F. Luzzaro, et al. (2007). "Spread in an Italian hospital of a clonal *Acinetobacter baumannii* strain producing the TEM-92 extended-spectrum beta-lactamase." Antimicrob Agents Chemother **51**(6): 2211-2214.

Enoch, D. A., C. Summers, et al. (2008). "Investigation and management of an outbreak of multidrug-carbapenem-resistant *Acinetobacter baumannii* in Cambridge, UK." Journal of Hospital Infection **70**(2): 109-118.

Evans, B. A., S. Brown, et al. (2007). "Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii*." Clin Microbiol Infect **13**(11): 1137-1138.

Falagas, M. E., K. N. Fragoulis, et al. (2005). "Nephrotoxicity of intravenous colistin: a prospective evaluation." International Journal of Antimicrobial Agents **26**(6): 504-507.

Falagas, M. E., P. I. Rafailidis, et al. (2010). "Colistin therapy for microbiologically documented multidrug-resistant Gram-negative bacterial infections: a retrospective cohort study of 258 patients." International Journal of Antimicrobial Agents **35**(2): 194-199.

Falagas, M. E., P. I. Rafailidis, et al. (2006). "Effectiveness and nephrotoxicity of colistin monotherapy vs. colistin–meropenem combination therapy for multidrug-resistant Gram-negative bacterial infections." Clinical Microbiology and Infection **12**(12): 1227-1230.

Fishbain, J. and A. Y. Peleg (2010). "Treatment of *Acinetobacter* infections." Clin Infect Dis **51**(1): 79-84.

Forster, D. H. and F. D. Daschner (1998). "*Acinetobacter* species as nosocomial pathogens." Eur J Clin Microbiol Infect Dis **17**(2): 73-77.

Fournier, P. E., D. Vallenet, et al. (2006). "Comparative genomics of multidrug resistance in *Acinetobacter baumannii*." PLoS Genet **2**(1): e7.

Fu, Y., J. Zhou, et al. (2010). "Wide dissemination of OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* clonal complex 22 in multiple cities of China." Journal of Antimicrobial Chemotherapy **65**(4): 644-650.

Garcia-Garmendia, J.-L., C. Ortiz-Leyba, et al. (2001). "Risk Factors for *Acinetobacter baumannii* Nosocomial Bacteremia in Critically Ill Patients: A Cohort Study." Clinical Infectious Diseases **33**(7): 939-946.

Garrison, M. W., R. Mutters, et al. (2009). "In vitro activity of tigecycline and comparator agents against a global collection of Gram-negative and Gram-positive organisms: tigecycline Evaluation and Surveillance Trial 2004 to 2007." Diagn Microbiol Infect Dis **65**(3): 288-299.

Gerner-Smidt, P. (1992). "Ribotyping of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex." J Clin Microbiol **30**(10): 2680-2685.

Gerner-Smidt, P., I. Tjernberg, et al. (1991). "Reliability of phenotypic tests for identification of *Acinetobacter* species." J Clin Microbiol **29**(2): 277-282.

Gordon, N. C., K. Png, et al. (2010). "Potent synergy and sustained bactericidal activity of a vancomycin-colistin combination versus multidrug-resistant strains of *Acinetobacter baumannii*." Antimicrob Agents Chemother **54**(12): 5316-5322.

Gordon, N. C. and D. W. Wareham (2009). "A review of clinical and microbiological outcomes following treatment of infections involving multidrug-resistant *Acinetobacter baumannii* with tigecycline." Journal of Antimicrobial Chemotherapy **63**(4): 775-780.

Hamouda, A. and S. G. Amyes (2004). "Novel *gyrA* and *parC* point mutations in two strains of *Acinetobacter baumannii* resistant to ciprofloxacin." J Antimicrob Chemother **54**(3): 695-696.

Henwood, C. J., T. Gatward, et al. (2002). "Antibiotic resistance among clinical isolates of *Acinetobacter* in the UK, and in vitro evaluation of tigecycline (GAR-936)." J Antimicrob Chemother **49**(3): 479-487.

Héritier, C., L. Poirel, et al. (2005). "Contribution of Acquired Carbapenem-Hydrolyzing Oxacillinases to Carbapenem Resistance in *Acinetobacter baumannii*." Antimicrobial Agents and Chemotherapy **49**(8): 3198-3202.

