Investigating Two Component Regulatory Systems for the Determination of Adaptive Responses in *A. baumannii.*

By

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Abstract

To investigate the role of the two component systems AdeRS and PmrAB in adaptation to the presence of antimicrobials, *adeRS* and *pmrAB* were deleted in multi-drug resistant *Acinetobacter baumannii* strain AYE. The effect of deleting these genes on antimicrobial susceptibility, growth, accumulation, virulence and ability to form a biofilm was investigated. The deletion of *adeRS* and *pmrAB* had no effect on bacterial growth or the accumulation of Hoechst 33342 (bis-benzimide). AYEΔ*pmrAB*, but not AYEΔ*adeRS*, accumulated significantly more norfloxacin than AYE. All strains accumulated more norfloxacin in the presence of the efflux inhibitor carbonyl cyanide m-chlorophenylhydrazone. AYEΔ*adeRS* and AYEΔ*pmrAB*, but not AYE, accumulated more norfloxacin in the presence of verapamil. AYEΔ*adeRS* was more susceptible than AYE to antibiotics and biocides and both strains in biofilm were more tolerant of biocides than their planktonic counterparts. Deletion of *pmrAB* had no effect on antibiotic susceptibility. AYE was more virulent than both AYEΔ*adeRS* and AYEΔ*pmrAB.* The results of this study suggest that in strain AYE, *adeRS* are not essential for efflux of norfloxacin or Hoechst 33342 and that *pmrAB* are involved in the accumulation of some compounds. Both AdeRS and PmrAB are important for virulence and AdeRS has a role in antimicrobial susceptibility.

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Abbreviation	Definition
***A. calcoaceticus-A. baumannii* complex**	Acinetobacter *genomic species 3, Acinetobacter genomic species 13TU, A. baumannii* and *A. calcoaceticus*
***A. baumannii* complex**	*A. baumannii, Acinetobacter* genomic species 3 and *Acinetobacter* genomic species 13TU
ABC	ATP-Binding Cassette
bp	Base pair
Cetylpyridinium chloride	Hexadecylpyridinium chloride monohydrate
CCCP	Carbonyl Cyanide m-Chlorophenylhydrazone
CFU	Colony Forming Units
gen. sp.	Genomic species
g	Gram
x g	Gravity
H_2O_2	Hydrogen peroxide
hr	Hour
HPA	Health Protection Agency
kb	Kilobase pair
L	Litre
LB	Luria Bertani
LPS	Lipopolysaccharide
Mb	Mega base pair
MBC	Minimum Bactericidal Concentration
MDR	Multiple Drug Resistance; Multiple Drug Resistant
mg	Milligram
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Millilitre
mol	Mole

ng	Nanogram
nm	Nanometre
NLS	Nuclear Localisation Signal
PAβN	Phenylalanine-Arginine Beta-Naphthylamide
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pmol	Picomole
RNAseq	RNA sequencing
RND	Resistance Nodulation Division
rpm	Revolutions per minute
SDW	Sterile Distilled Water
sec	Second
spp.	Species
TCS	Two Component System
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
Ω	Ohm
µF	Microfarad
µg	Microgram
µl	Microlitre
µM	Micromolar

1. Introduction

1.1 *Acinetobacter*

Acinetobacter, first described by Beijerinck in 1911, is a ubiquitous Gram negative coccobacillus of the family *Moraxellaceae* (Rossau *et al.*, 1991). Since identification, this organism has had many names such as *Bacterium anitratum* (Schaub and Hauber, 1948) and *Moraxella lwoffii* (Audureau, 1940). *Acinetobacter* has been classified into at least 15 different genera because it did not have enough unique phenotypic characteristics to allow it to be differentiated from phenotypically similar organisms (Juni, 1978). This bacterium became known as *Acinetobacter* in the 1950s (Brisou and Prévot, 1954).

A. baumannii, one of the most important nosocomial species, is generally considered to be a low-grade, opportunistic pathogen, as it typically only causes infection in individuals who are immunocompromised or have an underlying disease (Joly-Guillou, 2005). *A. baumannii* was not formally recognised or designated until 1986, prior to which *Acinetobacter spp.* were often ignored when identified in clinical samples as they remained susceptible to antibiotics such as gentamicin, ampicillin, nalidixic acid and minocycline (Joly-Guillou, 2005). However, *Acinetobacter* has since become an important nosocomial pathogen due to an increase in the number of genuine *A. baumannii* infections and in the number of multidrug-resistant strains, making treatment of these infections difficult (McConnell *et al.*, 2012). In addition, *A. baumannii* is able to survive in a range of environmental conditions, allowing it to persist in the hospital environment for years (Wendt *et al.*, 1997; Bergogne-Berezin and Towner, 1996). *A. baumannii* is phylogenetically related to *Pseudomonas*

aeruginosa, with 65% of its core genes having orthologues in *P. aeruginosa* PAO1 (Adams *et al.*, 2008).

1.1.1 Different Species of *Acinetobacter*

Most species of *Acinetobacter* are environmental and are not associated with human disease and only a few species are clinically important (Gordon and Wareham, 2010; Garnacho-Montero and Amaya-Villar, 2010; Towner, 2009). At the time of writing this thesis, 27 species have been formally identified (Table 1.1) and based on DNA-DNA hybridisation there are nine provisional species (McConnell *et al.*, 2012). Nemec *et al.* (2011) proposed species names for *Acinetobacter genomic species* (*gen. sp.*) 3 and *Acinetobacter gen. sp.* 13TU: *A. pittii* sp. nov.*,* and *A. nosocomialis* sp. nov. respectively. *A. pittii, A. nosocomialis*, *A. baumannii* and *A. calcoaceticus* cannot be distinguished phenotypically, since they are very closely related (Manchanda *et al.*, 2010). These four species are often grouped and referred to as the *A. calcoaceticus-A. baumannii* complex. When reporting incidents of *Acinetobacter* infection this is not a suitable grouping because *A. calcoaceticus* is an environmental strain, whereas the other three species are the most common causes of *Acinetobacter* infection (Peleg *et al.*, 2008). It is of interest to determine which species of the *A. calcoaceticus-A. baumannii* complex is responsible for infection as the different species may have different clinical outcomes and different treatment regimes may be required (Chuang *et al.*, 2011). For example, patients with *A. baumannii* bacteraemia are more likely to develop pneumonia than those with *A. nosocomialis* bacteraemia. *A. baumannii* is also associated with decreased antibiotic susceptibility and increased mortality than *A. nosocomialis* (Chuang *et al.*, 2011). However, the majority of laboratories that report data for this genus use phenotypic typing systems, which are less

Table 1.1 Species of the Genus *Acinetobacter*.

Species	Habitat	Clinical importance	Antimicrobial susceptibility	References
A. baumannii	No known natural habitat outside of hospital. Isolated rarely from environment. Not ubiquitous.	Most frequently isolated species from human clinical samples e.g. blood, respiratory tract	Often MDR	Towner, 2009; Adams *et al.*, 2008; Adams *et al.*, 2009; Bouvet and Grimont, 1986
A. baylyi	Activated sludge and soil	Has been identified as a pathogen for opportunistic infection		Dijkshoorn *et al.*, 2007; Chen *et al.*, 2008; Carr *et al.*, 2003
A. beijerinckii	Soil, water, humans	Has been isolated from clinical specimens e.g. faeces, sputum, throat swab		Nemec *et al.*, 2009
A. bereziniae				Nemec *et al.*, 2010
A. bouvetii	Activated sludge			Towner, 2009; Dijkshoorn *et al.*, 2007
A. brisouii	Wetland			Anandham *et al.*, 2010
A. calcoaceticus	Soil and water	Rarely isolated from human specimens	Sensitive	Nemec *et al.*, 2011; Towner, 2009
A. gerneri	Activated sludge			Towner, 2009; Dijkshoorn *et al.*, 2007
A. guillouiae				Nemec *et al.*, 2010
A. gyllenbergii	Human	Isolated from clinical specimens e.g. blood, urine, vaginal swab		Nemec *et al.*, 2009
A. haemolyticus	Humans	Isolated from clinical specimens		Bouvet and Grimont, 1986
A. indicus				Malhotra *et al.*, 2012
A. junii [a]	Humans, animals	Frequently isolated from human skin. Infection is rare and usually		de Breij *et al.*, 2010; Nemec *et al.*, 2009; Dijkshoorn *et al.*,

		benign.		2007; Towner, 2009; Bouvet and Grimont, 1986
A. johnsonii	Normal commensal skin flora of humans. Environment, soil, wastewater.	Infection is rare and usually benign.	Sensitive	Towner, 2009; Dijkshoorn *et al.*, 2007; Bouvet and Grimont, 1986
A. lwoffii	Normal commensal skin flora of humans.	Has been isolated from catheter-related bloodstream infections. Infections are usually not severe.	Usually sensitive. MDR isolates have been identified.	Tega *et al.*, 2007; de Breij *et al.*, 2010
A. nosocomialis[b]	Humans	One of the species most commonly associated with infection		Nemec *et al.*, 2011
A. parvus	Humans and animals	Has been isolated from human and animal non-sterile body sites, such as skin, eyes and ears and from human catheter-related bloodstream infections.		Nemec *et al.*, 2003
A. pitii[b]	Humans, soil, vegetables	One of the species most commonly associated with infection		Nemec *et al.*, 2011
A. radioresistens	Normal commensal skin flora of humans and animals Spoilage flora of food	Not associated with infection	Sensitive	Towner, 2009
A. rudis	Milk and wastewater			Vaz-Moreira *et al.*, 2011
A. schinleri	Humans	Has been isolated from outpatients. Clinical specimens include blood, vaginal swab, skin		Nemec *et al.*, 2001
A. soli	Soil	Implicated in bloodstream infection	MDR	Kim *et al.*, 2008; Pellegrino *et al.*, 2011

A. towneri	Activated sludge		Carr *et al.*, 2003
A. tandoii	Activated sludge		Carr *et al.*, 2003
A. tjernbergiae	Activated sludge		Carr *et al.*, 2003
A. ursingii	Humans	Has been isolated from seriously ill patients (e.g. blood, urine) and has potential to spread amongst patients	Nemec *et al.*, 2001
A. venetianus			Di Cello *et al.*, 1997

[a] *A. grimontii* is a synonym of *A. junii;* MDR, multi-drug resistant

reliable than molecular methods and meaning that the specific species of the *A. calcoaceticus-A. baumannii* complex cannot be reported (HPA, 2010).

1.1.1.1 AYE

A. baumannii AYE is a multi-drug resistant (MDR) clinical isolate which was cultured from a patient with pneumonia and a urinary tract infection in France in 2001 (Poirel *et al.*, 2003). AYE was epidemic in France in 2003-2006 and was associated with mortality in 26% of infected patients (Fournier *et al.*, 2006). However, it is important to note that it is difficult to determine whether mortality associated with *A. baumannii* is attributable to infection or another factor, such as an underlying disease.

AYE has an estimated genome size of 3.9 Mb and three plasmids of 5, 9, and 94 kb. No genes associated with antibiotic resistance or virulence have been identified on any of the plasmids (Vallenet *et al.*, 2008; Fournier *et al.*, 2006). Multidrug resistance in this strain is associated with AbaR1, a large antibiotic resistance island of 86 kb, which has a G+C content of 52.8% (the rest of its genome has a G+C content of 38.8%) and contains a cluster of antibiotic resistance genes and mobile genetic elements (Fournier *et al.*, 2006). AbaR1 is absent from drug susceptible isolates SDF, AB307-0294 and ATCC 17978 (Adams *et al.*, 2008). According to amino acid similarity, 99% of the AbaR1 genes are likely to have originated from other bacterial species such as *Pseudomonas, Salmonella, and Escherichia coli*. (Fournier *et al.*, 2006).

1.1.2 Physiology

Acinetobacter cells in logarithmic phase of growth are typically 1-1.5 µm x 1.5-2.5 µm in size. (Bergogne-Berezin and Towner, 1996). All species are obligate aerobes and non-

fermentive (Gordon and Wareham, 2010; Bergogne-Berezin and Towner, 1996). *Acinetobacter* have few nutritional requirements, and most strains are able to grow in a medium containing only ammonium or nitrate as a source of nitrogen and a single carbon source such as acetate, pyruvate or alcohol (Baumann *et al.*, 1968). However, most strains are unable to utilize glucose as a carbon source (Baumann *et al.*, 1968).

Although the name *Acinetobacter* means "non-motile rod" (Mussi *et al.*, 2010), motility has been observed in this genus. Mussi *et al.* (2010) identified motility in *A. baumannii* strain ATCC 17978, which was inhibited in response to blue light in a temperature-dependent manner. *Acinetobacter* have fimbriae, which are important for initial adhesion to biotic and abiotic surfaces, allowing subsequent formation of biofilms on these surfaces (Gordon and Wareham, 2010). Thirty per cent of *Acinetobacter* strains produce exopolysaccharide, a component of an extracellular capsule, and mature biofilms (Joly-Guillou, 2005). The capsule enables *A. baumannii* to resist human serum (Russo *et al.*, 2010).

1.1.3 Habitat

Acinetobacter as a genus are widely found in soil and water and can also be isolated from the skin of healthy individuals, food and animals (Table 1.1) (Turton *et al.*, 2006; Manchanda *et al.*, 2010; Gordon and Wareham, 2010; Munoz-Price and Weinstein, 2008). Although *A. baumannii* has been isolated from the environment, it is not a ubiquitous environmental organism (Towner, 2009) and does not appear to be carried by people in the community (Peleg *et al.*, 2008). This non-ubiquitous nature is also true for the other members of the *A. baumannii* complex: *A. pittii* and *A. nosocomialis*. These three species are generally isolated only from the hospital or patients who are infected or colonised with the organism (Towner, 2009).

1.1.4 Disease

1.1.4.1 Pathogenicity

As this organism is an opportunistic pathogen, patients who develop an *Acinetobacter* infection usually have an underlying condition such as renal dysfunction or trauma (burn or wound injuries) (Towner, 2009; Esterly *et al.*, 2011). *Acinetobacter* infections usually occur in patients in intensive care units with most cases in children younger than 1 year or older than 64 years of age, possibly due to the increased likelihood that these cohorts will require ICU treatment (HPA, 2010). Other risk factors are the use of invasive procedures such as mechanical ventilation and treatment with broad spectrum antibiotics (HPA, 2010).

The species most frequently isolated from human infections are *A. baumannii, A. pittii and A. nosocomialis* (Dijkshoorn *et al.*, 2007; de Breij *et al.*, 2010). *A. baumannii* is responsible for up to 10% of nosocomial infections (Adams *et al.*, 2008) and is most often associated with hospital-acquired pneumonia, since the airways are often the first and main site to be colonised (Knapp *et al.*, 2006; Lee *et al.*, 2006). Community-acquired *A. baumannii* infections are rare, although cases of community-acquired pneumonia caused by this organism have been reported, mainly among young alcoholics in tropical climates (Chen *et al.*, 2001). Other infections caused by *A. baumannii* include meningitis, bacteraemia, urinary tract infection and wound infection (Manchanda *et al.*, 2010). Wound infections are a problem in soldiers returning from war in Afghanistan and Iraq (Hujer *et al.*, 2006). Other species of *Acinetobacter* are only pathogenic in rare cases and, like *A. baumannii*, are usually isolated from patients who already have an underlying disease (Joly-Guillou, 2005; HPA, 2010). For example, *A. lwoffii,* a commensal organism carried by 20-25% of healthy individuals, was reported as a cause of bacteraemia in England, Wales and Northern Ireland

from 2005 to 2009. However, it is possible that some of these reported cases were actually due to blood culture contamination, as this species would not be expected to be a cause of infection (HPA, 2010).

A. baumannii easily survives in the hospital environment; it can survive periods of desiccation and nutrient starvation, and can grow at many temperatures and pH conditions (Bergogne-Berezin and Towner, 1996). *A. baumannii* is usually MDR, (McConnell *et al.*, 2012) and is able to form biofilms on both biotic and abiotic surfaces (de Breij *et al.*, 2010). All of these characteristics aid its survival (Tomaras *et al.*, 2008). However, the environmental signals and regulation of factors aiding survival and pathogenesis of this organism are largely unknown (Mussi *et al.*, 2010) and the reservoirs of infection within hospitals are poorly understood (Towner, 2009).

de Breij *et al.* (2010) reported that *A. baumannii* induced the production of less inflammatory cytokines from human macrophages than *A. junii*. They hypothesised that *A. baumannii* may be such a successful opportunistic pathogen as the weak immune response enables the organism to evade eradication from the host. However, the ability to adhere to, and form biofilms on, human airway epithelial cells did not vary between clinically relevant and less clinically relevant strains and species of *A. baumannii.*

1.1.4.2 Virulence

In order to cause infection, *Acinetobacter* must survive the iron-limiting conditions inside the host, where the free iron concentration (10^{-8}M) is less than that required for bacterial survival (Goel and Kapil, 2001). *A. baumannii* secrete a catechol-type siderophore, acinetobactin, and express outer membrane iron-regulated iron receptors (BauA for

acinetobactin) to take up the siderophore-iron complex (Goel and Kapil, 2001; Zimbler *et al.*, 2009). *A. baumannii* species, such as AYE, also express a hemin utilisation system, enabling these bacteria to obtain iron from multiple sources during infection (Zimbler *et al.*, 2009). Gaddy *et al.* (2012) investigated the role of acinetobactin in infection and virulence of *A. baumannii*. A functional acinetobactin-mediated iron acquisition sytem was required for bacterial survival and full virulence in *Galleria mellonella* and murine sepsis models of infection. Acinetobactin was not required for initial interaction of *A. baumannii* with human epithelial cells, but it was required for infection and intracellular persistence.

Outer membrane protein A (OmpA (previously known as Omp38)), the most abundant surface protein of *A. baumannii,* is likely to be an importance virulence factor of the organism (Choi *et al.*, 2008). OmpA has been implicated in cytotoxicity and apoptosis of host cells; it has been shown to induce macrophage and dendritic cell death *in vitro* and frog embryonic death *in vivo* (Choi *et al.*, 2008; Lee *et al.*, 2010; Choi *et al.*, 2005). The protein directly binds to the surface of eukaryotic cells, suggesting that it may be involved in the interaction of *A. baumannii* with host cells. It translocates to the nucleus via a novel nuclear localisation signal (NLS) and induces apoptosis by targeting mitochondria (Choi *et al.*, 2005; Choi *et al.*, 2008). Interestingly, the specific NLS known to target the nucleus is only conserved among species of the *A. baumannii* complex. The NLS in environmental strains such as *A. baylii* and *A. radioresistens* differ from that of *A. baumannii* by one amino acid (Choi *et al.*, 2008). OmpA inhibits complement-mediated cell lysis and death of dendritic cells may lead to defective T- cell responses against the organism. Therefore, OmpA may have a detrimental effect on adaptive immunity in the host (Lee *et al.*, 2010).

Phospholipase D has also been identified as a virulence factor in *A. baumannii.* Insertion of a transposon in the phospholipase D gene (*pld*) in a clinical isolate resulted in decreased epithelial cell invasion (*in vitro*) and decreased bacteraemia and infection of visceral organs in a mouse model of infection (Jacobs *et al.*, 2010).

Epidemiology

Different clones are epidemic in different regions of the world and even different parts of a country. European clones I, II and III are 3 major lineages of *A. baumannii*, which are frequently implicated in outbreaks throughout Europe and other parts of the world, such as the USA and South Africa (de Breij *et al.*, 2010; van Dessel *et al.*, 2004; Diancourt *et al.*, 2010). Three widespread clones of *A. baumannii* have been responsible for infections in UK hospitals: OXA-23 Clones 1 and 2 and the "South East" (SE) clone (Turton *et al.*, 2006).

A. baumannii infections in war repatriates have been attributed to contaminated soil at the time of injury. However, this is now debated since this organism is now thought to be rare in the environment (Dijkshoorn *et al.*, 2007). Scott *et al.* (2007) isolated *A. baumannii* from the skin of 1 out of 160 (0.6%) patients, 1 out of 49 (2%) soil samples, but all (7) of the treatment areas tested, suggesting that infection is acquired in the hospital after injury.