Higgins, P. G., M. Lehmann, et al. (2010). "*gyrB* Multiplex PCR To Differentiate between *Acinetobacter calcoaceticus* and *Acinetobacter* Genomic Species 3." Journal of Clinical Microbiology **48**(12): 4592-4594.

Higgins, P. G., H. Wisplinghoff, et al. (2007). "A PCR-based method to differentiate between *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU." Clinical Microbiology and Infection **13**(12): 1199-1201.

Higgins, P. G., H. Wisplinghoff, et al. (2004). "In vitro activities of the beta-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam alone or in combination with beta-lactams against epidemiologically characterized multidrug-resistant *Acinetobacter baumannii* strains." Antimicrob Agents Chemother **48**(5): 1586-1592.

Holloway, K. P., N. G. Rouphael, et al. (2006). "Polymyxin B and Doxycycline Use in Patients with Multidrug-Resistant *Acinetobacter baumannii* Infections in the Intensive Care Unit." The Annals of Pharmacotherapy **40**(11): 1939-1945.

Hornsey, M., N. Loman, et al. (2011). "Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy." Journal of Antimicrobial Chemotherapy **66**(7): 1499-1503.

Huang, L., L. Sun, et al. (2008). "Differential susceptibility to carbapenems due to the AdeABC efflux pump among nosocomial outbreak isolates of *Acinetobacter baumannii* in a Chinese hospital." Diagnostic Microbiology and Infectious Disease **62**(3): 326-332.

Huang, Z. M., P. H. Mao, et al. (2004). "Study on the molecular epidemiology of SHV type beta-lactamase-encoding genes of multiple-drug-resistant A*cinetobacter baumannii*." Zhonghua Liu Xing Bing Xue Za Zhi **25**(5): 425-427.

Huda, N., E.-W. Lee, et al. (2003). "Molecular Cloning and Characterization of an ABC Multidrug Efflux Pump, VcaM, in Non-O1 *Vibrio cholerae*." Antimicrobial Agents and Chemotherapy **47**(8): 2413-2417.

Hujer, K. M., A. M. Hujer, et al. (2006). "Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center." Antimicrob Agents Chemother **50**(12): 4114-4123.

Hwa, W. E., G. Subramaniam, et al. (2010). "Iron regulated outer membrane proteins (IROMPs) as potential targets against carbapenem-resistant *Acinetobacter* spp. isolated from a Medical Centre in Malaysia." Indian J Med Res **131**: 578-583.

Ikonomidis, A., S. Pournaras, et al. (2006). "Discordance of meropenem versus imipenem activity against *Acinetobacter baumannii*." International Journal of Antimicrobial Agents **28**(4): 376-377.

Janssen, P., K. Maquelin, et al. (1997). "Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting." Int J Syst Bacteriol **47**(4): 1179-1187.

Jawad, A., H. Seifert, et al. (1998). "Survival of *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates." J Clin Microbiol **36**(7): 1938-1941.

Johnson, E. N., T. C. Burns, et al. (2007). "Infectious complications of open type III tibial fractures among combat casualties." Clin Infect Dis **45**(4): 409-415.

Kallel, H., M. Bahloul, et al. (2006). "Colistin as a salvage therapy for nosocomial infections caused by multidrug-resistant bacteria in the ICU." International Journal of Antimicrobial Agents **28**(4): 366-369.

Karageorgopoulos, D. E. and M. E. Falagas (2008). "Current control and treatment of multidrug-resistant *Acinetobacter baumannii* infections." Lancet Infect Dis **8**(12): 751-762.

Kempf, M. and J.-M. Rolain (2012). "Emergence of resistance to carbapenems in *Acinetobacter baumannii* in Europe: clinical impact and therapeutic options." International Journal of Antimicrobial Agents **39**(2): 105-114.

Kim, B. N., A. Y. Peleg, et al. (2009). "Management of meningitis due to antibiotic-resistant *Acinetobacter* species." Lancet Infect Dis **9**(4): 245-255.