Potential sources of *A. baumannii* infection within the hospital include ventilators, bed linen, floor mops and computer keyboards. Once a patient is infected with the organism, they then become a reservoir of infection. Spread of infection is often thought to occur via direct contact, in which case the hands of hospital staff can sometimes be an important transmission vector (Joly-Guillou, 2005; Dijkshoorn *et al.*, 2007). Air transmission has also been implicated (Garnacho-Montero and Amaya-Villar, 2010).

The ability of *Acinetobacter spp.* to survive in desiccated conditions plus their resistance to antibiotics are thought to be of importance in their ability to persist in the hospital and cause outbreaks (Jawad 1998). Jawad *et al.* (1998) reported that there was no significant difference between the survival of outbreak and sporadic cells under desiccated conditions and that any strain may cause infection when the conditions are favourable. The outbreak strains investigated were more resistant than sporadic strains to aminoglycosides, fluoroquinolones and β-lactams, suggesting that antimicrobial resistance affects the likelihood of a strain to cause infection and also to become epidemic (Jawad *et al.*, 1998). In support of this, Higgins *et al.* (2004) reported that fluoroquinolone resistant isolates of *A. baumannii* were isolated from multiple patients during a hospital outbreak, whereas isolates with lower fluoroquinolone minimum inhibitory concentrations (MICs) were not isolated from more than one patient. *A. baumannii* is more resistant to desiccation than other species of *Acinetobacter* and this may contribute to its higher ability to cause infection than species that are more sensitive to desiccation (Jawad *et al.*, 1996).

Biofilm-forming *A. baumannii* strains have been found to survive longer than non-biofilm-forming strains. In a study by Espinal *et al.* (2012), biofilm-forming strains survived up to 36 days, whereas non-biofilm-forming strains survived a maximum of 15 days. In dry conditions, the non-biofilm-forming strains appeared to be dehydrated in contrast to the biofilm-forming strains. Therefore, the ability of *A. baumannii* to form biofilms may increase the ability of this bacterium to persist in the hospital.

Incomplete disinfection of hospital contaminated dry surfaces may also contribute to repeated outbreaks of *A. baumannii* (Jawad *et al.*, 1996). *A. baumannii* is able to survive in the presence of disinfectants when the concentration or exposure time of the agent fall

below the manufacturer's instructions (Wisplinghoff *et al.*, 2007). However, several studies have indicated that the development of disinfectant-resistance in *A. baumannii* does not usually occur after exposure to the agent and that the MICs of biocides for this bacterium are usually below the in-use concentrations (Martró *et al.*, 2003; Wisplinghoff *et al.*, 2007; Kawamura-Sato *et al.*, 2008; Kawamura-Sato *et al.*, 2010). Therefore, biocide resistance in *A. baumannii* is not considered to be involved in epidemic spread of *A. baumannii* infection, but incorrect disinfectant use does increase the chance of an outbreak (Wisplinghoff *et al.*, 2007).

It has been proposed that once an *Acinetobacter* infection is detected within a hospital, the number of colonised patients is already high, and that it is too late to prevent an outbreak (Joly-Guillou, 2005). However, outbreaks have been prevented by implementing patient isolation or ward closures for up to four weeks and in one report, *A. baumannii* was eradicated from a London teaching hospital without closure of the ward or isolation of patients (Towner, 2009). Instead, control measures included improved hand decontamination by implementing the use of alcohol gels, the use of closed tracheal suction for patients on mechanical ventilation and the administration of nebulised colistin to patients who were suspected to have ventilator-associated pneumonia caused by *Acinetobacter* (Wilks *et al.*, 2006).

1.1.5 Treatment

The scientific evidence used to treat *A. baumannii* infections is based on *in vitro* data, experimental models and case studies, since there have been very few randomised trials to identify the safest and most efficacious drug for treatment (Garnacho-Montero and Amaya-Villar, 2010). *In vitro* antimicrobial susceptibility assays are mainly used to guide the

treatment of infections caused by *Acinetobacter spp*., but it is suggested that these assays are less reliable than for other bacteria (Kulah *et al.*, 2009). This is because there is limited clinical evidence for the efficacy of different antibiotics against *A. baumannii* infection, rendering the antibiotic resistance recommended breakpoint conentrations unreliable (Perez *et al.*, 2007).

Treatment of *Acinetobacter* infections is difficult since clinically significant species belonging to the genus are frequently resistant to most commonly used agents, including aminoglycosides, broad-spectrum β-lactams and the quinolones (Towner, 2009; Van Looveren and Goossens, 2004). Until the 1970s, gentamicin, minocycline, ampicillin, carbenicillin and nalidixic acid could be used alone or in combination to treat nosocomial *Acinetobacter* infections. Unfortunately, resistance to these agents began to arise between 1971 and 1974 (Bergogne-Berezin and Towner, 1996).

Carbapenems

Carbapenems are the drugs of choice for susceptible strains (MIC ≤ 2) (Gordon and Wareham, 2010; Bergogne-Berezin and Towner, 1996; Neonakis *et al.*, 2011). Until the early 1990s, 100% of isolates remained susceptible to imipenem (Bergogne-Berezin and Towner, 1996). However, resistance to carbapenems (MIC of imipenem and meropenem, 8 μg/ml; MIC of doripenem, 4 μg/ml), as well as other agents is now widespread, leaving few treatment options (Lopez-Rojas *et al.*, 2011) When deciding upon appropriate treatment for *Acinetobacter* infections, it is recommended that the MIC of imipenem for *Acinetobacter* is determined (Garnacho-Montero and Amaya-Villar, 2010) since this drug has high success rates against *A. baumannii* with low levels of resistance to imipenem (MIC, 8 μg/ml), but is inactive *in vivo* when the organism has a high level of resistance (MIC $\geq$512 μg/ml) (Montero

et al., 2004). Another carbapenem, doripenem, is less efficacious than imipenem (when strains have only a low level resistance to imipenem (MIC, 8µg/ml), but is more effective in strains carrying the OXA-58 gene, which encodes a β-lactamase (Section 1.2.2.1). Doripenem is not a commonly used drug in the treatment of *A. baumannii* infections and more clinical experience is required before it will become routinely used (Garnacho-Montero and Amaya-Villar, 2010).

A new broad spectrum carbapenem, ertapenem, was licensed for use in Europe in 2002. However, despite the past efficacy of carbapenems, this newer drug is unfortunately ineffective in the treatment of *Acinetobacter spp.* infections (Burkhardt *et al.*, 2007).

Fluoroquinolones

Although fluoroquinolones were once effective against *A. baumannii* infections, resistance has rapidly emerged against these agents since 1990 (Towner, 2009). Higgins *et al.* (2004) reported a case of *A. baumannii* with mutations in *gyrA* and *parC*, which encode the A sub-units of the targets of fluoroquinolones (a subunit of DNA gyrase and topoisomerase IV, respectively) and upregulated *adeB* expression, conferring increased efflux, which were thought to have occurred after ciprofloxacin exposure. Newer fluoroquinolones such as moxifloxacin retain greater activity than ciprofloxacin for Acinetobacter, but growth has been reported to occur in the presence of moxifloxacin (Towner, 2009), suggesting that resistant mutants are rapidly selected. For these reasons, fluoroquinolones are not the agents of choice for the treatment of *A. baumannii* infections.

Polymyxins

Polymyxin E (colistin), an agent used only as a last resort due to its high toxicity, is the last remaining drug with high activity against *A. baumannii* (Fernandez-Reyes *et al.*, 2009). Colistin and other polymyxins were abandoned in the 1960s and 1970s due to the nephrotoxicity and neurotoxicity associated with treatment (Munoz-Price and Weinstein, 2008). However, cases of nephrotoxicity are now less frequently reported and neurotoxicity is uncommon (Garnacho-Montero and Amaya-Villar, 2010). This could be due to inappropriate dosing in the past (Li *et al.*, 2006) or the retrospective nature of more recent studies, which rely upon appropriate documentation of toxicity when it occurs (Paksu *et al.*, 2012).

Colistin does not penetrate into respiratory secretions efficiently, so is not suitable for treatment of pneumonia, although nebulised colistin overcomes this problem (Livermore, 2005; Peleg *et al.*, 2007b). A side effect of using nebulised colistin is bronchoconstriction, although this can be decreased by using intravenous colistin at the same time (Garnacho-Montero and Amaya-Villar, 2010).

Polymyxins show promising activity against MDR *A. baumannii* when used in combination with other agents such as meropenem or rifampicin. It is advised that colistin is not used alone since heteroresisistance (presence of a resistant sub-population in an otherwise susceptible population (Cai *et al.*, 2012)) is higher amongst isolates previously exposed to colistin than those never exposed to the agent (Hawley *et al.*, 2008). Nonetheless, colistin is currently considered a safe and suitable agent for the treatment of *A. baumannii* infections (Lopez-Rojas *et al.*, 2011). Colistin-resistant outbreaks of *Acinetobacter* are rare, possibly due to the decreased fitness associated with resistance to the antibiotic (Section 1.2.2.3) (Lopez-

Rojas *et al.*, 2011). Furthermore, mutations that compensate the fitness burden in the mutants do not seem to be easily acquired (it has been postulated that colistin may have multiple targets), meaning that the fitness cost is generally not overcome (Fernandez-Reyes *et al.*, 2009). Colistin also does not promote cross resistance to other agents (Garnacho-Montero and Amaya-Villar, 2010).

Tigecycline

Tigecycline can be used to treat infections caused by colistin-resistant strains of *A. baumannii* (Garnacho-Montero and Amaya-Villar, 2010). Tigecycline is a broad spectrum derivative of minocycline (glycylcycline), and activity is not affected by tetracycline-specific efflux pumps or tetracycline resistance determinants that protect the ribosome (Peleg *et al.*, 2007b; Livermore, 2005).

There have been few clinical reports of tigecycline resistance (Peleg *et al.*, 2007b), but in 2005, there was a report of resistance which emerged during phase III trials (unpublished) (Livermore, 2005). Tigecycline non-susceptible (MIC > 0.5) *A. baumannii* have also been isolated from hospitals in London (MIC, 4-6 μg/ml) (Livermore, 2005). Peleg *et al.* (2007b) also reported two cases of bloodstream infection caused by tigecycline non-susceptible isolates of *A. baumannii* in the USA. The bacteraemia developed following treatment with tigecycline, and led to a cautionary report about the use of tigecycline for *A. baumannii* bloodstream infection.

The sub-inhibitory serum concentrations (mean maximum steady state concentration, 0.63 μg/ml) can allow the development of *Acinetobacter* bloodstream infection, exemplified by the above cases (Peleg *et al.*, 2007b). Fishbain and Peleg, (2010) recommended that

tigecycline should not be used for *A. baumannii* for which the MIC exceeds 1 μg/ml. Tigecycline is also not suitable for urinary tract infections as little active drug is excreted in urine; excretion is mostly biliary (Livermore, 2005). However, tigecycline is efficacious in the treatment of tissue infections as the level of transfer of tigecycline from the blood to the tissues is very high (Livermore, 2005; Peleg *et al.*, 2007b).

Sulbactam

Sulbactam (β-lactamase inhibitor) in combination with ampicillin, has been reported to be successful in the treatment of *Acinetobacter* infections (Levin *et al.*, 2003). Early studies showed high in vitro activity against *Acinetobacter*, including against multidrug resistant strains (Karageorgopoulos and Falagas, 2008). The activity of sulbactam in combination with a β-lactam has shown comparable activity to that of imipenem in ventilator-associated pneumonia and bacteraemia caused by MDR strains of *A. baumannii* (Wood *et al.*, 2002; Cisneros *et al.*, 1996). Furthermore, it has been suggested that this agent may be more effective in treating pneumonia than colistin, and is considered to be a safe choice for therapy (Levin *et al.*, 2003). However, the efficacy of this compound has declined, possibly due to its increased use (Karageorgopoulos and Falagas, 2008).

1.2. Antimicrobial resistance

1.2.1. Antibiotic and biocide resistance in Gram Negative Bacteria

1.2.1.1. Chromosomal vs. Transmissible Resistance

Resistance determinants can be found on the bacterial chromosome, or on mobile genetic elements such as plasmids, transposons and integrons. Mobile genetic elements allow horizontal transfer of resistance genes between strains and species of bacteria (Biliouris *et*

al., 2011). Resistance genes transferred on mobile elements can also become incorporated into the chromosome (Burrus and Waldor, 2004). Likewise, although chromosomally encoded resistance is acquired via vertical gene transfer, it can become transmissible. For example, some chromosomally encoded β-lactamases have migrated onto plasmids and integrons, facilitating their dissemination (Gootz, 2006). Topoisomerase genes are located on the chromosome, and have not been found on mobile genetic elements. However, transmissible resistance to fluoroquinolones has been described. For instance, the *qnr* gene, whose product protects DNA gyrase against fluoroquinolones, has been identified on plasmids predominantly in *K. pneumoniae* (Martinez-Martinez *et al.*, 1998). Other resistance determinants, such as tetracycline resistance genes, are often associated with transmissible elements (Biswas *et al.*, 2008).

1.2.1.2. Brief Overview of Mechanisms of Resistance

Antibiotic resistance can be acquired either due to the uptake of DNA containing resistance genes or due to evolutionary mutations in the organism's genome (Martinez *et al.*, 2009). Bacteria can develop resistance against one agent, or against a broad spectrum of agents. A bacterium is said to be MDR if it is resistant to drugs belonging to at least three different classes of antibiotic (Piddock, 2006). MDR is often due to the action of a combination of mechanisms, each conferring resistance to a different agent (Gootz, 2006), although one mechanism alone, such as overexpression of a multi-drug efflux pump, can be responsible (Piddock, 2006). Some bacteria can express all mechanisms of resistance described to date, such as enzymatic inactivation of the antibiotic, mutation(s) in the structural or regulatory genes of the target protein, decreased permeability of the outer membrane and transport of the agent out of the cell by efflux (Strateva and Yordanov, 2009; Gootz, 2006).

Resistance to β-lactams is often due to the presence of β-lactamase enzymes, which hydrolyse the β-lactam ring, the active constituent of the enzyme (Walsh, 2000). Aminoglycoside resistance can occur due to enzymatic modification of the agent, which reduces the affinity of the agent for its target (Walsh, 2000). Aminoglycoside phosphotransferases, acetyltransferases, and nucleotidyltransferases inactivate aminoglycosides by catalysing the transfer of a phosphate group, acetyl group or adenosine monophosphate (AMP), respectively to the antibiotic (Ramirez and Tolmasky, 2010).

Fluoroquinolone resistance can be a result of mutations in chromosomal genes which lead to an alteration of their target (Marcusson *et al.*, 2009). Mutations can occur in *gyrA* or *gyrB* which result in alteration of DNA gyrase, or in *parC* or *parE*, which encode subunits of topoisomerase IV (Hooper, 2001). Clinically relevant resistance to fluoroquinolones in Gram negative bacteria is usually the result of a stepwise accumulation of mutations including those resulting in alteration of the target and up-regulation of efflux (Komp Lindgren *et al.*, 2003). Fluoroquinolone-resistance can also arise due to enzymatic inactivation of the antibiotic, by a variant of an aminoglycoside acetyltransferase, which recognises some fluoroquinolones (Robicsek *et al.*, 2006).

Innate resistance in Gram negative bacteria is often due to the synergistic effect of the low permeability of the outer membrane and active efflux, which results in a decreased intracellular drug concentration, allowing survival in the presence of these compounds (Nikaido, 1994; Piddock, 2006). All Gram negative bacteria studied to date have porins in their outer membrane, which can be non-specific or specific for the solutes they allow through (Nikaido, 2003). The intrinsic resistance of *P. aeruginosa* is at least in part due to the low permeability of its outer membrane (Strateva and Yordanov, 2009). However, it has

been reported that the low permeability alone would not prevent the accumulation of toxic concentrations of antibiotic; efflux is also important (Livermore and Davy, 1991; Nikaido, 1994).

Both antimicrobial resistant and antimicrobial susceptible bacteria express efflux pumps (Piddock, 2006). Resistance can arise in a susceptible organism due to the up-regulation of an efflux pump, which can be induced by environmental signals or a mutation(s) in a regulatory gene (Levy, 2002; Piddock, 2006). For example, transcription of *tetA*, which encodes a tetracycline-specific efflux pump, is dependent on the presence of tetracycline for activation (Levy and McMurry, 1978). Alternatively, resistance can be due to a mutation(s) in an efflux pump gene, which makes export more efficient (Blair and Piddock, 2009; Piddock, 2006). Efflux pumps such as CmlA, which transports chloramphenicol, have a specific substrate (Coyne *et al.*, 2010b). Other pumps, such as AcrAB-TolC of the resistance nodulation division (RND) family, export a wide range of structurally diverse substrates, and are the most important family in conferring multidrug resistance to Gram negative bacteria (Piddock, 2006). RND efflux pumps are tripartite pumps, which use the proton motive force to transport substrates from the periplasm into the extracellular space (Blair and Piddock, 2009; Piddock, 2006), (Figure 1.1). There are four other families of efflux pump; the major facilitator superfamily (MFS), staphylococcal multiresistance (SMR), multidrug and toxic compound extrusion (MATE) and ATP binding cassette (ABC) families. Efflux pumps alone often lead to lower levels of resistance than other mechanisms, but nevertheless, they are often reported to be important in contributing to MDR. It has been proposed that efflux is the first step in MDR, since it lowers the concentration of drug inside the cell, increasing the

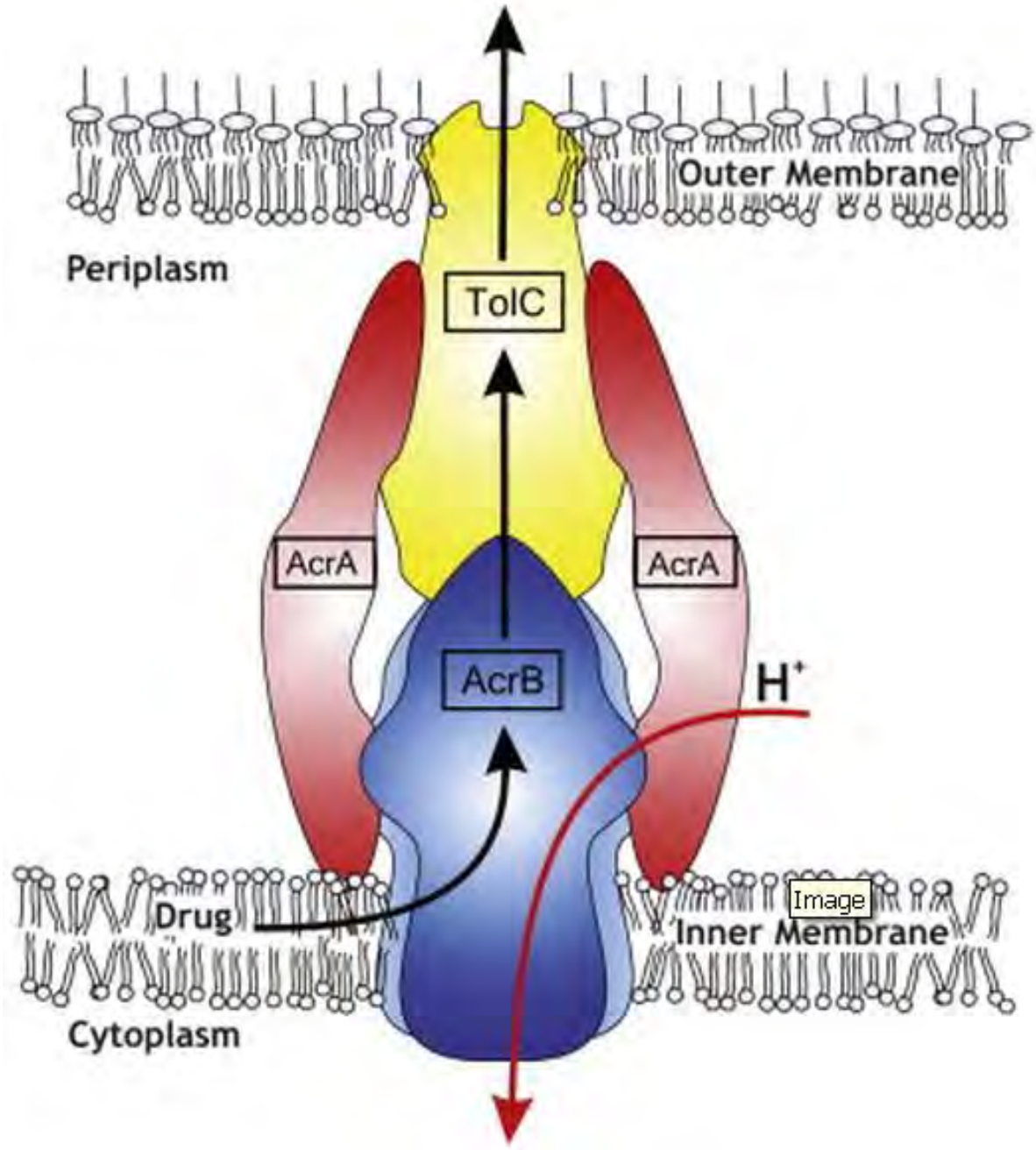
MIC of the agent. This enables the organism to survive long enough for other, more specific modifications or mutations to occur (Piddock, 2006).