Ko, K. S., J. Y. Suh, et al. (2007). "High rates of resistance to colistin and polymyxin B in subgroups of *Acinetobacter baumannii* isolates from Korea." Journal of Antimicrobial Chemotherapy **60**(5): 1163-1167.

Koh, T. H., T. T. Tan, et al. (2011). "*Acinetobacter calcoaceticus- Acinetobacter baumannii* complex species in clinical specimens in Singapore." Epidemiol Infect: 1-4.

Kuo, L. C., C. C. Lai, et al. (2007). "Multidrug-resistant *Acinetobacter baumannii* bacteraemia: clinical features, antimicrobial therapy and outcome." Clinical Microbiology and Infection **13**(2): 196-198.

La Scola, B., V. A. Gundi, et al. (2006). "Sequencing of the rpoB gene and flanking spacers for molecular identification of *Acinetobacter* species." J Clin Microbiol **44**(3): 827-832.

Lalande, M. E., V. Ling, et al. (1981). "Hoechst 33342 dye uptake as a probe of membrane permeability changes in mammalian cells." Proc Natl Acad Sci U S A **78**(1): 363-367.

Lee, Y.-T., C.-P. Fung, et al. (2011). "Outbreak of imipenem-resistant *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex harboring different carbapenemase gene-associated genetic structures in an intensive care unit." Journal of Microbiology, Immunology and Infection(0).

Lesho, E., G. Wortmann, et al. (2005). "Fatal *Acinetobacter baumannii* Infection with Discordant Carbapenem Susceptibility." Clinical Infectious Diseases **41**(5): 758-759.

Lessel, E. F. (1971). "International Committee on Nomenclature of Bacteria: Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria." International Journal of Systematic Bacteriology **21**(2): 213-214.

Levin, A. S. (2002). "Multiresistant *Acinetobacter* infections: a role for sulbactam combinations in overcoming an emerging worldwide problem." Clinical Microbiology and Infection **8**(3): 144-153.

Levin, A. S., C. E. Levy, et al. (2003). "Severe nosocomial infections with imipenem-resistant *Acinetobacter baumannii* treated with ampicillin/sulbactam." International Journal of Antimicrobial Agents **21**(1): 58-62.

Lin, L., B.-D. Ling, et al. (2009). "Distribution of the multidrug efflux pump genes, *adeABC*, *adeDE* and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii–Acinetobacter calcoaceticus* complex." International Journal of Antimicrobial Agents **33**(1): 27-32.

Lomovskaya, O., M. S. Warren, et al. (2001). "Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy." Antimicrobial Agents and Chemotherapy **45**(1): 105-116.

Lomovskaya, O. and W. Watkins (2001). "Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria." Journal of molecular microbiology and biotechnology **3**(2): 225-236.

Magnet, S., P. Courvalin, et al. (2001). "Resistance-Nodulation-Cell Division-Type Efflux Pump Involved in Aminoglycoside Resistance in *Acinetobacter baumannii* Strain BM4454." Antimicrobial Agents and Chemotherapy **45**(12): 3375-3380.

Mak, J. K., M. J. Kim, et al. (2009). "Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*." J Antimicrob Chemother **63**(1): 47-54.

Marchand, I., L. Damier-Piolle, et al. (2004). "Expression of the RND-Type Efflux Pump AdeABC in *Acinetobacter baumannii* Is Regulated by the AdeRS Two-Component System." Antimicrobial Agents and Chemotherapy **48**(9): 3298-3304.

Marti, S., F. Fernandez-Cuenca, et al. (2006). "Prevalence of the tetA and tetB genes as mechanisms of resistance to tetracycline and minocycline in *Acinetobacter baumannii* clinical isolates." Enferm Infecc Microbiol Clin **24**(2): 77-80.

Matthaiou, D. K., A. Michalopoulos, et al. (2008). "Risk factors associated with the isolation of colistin-resistant gram-negative bacteria: a matched case-control study." Crit Care Med **36**(3): 807-811.

Moffatt, J. H., M. Harper, et al. (2010). "Colistin Resistance in *Acinetobacter baumannii* Is Mediated by Complete Loss of Lipopolysaccharide Production." Antimicrobial Agents and Chemotherapy **54**(12): 4971-4977.