Unlike antibiotics, biocides often have many targets within the bacterial cell (Russell, 2003). Therefore, biocide resistance in Gram negative bacteria is often due to intrinsic mechanisms, such as low permeability of the outer membrane, the ability to sporulate and biofilm formation (McDonnell and Russell, 1999). The most common acquired resistance mechanisms are a change in permeability of the cell membrane or increased efflux (Poole, 2002). Adaptation via the acquisition of mutations or alteration of the biocide target is not so common because alterations to multiple targets would usually have to occur (Poole, 2002). However, resistance to triclosan, which has a specific target (FabI, involved in fatty acid synthesis), can arise due to mutations in the *fabI* gene (Webber *et al.*, 2008).

1.2.2 Antibiotic Resistance and Mechanisms in *Acinetobacter baumannii*

A. baumannii is the most resistant species of *Acinetobacter* and is typically resistant to many antibiotics, disinfectants and antiseptics (Garnacho-Montero and Amaya-Villar, 2010). The main reason for resistance in this species is the acquisition of genes encoding drug-inactivating enzymes located on mobile genetic elements, such as plasmids, integrons or transposons (Adams *et al.*, 2008; Fournier *et al.*, 2006). Other determinants of resistance are mutations in chromosomal genes and overexpression of chromosomal genes encoding efflux systems (Coyne *et al.*, 2010a; Coyne *et al.*, 2010b) . Little is known about the permeability of the outer membrane in *A. baumannii* or its outer membrane porins (Vila *et al.*, 2007). Only a few porins have been reported in this bacterium, such as OmpW, HMP-MB, CarO and OprD (Mussi *et al.*, 2005; Vila *et al.*, 2007).

Figure 1.1 Structure of the AcrAB-TolC RND Efflux Pump (Blair and Piddock, 2009).



1.2.2.1 β-lactams

The most common mechanism of β-lactam resistance is inactivation of the drug by β-lactamase enzymes, which can be either chromosomally- or plasmid-encoded (Roca *et al.*, 2012).

Two intrinsic β-lactamases are present in most strains of *A. baumannii*. These are the chromosomally-encoded AmpC and OXA-51-like β-lactamases, which must be up-regulated to provide resistance (Roca *et al.*, 2012). As observed in several other bacterial species, inducible expression of AmpC does not occur in *Acinetobacter* (Peleg *et al.*, 2008). Increased expression is due to the upstream presence of an insertion sequence, IS*Aba1*, which provides a promoter for gene expression (Livermore, 2009; Turton *et al.*, 2006). This element has also been found upstream of carbapenemase genes such as $bla_{\text{OXA-51,}}$ $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-27}}$ and detected in all representatives of most of the outbreak strains and clones, including OXA-23 clones 1 and 2, T-strain (associated with infection in the Midlands, UK) and SE clone. It is not present in European clone 1 (Turton *et al.*, 2006).

Turton et al. (2006) found that isolates that had ISAba1 upstream of their blaOXA-51-like gene were resistant to imipenem (MIC 4-8 μg/ml) and/or meropenem (MIC 16- >32 μg/ml). By contrast, the bla gene in susceptible isolates (MIC <4 μg/ml) did not have the IS upstream. Carbapenem-resistant isolates have been identified, which possess blaOXA-51-like but have the IS associated with other carbapenemase genes. However, in isolates whose only cabapenemase gene is blaOXA-51-like, ISAba1 must be present for imipenem and/or meropenem resistance, suggesting that this IS is acting as a promoter for the gene, and is essential for expression.

β-lactam resistance is not attributable to the action of efflux pumps alone (Coyne *et al.*, 2010b). However, overexpression of the RND efflux pump AdeABC in conjunction with the action of carbapenemases has been reported to confer high levels of resistance to β-lactams (Giamarellou *et al.*, 2008). AdeIJK has also been implicated in intrinsic β-lactam resistance (Damier-Piolle *et al.*, 2008), whilst AdeFGH does not transport this class of antibiotic, and therefore does not contribute to resistance (Coyne *et al.*, 2010c). A decrease in outer membrane permeability (due to loss of or decreased expression of outer membrane proteins), resulting in reduced uptake of the agent (Mussi *et al.*, 2005) or changes in the structure or expression of penicillin-binding proteins have also been associated with resistance to β-lactams (Vashist *et al.*, 2011).

Secretion of outer membrane vesicles, which harbour the β-lactamase gene, $bla_{\text{OXA-24}}$ have been identified in *A. baumannii* and could result in dissemination of β-lactam resistance between *A. baumannii* and also to other bacteria (Rumbo *et al.*, 2011).

1.2.2.2 Fluoroquinolones

Acinetobacter isolates in France were susceptible to fluoroquinolones when these agents were first introduced as a treatment strategy. However, within five years of their introduction, 75-80% of isolates in this country became insusceptible to pefloxacin and other drugs of this class (Bergogne-Berezin and Towner, 1996).

As for other Gram negative bacteria, mutations in *gyrA* (Vila *et al.*, 1995) and *parC* (Vila *et al.*, 1997) have been implicated in fluoroquinolone resistance in *A. baumannii*. Vila *et al.,* (1995) identified a Ser83 to Leu mutation in *gyrA* of 15 *A. baumannii* clinical isolates. However, the MIC of ciprofloxacin for these isolates ranged from 4 to 128 μg/ml. Vila *et al.*, (1997) investigated the reason for the varying fluoroquinolone susceptibility in these

isolates. They identified that the isolates with higher resistance (MIC≥ 32 μg/ml) also had a Ser80 to Leu mutation in *parC*, suggesting that an accumulation of mutations in both *gyrA* and *parC* is required to achieve high fluoroquinolone resistance. Mutations in *parC*, in the absence of *gyrA* mutations have not been identified, suggesting that DNA gyrase is the primary target of fluoroquinolones in *A. baumannii* (Vila *et al.*, 1997).

Fluoroquinolone resistance can also be conferred by increased activity of the RND efflux pumps, AdeABC, AdeIJK and AdeFGH and the MATE efflux pump, AbeM (Magnet *et al.*, 2001; Damier-Piolle *et al.*, 2008; Coyne *et al.*, 2010c; Su *et al.*, 2005). In particular, AdeFGH confers high-level resistance to fluoroquinolones (Coyne *et al.*, 2010c). The MICs of norfloxacin, ciprofloxacin and moxifloxacin for two mutants (BM4454Δ*adeABC*Δ*adeIJK*), which overexpressed AdeFGH were >256 μg/ml, >32 μg/ml and 12 μg/ml, respectively and increased by at least 16-fold, compared with their parent (Coyne *et al.*, 2010c).

1.2.2.3 Polymyxins

Colistin resistance in *A. baumannii* has only been associated with sporadic outbreaks, which is thought to be due to the decreased growth and virulence associated with colistin resistance (Lopez-Rojas *et al.*, 2011). In a study by Lopez-Rojas *et al.* (2011), a colistin-resistant derivative of *A. baumannii* ATCC 19606 had a longer generation time than its parent and mice infected with the resistant derivative survived twice as long as those infected with ATCC 19606.

Resistance to colistin is usually due to remodelling of lipopolysaccharide (LPS) in the outer membrane by PmrC, which decreases the interaction and translocation of the agent across the membrane (Section 1.3.2.1.2) (Fernandez-Reyes *et al.*, 2009; Beceiro *et al.*, 2011). This resistance mechanism can be attributed to mutations in one or both of the two-component

system genes, *pmrAB*, which regulate *pmrC* expression (Section 1.3.2.1.2) (Adams *et al.*, 2009).

A loss of LPS, resulting from a mutation or deletion in one of the lipid A biosynthesis genes, *lpxA, lpxC* or *lpxD* has been implicated in colistin resistance in colistin-resistant (MIC > 128 µg/ml) derivatives of *A. baumannii* ATCC 19606 (Moffatt *et al.*, 2010). Disruption of *lpxA* or *lpxC* by the insertion sequence IS*Aba11* in can also result in loss of LPS and colistin resistance (Moffatt *et al.*, 2011).

Decreased expression of OmpW, an outer membrane protein has also been reported in a colistin resistant *A. baumannii* isolate (Vila *et al.*, 2007; Fernandez-Reyes *et al.*, 2009).

1.2.2.4 Aminoglycosides

Acinetobacter spp. exhibit a higher level of aminoglycoside resistance than most other Gram negative bacteria, and strains with high levels of resistance have existed since the late 1970s (Towner, 2009; Van Looveren and Goossens, 2004). This genus possesses all three classes of aminoglycoside enzymes with multiple aminoglycoside-resistance genes present in some strains (Bergogne-Berezin and Towner, 1996; Towner, 2009). In addition to modification of the drug, efflux pumps such as AdeABC and AdeT have also been reported to mediate resistance to aminoglycosides (Giamarellou *et al.*, 2008; Adams *et al.*, 2010; Marchand *et al.*, 2004).

1.2.2.5. Tetracycline, Chloramphenicol and Tigecycline

The main mechanisms of tetracycline resistance are the expression of an efflux pump (TetA-E and TetK) or a ribosomal protection system (TetO and TetM) (Vila *et al.*, 2007; Peleg *et al.*, 2007b).

cat genes, which encode chloramphenicol acetyltransferases have been identified on an integron and resistance island in *A. baumannii* and confer chloramphenicol resistance by enzymatic modification (Fournier *et al.*, 2006; Turton *et al.*, 2005). Chloramphenicol resistance can also be a result of efflux by CraA, a MFS family efflux pump (Roca *et al.*, 2009).