Moreira Silva, G., L. Morais, et al. (2011). "Pneumonia adquirida na comunidade numa crianca saudavel por Acinetobacter." Revista Portuguesa de Pneumologia **Epub ahead of print**.

Motaouakkil, S., B. Charra, et al. (2006). "Colistin and rifampicin in the treatment of nosocomial infections from multiresistant *Acinetobacter baumannii*." Journal of Infection **53**(4): 274-278.

Murray, C. K., S. A. Roop, et al. (2006). "Bacteriology of War Wounds at the Time of Injury." Military Medicine **171**(9): 826-829.

Naas, T., P. Bogaerts, et al. (2006). "Emergence of PER and VEB extended-spectrum β-lactamases in *Acinetobacter baumannii* in Belgium." Journal of Antimicrobial Chemotherapy **58**(1): 178-182.

Nagano, N., Y. Nagano, et al. (2004). "Nosocomial transmission of CTX-M-2 beta-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward." J Clin Microbiol **42**(9): 3978-3984.

Nemec, A., L. Krizova, et al. (2011). "Genotypic and phenotypic characterization of the *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii* sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis* sp. nov. (formerly *Acinetobacter* genomic species 13TU)." Research in Microbiology **162**(4): 393-404.

Neonakis, I. K., D. A. Spandidos, et al. (2011). "Confronting multidrug-resistant *Acinetobacter baumannii*: a review." Int J Antimicrob Agents **37**(2): 102-109.

Oliveira, M. S., G. V. B. Prado, et al. (2008). "Ampicillin/sulbactam compared with polymyxins for the treatment of infections caused by carbapenem-resistant *Acinetobacter* spp." Journal of Antimicrobial Chemotherapy **61**(6): 1369-1375.

Oncul, O., O. Keskin, et al. (2002). "Hospital-acquired infections following the 1999 Marmara earthquake." J Hosp Infect **51**(1): 47-51.

Pagès, J.-M., M. Masi, et al. (2005). "Inhibitors of efflux pumps in Gram-negative bacteria." Trends in Molecular Medicine **11**(8): 382-389.

Park, Y. K., G. H. Lee, et al. (2010). "A single clone of *Acinetobacter baumannii*, ST22, is responsible for high antimicrobial resistance rates of Acinetobacter spp. isolates that cause bacteremia and urinary tract infections in Korea." Microb Drug Resist **16**(2): 143-149.

Peleg, A. Y. (2007). "Optimizing therapy for *Acinetobacter baumannii*." Semin Respir Crit Care Med **28**(6): 662-671.

Peleg, A. Y., J. Adams, et al. (2007). "Tigecycline Efflux as a Mechanism for Nonsusceptibility in *Acinetobacter baumannii*." Antimicrobial Agents and Chemotherapy **51**(6): 2065-2069.

Peleg, A. Y., H. Seifert, et al. (2008). "*Acinetobacter baumannii*: emergence of a successful pathogen." Clin Microbiol Rev **21**(3): 538-582.

Petersen, K., S. C. Cannegieter, et al. (2011). "Diversity and clinical impact of *Acinetobacter baumannii* colonization and infection at a military medical center." J Clin Microbiol **49**(1): 159-166.

Petersen, K., M. S. Riddle, et al. (2007). "Trauma-related infections in battlefield casualties from Iraq." Ann Surg **245**(5): 803-811.

Piddock, L. J. V. (2006). "Clinically Relevant Chromosomally Encoded Multidrug Resistance Efflux Pumps in Bacteria." Clinical Microbiology Reviews **19**(2): 382-402.

Poirel, L., O. Menuteau, et al. (2003). "Outbreak of Extended-Spectrum β-Lactamase VEB-1-Producing Isolates of *Acinetobacter baumannii* in a French Hospital." Journal of Clinical Microbiology **41**(8): 3542-3547.

Poirel, L. and P. Nordmann (2006). "Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology." Clin Microbiol Infect **12**(9): 826-836.

Poulakou, G., F. V. Kontopidou, et al. (2009). "Tigecycline in the treatment of infections from multi-drug resistant gram-negative pathogens." Journal of Infection **58**(4): 273-284.

Pournaras, S., A. Markogiannakis, et al. (2006). "Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-58 carbapenemase in an intensive care unit." Journal of Antimicrobial Chemotherapy **57**(3): 557-561.