Tigecycline resistance has been associated with increased efflux by AdeABC. Peleg *et al.* (2007a), showed that exposure of resistant clinical isolates B46 and C75 (MIC of tigecycline 4 and 6 μg/ml, respectively) to PAβN (efflux pump inhibitor) increased their susceptibility to tigeycline (MIC reduced to 1 and 4 μg/ml, respectively). Furthermore, *adeB* expression in B46 and C75 was 54- and 40-fold higher than that in a tigecycline-susceptible laboratory strain, suggesting that AdeABC was at least partly responsible for tigecycline resistance. However, the contribution of other efflux pumps cannot be ruled out, particularly since the AdeABC efflux system was not disrupted in this study.

1.2.2.6. Biocides

Efflux has been associated with biocide resistance in *A. baumannii.* Transporters reported to be involved are AdeABC, AdeIJK and the SMR efflux pump QacE (Rajamohan *et al.*, 2010). An MFS family efflux pump, AmvA has also been described, which conferred significant increase in resistance to disinfectants, detergents and dyes in *A. baumannii* AC0037, an MDR clinical isolate (Rajamohan *et al.*, 2010a). AmvA expressed in *E. coli* conferred only a subtle decrease in susceptibility to antibiotics, and appears to be more specific for biocides. AbeS is an MFS pump, which transports biocides (Srinivasan *et al.*, 2011). An *A. baumannii* AC0037 mutant, in which *abeS* had been deleted, exhibited four-fold increased susceptibility to benzalkonium chloride compared with its parent and more than 16-fold increased susceptibility to the detergents, deoxycholate and sodium dodecyl sulphate.

A. baumannii strains, which form strong biofilms, have been reported to be more tolerant of biocides than strains that form a weaker biofilm (Rajamohan et al., 2009). Biofilm formation can be induced by the presence of biocides at sub-MIC concentration or by the presence of aminoglycosides (Hoffman et al., 2005; Rajamohan et al., 2009). Integrons commonly carry aminoglycoside-modifying enzymes and the presence of class one integrons correlates with the ability of *A. baumannii* strains to form strong biofilms and with biocide resistance.

It is hypothesized that bacterial resistance to biocides could arise due to exposure to sub-inhibitory concentrations of these compounds (Wendt *et al.*, 1997). Fuangthong *et al.,* (2011) reported that exposure of *A. baylyi* to sublethal concentrations (0.000001%) of chlorhexidine for 30 minutes induced adaptive resistance to lethal concentrations (0.00007%) of the biocide. Efflux was implicated in this adaptation, since the MIC of chlorhexidine for this bacterium decreased in the presence of PAβN (50 μg/ml). Kawamura-Sato (2008) reported that repeated exposure of *Acinetobacter spp.* to sub-inhibitory levels of chlorhexidine gluconate resulted in increased resistance (an increase in MIC of up to 10-fold). However, It has been reported that clinical isolates of *A. baumannii* do not become resistant to working concentrations of biocides over time (Martró *et al.*, 2003; Wisplinghoff *et al.*, 2007) and that whilst MICs may increase after exposure, they usually remain below the in-use concentrations (Kawamura-Sato *et al.*, 2008).

1.2.2.7. Multidrug Resistance

In the healthcare environment, more than two-thirds of *A. baumannii* infections are MDR (Adams *et al.*, 2010) and some strains are resistant to almost all antibacterial agents (Fournier *et al.*, 2006). Not all strains of *A. baumannii* are MDR; SDF, isolated from a body louse, is antibiotic susceptible and ATCC 17978 is susceptible to many antibiotics (Adams *et al.*, 2008). Efflux systems are an important determinant of MDR and are involved in both

antibiotic and biocide resistance (Coyne *et al.*, 2010b; Rajamohan *et al.*, 2010). Resistance islands, such as AbaR1, have been found in all sequenced *A. baumannii* genomes known to contain multiple resistance determinants (Fournier *et al.*, 2006). These genomic regions also contribute to MDR (Adams *et al.*, 2008).

1.2.2.7.1. Efflux

Efflux pumps transport toxic compounds, such as antibiotics, biocides and heavy metals out of the cell and can lead to resistance to these compounds (Mitscher, 2005; Rajamohan *et al.*, 2010; Levy, 2002). They are also implicated in virulence, cell homeostasis and quorum sensing (Piddock, 2006). Efflux pump-mediated resistance in *A. baumannii* is generally associated with the RND and MFS families of efflux pump (Vila *et al.*, 2007).

AdeABC, an RND Efflux Pump

The *adeABC* operon present on the chromosome, encodes components of an efflux pump of the RND family (Magnet *et al.*, 2001). *adeA* encodes the inner membrane fusion protein, *adeB* encodes the trans-membrane pump and *adeC* encodes the outer membrane component (Magnet *et al.*, 2001). AdeA and AdeB share 55% similiarity and 68% similiarity to AcrA and AcrB of *E. coli*, respectively (Piddock, 2006). *adeABC* are not present in all strains and appear to be associated with clinical isolates, rather than environmental strains (Huys *et al.*, 2005). Of 116 strains investigated by Nemec *et al.* (2007b), 68 lacked at least one of the genes in the *adeABC* operon, with 15 of these lacking *adeB*.

AdeABC plays a major role in MDR in cells which overexpress it (Coyne *et al.*, 2010a). It has broad specificity and was responsible for decreased susceptibility to β-lactams, fluoroquinolones, tetracycline, chloramphenicol, erythromycin and aminoglycosides in a clinical isolate, BM4454 (Magnet *et al.*, 2001). MDR in BM4454 was lost when *adeB* was

disrupted, suggesting that AdeB is essential for the function of this efflux pump (Magnet *et al.*, 2001).

Other RND Efflux Pumps

Although AdeABC is often implicated in MDR, other RND efflux pumps also contribute, such as AdeIJK and AdeFGH (Damier-Piolle *et al.*, 2008; Coyne *et al.*, 2010c). *adeIJK* genes have been found in all strains examined so far (Coyne *et al.*, 2010b). It has been suggested that AdeIJK has a larger role in intrinsic low-level antibiotic resistance (base-line resistance in the absence of regulation) than AdeABC in *A. baumannii* (Damier-Piolle *et al.*, 2008). However, AdeIJK is not thought to be involved in increased resistance as a result of enhanced efflux since the pump was toxic when overexpressed in *A. baumannii* strains BM4454 and CIP 70-10 (growth was impaired) (Damier-Piolle *et al.*, 2008). AdeIJK and AdeABC have overlapping substrate profiles and appear to have a synergistic contribution to tetracycline, minocycline and tigecycline resistance. It is thought that if one efflux system were impaired, the other could transport at least some of the toxic compounds. An exception is ethidium bromide, which is not a substrate for AdeIJK (Damier-Piolle *et al.*, 2008). Rajamohan, Srinivasan *et al.* (2010b) described decreased biocide (SDS, benzalkonium chloride and tetraphenylphosphonium chloride) susceptibility in four *A. baumannii* clinical isolates due to overexpression of AdeABC and AdeIJK. Inactivation of *adeB* or *adeJ* in one of these resistant isolates (AC0037) resulted in increased susceptibility to disinfectants, but not a complete loss of resistance, suggesting that other efflux pumps contributed to the resistant phenotype. AdeFGH is cryptic and therefore does not contribute to intrinsic resistance in *A. baumannii* (Coyne *et al.*, 2010c). However, it can give rise to MDR, when overexpressed (Coyne *et al.*, 2010c).

Non-RND efflux pumps

AmvA, a MFS family efflux pump, has been reported to confer resistance to antibiotics, dyes, antiseptics and disinfectants in *A. baumannii* isolate AC0037 (Rajamohan *et al.*, 2010a). CmlA, a MFS efflux pump, confers chloramphenicol resistance and has been identified in the resistance island of *A. baumannii* AYE (Fournier *et al.*, 2006). SMR efflux pumps encoded by *qac* genes remove quaternary ammonium compounds from the cell, and are involved in biocide resistance (Coyne *et al.*, 2010b; Rajamohan *et al.*, 2010). AbeS a member of the SMR family, confers low level resistance to antibiotics, dyes and detergents (Srinivasan *et al.*, 2009). The MATE family pump AbeM, confers resistance to fluoroquinolones although the increase in resistance attributable to this pump is thought to be small (Giamarellou *et al.*, 2008; Coyne *et al.*, 2010b).

1.3. Two Component Systems

When there is a change to the extracellular environment of a cell or in the cellular homeostasis, it is important that the change is detected so that any necessary modifications to the intracellular environment can be made (Mitrophanov and Groisman, 2008; Mascher *et al.*, 2006). Extracellular information is tranduced to intracellular proteins by cellular signalling. A widespread type of signalling, found in prokaryotes and eukaryotes, is protein phosphorylation. Two component systems (TCSs) are the predominant type of such signalling in prokaryotes (West and Stock, 2001; Mitrophanov and Groisman, 2008). These systems are also found in plants and fungi, but are absent from animals and humans (Zhang and Hendrickson, 2010; West and Stock, 2001). Some bacteria such as *Myxococcus xanthus,* a soil bacterium have at least 150 two-component pathways (Vos and Velicer, 2006), which has 146 histidine kinases (Bell *et al.*, 2010; Mascher *et al.*, 2006). Other bacteria have far fewer TCSs; *P. aeruginosa* has around 70 TCSs (Goodman *et al.*, 2009) whilst *Mycoplasma*

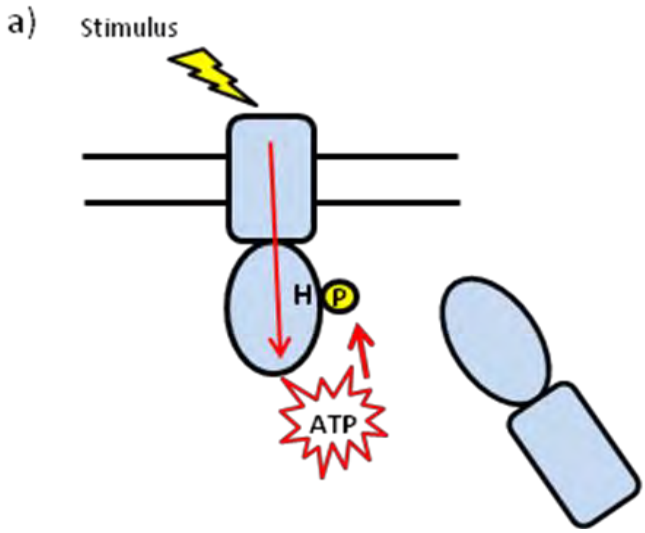
pneumoniae, the smallest organism known to be capable of self replication (Pitcher and Nicholas, 2005), has no TCS genes (Musatovova *et al.*, 2006). However, it is rare that bacteria have no TCSs as these pathways play a large role in adaptation to changing conditions (Wolanin *et al.*, 2002; Mascher *et al.*, 2006). The larger the number of environments that a bacterium is able to inhabit, the larger the genome generally is, and the larger the number of TCSs the organism usually has. For example, soil bacteria tend to have more TCSs than enteric bacteria (Krell *et al.*, 2009; Goodman *et al.*, 2009).