Poutanen, S. M., M. Louie, et al. (1997). "Risk factors, clinical features and outcome of *Acinetobacter* bacteremia in adults." Eur J Clin Microbiol Infect Dis **16**(10): 737-740.

Rajamohan, G., V. B. Srinivasan, et al. "Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*." J Antimicrob Chemother **65**(9): 1919-1925.

Ravasi, P., A. S. Limansky, et al. (2011). "IS*Aba*825, a Functional Insertion Sequence Modulating Genomic Plasticity and *bla*OXA-58 Expression in *Acinetobacter baumannii*." Antimicrobial Agents and Chemotherapy **55**(2): 917-920.

Reid, G. E., S. A. Grim, et al. (2007). "Rapid Development of *Acinetobacter baumannii* Resistance to Tigecycline." Pharmacotherapy **27**(8): 1198-1201.

Reinert, R. R., D. E. Low, et al. (2007). "Antimicrobial susceptibility among organisms from the Asia/Pacific Rim, Europe and Latin and North America collected as part of TEST and the in vitro activity of tigecycline." Journal of Antimicrobial Chemotherapy **60**(5): 1018-1029.

Roca, I., P. Espinal, et al. (2011). "First Identification and Characterization of an AdeABC-Like Efflux Pump in *Acinetobacter* Genomospecies 13TU." Antimicrobial Agents and Chemotherapy **55**(3): 1285-1286.

Roca, I., P. Espinal, et al. (2012). "The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug resistant menace." Frontiers in Microbiology **3**.

Roca, I., S. Marti, et al. (2009). "CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*." Antimicrob Agents Chemother **53**(9): 4013-4014.

Rossau, R., A. Van Landschoot, et al. (1991). "Taxonomy of *Moraxellaceae* fam. nov., a New Bacterial Family To Accommodate the Genera *Moraxella, Acinetobacter,* and *Psychrobacter* and Related Organisms." International Journal of Systematic Bacteriology **41**(2): 310-319.

Ruiz, M., S. Marti, et al. (2007). "Prevalence of IS(Aba1) in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates." FEMS Microbiol Lett **274**(1): 63-66.

Ruzin, A., D. Keeney, et al. (2007). "AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex." J Antimicrob Chemother **59**(5): 1001-1004.

Saballs, M., M. Pujol, et al. (2006). "Rifampicin/imipenem combination in the treatment of carbapenem-resistant *Acinetobacter baumannii* infections." Journal of Antimicrobial Chemotherapy **58**(3): 697-700.

Sato, K. and T. Nakae (1991). "Outer membrane permeability of *Acinetobacter calcoaceticus* and its implication in antibiotic resistance." Journal of Antimicrobial Chemotherapy **28**(1): 35-45.

Schafer, J. J., D. A. Goff, et al. (2007). "Early Experience with Tigecycline for Ventilator-Associated Pneumonia and Bacteremia Caused by Multidrug-Resistant *Acinetobacter baumannii*." Pharmacotherapy **27**(7): 980-987.

Scott, P., G. Deye, et al. (2007). "An outbreak of multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq." Clin Infect Dis **44**(12): 1577-1584.

Seifert, H., L. Dijkshoorn, et al. (1997). "Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods." J Clin Microbiol **35**(11): 2819-2825.

Seifert, H., A. Strate, et al. (1995). "Nosocomial bacteremia due to *Acinetobacter baumannii*. Clinical features, epidemiology, and predictors of mortality." Medicine (Baltimore) **74**(6): 340-349.

Sheng, W. H., J. T. Wang, et al. (2011). "Comparative in vitro antimicrobial susceptibilities and synergistic activities of antimicrobial combinations against carbapenem-resistant *Acinetobacter* species: *Acinetobacter baumannii* versus *Acinetobacter* genospecies 3 and 13TU." Diagn Microbiol Infect Dis **70**(3): 380-386.

Siau, H., K. Y. Yuen, et al. (1999). "*Acinetobacter* bacteremia in Hong Kong: prospective study and review." Clin Infect Dis **28**(1): 26-30.

Srinivasan, V. B., G. Rajamohan, et al. (2009). "Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*." Antimicrob Agents Chemother **53**(12): 5312-5316.