TCSs are involved in basic housekeeping functions such as transition to stationary phase, competence, metabolism, adaptation to a lack of nutrients, sporulation and osmoregulation. They are also involved in other adaptive responses such as expression of toxins and antimicrobial resistance (Mitrophanov and Groisman, 2008; West and Stock, 2001). For example, mutations in *pmrAB* have been implicated in colistin resistance in *P. aeruginosa, A. baumannii* and *Salmonella* (Lopez-Rojas *et al.*, 2011). It is the involvement of TCSs in antimicrobial resistance that is of relevance to this project.

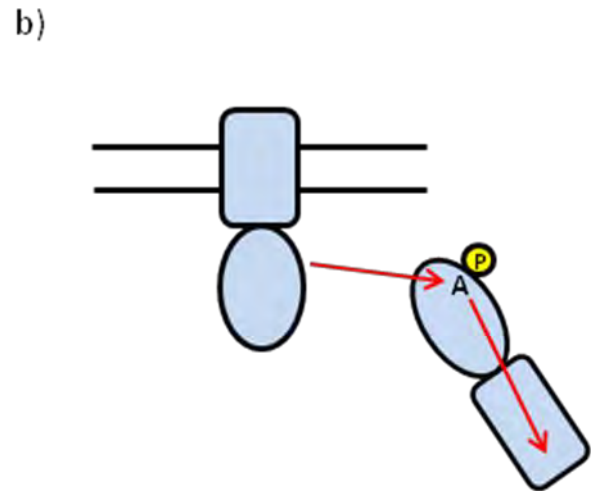
1.3.1. Signal Transduction

Signalling via TCSs requires a sensor protein to detect a signal and a regulatory protein to mediate an intracellular response to adapt to the changed environment (Figure 1.2) (Mitrophanov and Groisman, 2008). The sensor protein is a histidine kinase which detects an extracellular stimulus and activates or inactivates a response regulator (Figure 1.2) (Wieczorek *et al.*, 2008). Histidine kinases are most often homodimeric transmembrane proteins which, in Gram negative bacteria, comprise a periplasmic sensor domain and a cytoplasmic kinase domain, but they can alternatively be cytosolic proteins (West and Stock, 2001; Khorchid and Ikura, 2006; Zhang and Hendrickson, 2010). The cytoplasmic domain contains a conserved histidine residue which accepts a phosphoryl group from ATP during

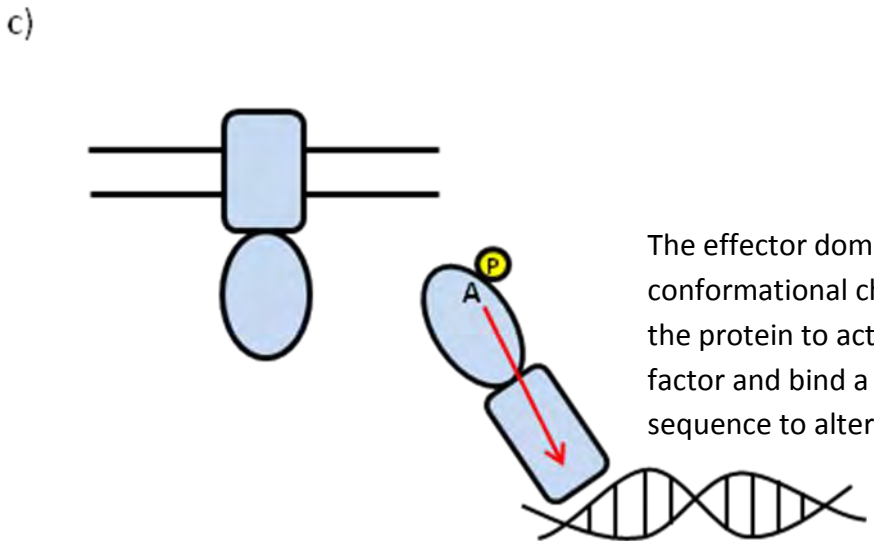
Figure 1.2. Transcription Modification in Response to an Extracellular Stimulus (An example of signal transduction through a two component system).



A stimulus is detected by the sensor domain of the histidine kinase. The signal is transduced to the kinase domain which binds ATP and catalyses the transfer of a phosphate group to the conserved histidine residue.



The kinase domain catalyses the transfer of the phosphate to the conserved aspartate residue in the receiver domain of the response regulator. The signal is tranduced to the effector domain.



The effector domain undergoes a conformational change which enables the protein to act as a transcription factor and bind a specific promoter sequence to alter gene transcription.

signal tranduction (Khorchid and Ikura, 2006; Wolanin *et al.*, 2002). Once the sensor domain has detected a stimulus, usually a chemical ligand (Zhang and Hendrickson, 2010), the histidine kinase autophosphorylates at a conserved histidine residue and then catalyses the transfer of the phosphate to the receiver domain of the response regulator (Marchand *et al.*, 2004). It is thought that the binding of a ligand induces a conformational change in the histidine kinase. This conformational change is proposed to propagate the signal from its sensor domain to the kinase domain (Khorchid and Ikura, 2006; Zhang and Hendrickson, 2010). Histidine kinases, particularly cytosolic histidine kinases, often have duplicated domains which allow responses to be made to multiple signals. For example, KinA, a soluble histidine kinase involved in DNA replication, has three sensor domains (Krell *et al.*, 2009; Mascher *et al.*, 2006).

The response regulator is responsible for bringing about the changes inside the cell, often by binding directly to DNA and modifying gene expression, since the majority of these proteins are transcription factors (Figure 1.2) (Wieczorek *et al.*, 2008; West and Stock, 2001; Zhang and Hendrickson, 2010). Alternatively, the response regulator can catalyse reactions, bind RNA or interact with other proteins (Mitrophanov and Groisman, 2008). The response regulator has two or more domains. All response regulators have a receiver domain which is connected to an effector domain by a flexible linker (Marchand *et al.*, 2004). The receiver domain accepts a phosphoryl group from the kinase domain of the histidine kinase and transduces the signal to the effector domain, which mediates an intracellular response. Many response regulators are dependent on phosphorylation for activation, since phosphorylation of a conserved Asp residue in the receiver domain brings about a conformational change which is necessary for DNA binding (Marchand *et al.*, 2004; Mitrophanov and Groisman, 2008; Zhang and Hendrickson, 2010; Mascher *et al.*, 2006). In

the PhoB response regulator from *E. coli*, an α-5 helix in the receiver domain inhibits DNA binding of the effector domain, which is otherwise constitutively active (Allen *et al.*, 2001). Phosphorylation of the receiver domain relieves this inhibition (Allen *et al.*, 2001).The receiver domain also has autophosphatase activity, further controlling the activity of the effector domain and determining the half life of the response regulator, which can range from seconds to hours and correlates with the protein's physiological activity (West and Stock, 2001). The strength of the response following the detection of an extracellular stimulus is determined by the concentration of phosphorylated response regulator (Zhang and Hendrickson, 2010). The level of conservation of the various domains in TCS proteins reflects the function of these systems. The histidine kinase sensor and response regulator effector domains are highly variable due to the wide range of signals detected and responses brought about by TCSs. By contrast, the kinase domains of histidine kinases and the receiver domains of response regulators are highly conserved in both sequence and tertiary structure suggesting a conserved mechanism of signal transduction between the proteins (West and Stock, 2001; Zhang and Hendrickson, 2010).

TCSs often comprise more than two proteins

Many TCSs do not comprise only one histidine kinase and one response regulator. One histidine kinase may transduce a signal to multiple response regulators or one response regulator may be able to receive a signal from multiple histidine kinases (West and Stock, 2001). This allows a larger number of signals and/or responses to be incorporated into the signal transduction and more regulation to be obtained (West and Stock, 2001). In addition to histidine kinases and response regulators, most TCSs comprise auxiliary proteins, (West and Stock, 2001) which post-translationally modify the activity of their cognate histidine kinase or response regulator, (Mitrophanov and Groisman, 2008). Auxiliary proteins enable

more complex signalling to be obtained as they can connect TCSs which would otherwise function independently, as well as enabling more complexity within a single TCS (Mitrophanov and Groisman, 2008). Auxiliary proteins are usually synthesised following a different stimulus to that detected by the histidine kinase. These proteins can therefore bring about changes in gene expression in response to a wider variety of signals than those specific for the TCS (Mitrophanov and Groisman, 2008). Components of a TCS can also transduce or receive signals if their usual partner is unavailable. In an *E. coli phoR* mutant, the histidine kinase CreC was found to replace PhoR in the activation of its response regulator, PhoB (Zhou *et al.* 2003). Such crosstalk enables the continuation of response and may be evidence of integrated cellular processes.

There is some evidence of crosstalk between components of different TCSs *in vitro*. It is rare *in vivo* as specificity within a TCS is usually tightly regulated to ensure that a histidine kinase only activates its cognate response regulator. (Bell *et al.*, 2010; Mitrophanov and Groisman, 2008; West and Stock, 2001). However, crosstalk does exist, such as between the YycFG and PhoPR TCSs in *B. subtilis* (Tomaras *et al.*, 2008). A histidine kinase has a strong kinetic preference for its cognate response regulator, with molecular recognition being the primary mechanism for specificity (Bell *et al.*, 2010). Small molecule donors more readily transfer their phosphate group to a response regulator than a non-cognate histidine kinase (West and Stock, 2001), but nonetheless, this questions the exact specificity of TCSs.