Stansly, P. G., R. G. Shepherd, et al. (1947). "Polymyxin: a new chemotherapeutic agent." Bull Johns Hopkins Hosp **81**(1): 43-54.

Su, X. Z., J. Chen, et al. (2005). "AbeM, an H+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters." Antimicrob Agents Chemother **49**(10): 4362-4364.

Tomás, M. d. M., A. Beceiro, et al. (2005). "Cloning and Functional Analysis of the Gene Encoding the 33- to 36-Kilodalton Outer Membrane Protein Associated with Carbapenem Resistance in *Acinetobacter baumannii*." Antimicrobial Agents and Chemotherapy **49**(12): 5172-5175.

Towner, K. J. (2009). "*Acinetobacter*: an old friend, but a new enemy." J Hosp Infect **73**(4): 355-363.

Towner, K. J., K. Levi, et al. (2008). "Genetic diversity of carbapenem-resistant isolates of *Acinetobacter baumannii* in Europe." Clin Microbiol Infect **14**(2): 161-167.

Turton, J. F., M. E. Ward, et al. (2006). "The role of ISAba1 in expression of OXA carbapenemase genes in *Acinetobacter baumannii*." FEMS Microbiol Lett **258**(1): 72-77.

Turton, J. F., N. Woodford, et al. (2006). "Identification of *Acinetobacter baumannii* by detection of the blaOXA-51-like carbapenemase gene intrinsic to this species." J Clin Microbiol **44**(8): 2974-2976.

Urban, C., E. Go, et al. (1995). "Interaction of sulbactam, clavulanic acid and tazobactam with penicillin-binding proteins of imipenem-resistant and -susceptible *Acinetobacter baumannii*." FEMS Microbiology Letters **125**(2-3): 193-197.

van den Berg van Saparoea, H. B., J. Lubelski, et al. (2005). "Proton motive force-dependent Hoechst 33342 transport by the ABC transporter LmrA of *Lactococcus lactis*." Biochemistry **44**(51): 16931-16938.

van den Broek, P. J., J. Arends, et al. (2006). "Epidemiology of multiple *Acinetobacter* outbreaks in The Netherlands during the period 1999-2001." Clin Microbiol Infect **12**(9): 837-843.

van Dessel, H., L. Dijkshoorn, et al. (2004). "Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals." Research in Microbiology **155**(2): 105-112.

Vaneechoutte, M., L. Dijkshoorn, et al. (1995). "Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis." J Clin Microbiol **33**(1): 11-15.

Vasilev, K., G. Reshedko, et al. (2008). "A Phase 3, open-label, non-comparative study of tigecycline in the treatment of patients with selected serious infections due to resistant Gram-negative organisms including *Enterobacter* species, *Acinetobacter baumannii* and *Klebsiella pneumoniae*." J Antimicrob Chemother **62 Suppl 1**: i29-40.

Vila, J., S. Marti, et al. (2007). "Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*." J Antimicrob Chemother **59**(6): 1210-1215.

Wareham, D. W., N. C. Gordon, et al. (2011). "In vitro activity of teicoplanin combined with colistin versus multidrug-resistant strains of *Acinetobacter baumannii*." J Antimicrob Chemother **66**(5): 1047-1051.

Wendt, C., B. Dietze, et al. (1997). "Survival of *Acinetobacter baumannii* on dry surfaces." J Clin Microbiol **35**(6): 1394-1397.

Yang, S. C., W. J. Chang, et al. (2010). "Prevalence of antibiotics resistance and OXA carbapenemases genes in multidrug-resistant *Acinetobacter baumannii* isolates in central Taiwan." European Journal of Clinical Microbiology & Infectious Diseases **29**(5): 601-604.

Yu, E. W., J. R. Aires, et al. (2005). "A Periplasmic Drug-Binding Site of the AcrB Multidrug Efflux Pump: a Crystallographic and Site-Directed Mutagenesis Study." Journal of Bacteriology **187**(19): 6804-6815.

Yun, S. H., C. W. Choi, et al. (2008). "Proteomic analysis of outer membrane proteins from *Acinetobacter baumannii* DU202 in tetracycline stress condition." Journal of microbiology **46**(6): 720-727.