1.3.2. Two Component Systems in *Acinetobacter*

Nineteen TCSs have been identified in *Acinetobacter baumannii* strain AB0057 (Adams *et al.*, 2008), the best characterised of which is AdeRS. AdeS is the histidine kinase and AdeR is the response regulator, a transcriptional activator which controls the expression of the *adeABC* operon (Peleg *et al.*, 2007a; Wieczorek *et al.*, 2008). Other TCSs include PmrAB, associated

with colistin resistance (Adams *et al.*, 2009) and BfmRS involved in biofilm formation and cellular morphology (Tomaras *et al.*, 2008). Two TCSs involved in heavy metal resistance have been identified, one of which is CusSR (Adams *et al.*, 2008).

1.3.2.1 TCSs in Antibiotic and Biocide Resistance

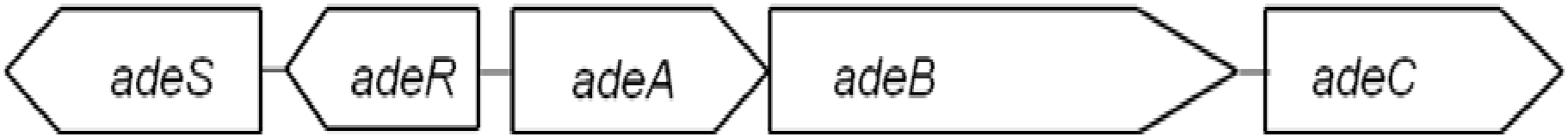
1.3.2.1.1 AdeRS

Expression of *adeABC* is regulated by AdeRS (Figure 1.3) (Marchand *et al.*, 2004). It is unusual for efflux pump genes to be controlled by a TCS; they are usually regulated by specific regulatory proteins whose genes are adjacent to the pump genes (Magnet *et al.*, 2001). However, regulation of efflux by a TCS has been reported in other bacteria such as *S. aureus* (NorA pump) (Fournier *et al.*, 2000).

Magnet *et al.* (2001) identified and characterised the *adeRS* and *adeABC* operons (Figure 1.3). The gene products shared sequence homology to sensor kinases (AdeS) and response regulators (specifically transcriptional activators) (AdeR), and it was hypothesised that the AdeABC efflux pump was regulated by a TCS comprising these proteins (Magnet *et al.*, 2001; Marchand *et al.*, 2004).

To investigate the hypothesis that alterations in AdeRS were responsible for AdeABC expression in a MDR clinical isolate BM4454, Marchand *et al.* (2004) investigated the sequences of *adeR* and *adeS* in MDR derivatives of CIP 70-10, a susceptible reference strain of *A. baumannii*. Point mutations were identified in both genes and these were hypothesised to be responsible for the overexpression of AdeABC and resistance to aminoglycosides in the mutants. The amino acid substitution identified in *adeS* (T153M; Table 1.2) was located four amino acids downstream of the conserved histidine residue (H149, the site of autophosphorylation) in the kinase domain and led to constitutive resistance. Amino acid

Figure 1.3 *adeRS* Control Expression of *adeABC.* Adapted from (Marchand *et al.*, 2004).



The *adeRS* open reading frames are upstream of the *adeABC* operon and divergently transcribed.

Table 1.2 Mutations in *adeS* Associated with Multi-Drug Resistance.

Amino acid substitution	Phenotype	Strain(s)	Source of Strain	Reference
G30D	MDR, overexpression of AdeABC (10- to 30- fold higher in BMK4665 than in BM4587) Reduced susceptibility to aminoglycosides (12- fold increase in MIC than BM4587)	BM4665 Spontaneous resistant mutant	Mutant of susceptible clinical isolate BM4587, obtained on gentamicin	Coyne *et al.*, 2010a
G103D	MDR Decreased tigecycline susceptibility (MIC = 64 µg/ml; MIC for AB210 = 0.5 µg/ml) Increased ceftazidime susceptibility (MIC = 16 µg/ml; MIC for AB210 = 64 µg/ml) Increased carbapenem susceptibility (at least 4-fold increased susceptibility to imipenem and meropenem compared with AB210) Increase (407-fold) in AdeABC expression compared with AB210	AB210-6	Laboratory mutant AB210 (parent) serially exposed to tigecycline	Hornsey *et al.*, 2010
Amino acid 94: Alanine in AB210, Valine in AB211.	AB211: increase in tigecycline resistance, overexpression of AdeABC (compared with AB210)	AB211 and AB210 (OXA-23 clone 1)	Isolated from a patient before (AB210) and after (AB211) treatment with tigecycline	Hornsey *et al.*, 2010; Hornsey *et al.*, 2011
T153M	Indistinguishable resistance phenotype from BM4454 (MDR clinical isolate). Constitutive *adeABC* transcription.	BM4546	Spontaneous one step mutant of CIP 70-10 (susceptible reference strain), obtained on gentamicin	Marchand *et al.*, 2004

MDR, multidrug resistant

substitutions at this position in other histidine kinases, such as $VanS_D$ in *Enterococcus faecium* have been implicated in constitutive expression of their downstream genes (Depardieu *et al.*, 2009). The H box, which contains the conserved histidine is also responsible for kinase and phosphatase activities of the histidine kinase (Depardieu *et al.*, 2009). The constitutive resistance in the mutants was suggested to be the result of a defect in phosphatase activity, but not kinase activity of AdeS, as has been reported for other bacteria (Aiba *et al.*, 1989; Marchand *et al.*, 2004). If so, AdeR would not be inactivated by AdeS and this would lead to a higher expression of AdeABC (Marchand *et al.*, 2004). The amino acid substitution identified in AdeR (P116L; Table 1.3) was located in the α-helix (Section 1.3.1) of its receiver domain. The alteration in AdeR resulted in constitutive transcription of *adeABC,* which Marchand *et al.*, (2004) hypothesised was due to lack of inhibition by the α-helix. Coyne *et al.* (2010) identified a glycine to aspartate substitution in the sensor domain of AdeS (Table 1.2), which was hypothesised to be responsible for the overexpression of AdeABC since mutations in the sensor domain of other histidine kinases have been implicated in constitutive expression of their target genes (Depardieu *et al.*, 2009).

Mutations in AdeS or AdeR are not the only reason for AdeABC overexpression; the presence of the insertion sequence, IS*Aba1*, upstream of the AdeABC operon has also been implicated (Coyne *et al.*, 2010a; Ruzin *et al.*, 2007). Ruzin *et al.* (2007) investigated the role of AdeABC in reduced tigecycline susceptibility in *A. baumannii.* They identified that *adeS* was disrupted by IS*Aba1* in two isolates with increased tigecycline resistance (MIC = 4 g/ml), which had 27- and 37- fold increased expression of AdeABC compared with less resistant isolates (MIC = 1.5 µg/ml). However, in the less resistant strains, *adeS* was not disrupted. There have also been

Table 1.3 Mutations in *adeR* Associated with Multi-Drug Resistance.

Amino acid substitution	Phenotype/genotype	Strain	Source of Strain	Reference
P116L	MDR, Constitutive *adeABC* transcription	BM4547	Spontaneous one step mutant of CIP 70-10 (on gentamicin)	Marchand *et al.*, 2004
A91V (immediately upstream of putative -10 promoter seq of *adeABC*)	MDR Decreased tigecycline susceptibility (MIC = 64 μg/ml; MIC for AB210 = 0.5 μg/ml) Increased ceftazidime susceptibility (MIC = 16 μg/ml; MIC for AB210 = 64 μg/ml) Increased carbapenem susceptibility (at least 4-fold increased susceptibility to imipenem and meropenem compared with AB210) Increase (407-fold) in AdeABC expression compared with AB210	AB210-6	Laboratory mutant AB210 (parent) serially exposed to tigecycline	Hornsey *et al.*, 2010
Single missense mutation at nucleotide 58 (G→A) in isolates G and J compared with isolates A and F Led to D20N replacement in the acidic triad (active site for phosphorylation)	Overexpression (>7-fold) of *adeB* in isolates G and J compared with isolates A and F No difference in expression of *adeRS* No ISs identified in vicinity of *adeABC* Decreased susceptibility (according to Etest) to co-trimozazole (MIC unreported), tigecycline (MIC for isolates G and J, 16; MIC for isolates A and F, 4 μg/ml), meropenem (MIC for isolates G and J, ≥64; MIC for isolates A and F, ≤ 32 μg/ml) and levofloxacin (MIC for isolates G and J, ≥64; MIC for isolates A and F, 8 μg/ml). A missense mutation in the β-lactamase $bla_{OXA-164}$ (present in isolates A and F) was identified, which converted it to bla_{OXA-58} (present in isolates G and J).	*A. baumannii* post-therapy isolates (Strain not reported). Isolates A and F were obtained on days 18 and 26 of infection; isolates G and J were obtained on days 32 and 64.	Hospitalised patient.	Higgins *et al.*, 2010b

MDR, multidrug resistant

reports of MDR associated with increased *adeABC* expression, where mutations in *adeRS* were not identified (Table 1.4).

Environmental stimuli detected by AdeRS

The signals detected by AdeRS are unknown (Marchand *et al.*, 2004), although upregulation of AdeABC following antibiotic exposure has been reported (Hornsey *et al.*, 2010). Hornsey *et al.,* (2010) showed that exposure of a tigecycline-susceptible clinical isolate (AB210, MIC, 0.5 µg/ml) to tigecycline (0.5 x MIC, then doubling the concentration every 24 hr until no growth was observed) resulted in overexpression of AdeABC and resistance (MIC, 64 µg/ml) to the antibiotic. It was not investigated whether the resistance was inducible or stable (Michael Hornsey, personal correspondence). A mutant (AB210-6), selected by exposing AB210 to tigecycline, had a Gly103 to Asp substitution in AdeS, and an Ala91 to Val substitution in AdeR (Table 1.2; Table 2.1), which were not present in the parental strain, and so were hypothesised to be responsible for increased AdeABC expression and decreased tigecycline susceptibility in the mutant. In the same study, compared with the pre-therapy isolate (AB210), a post tigecycline therapy clinical isolate (AB211) was also resistant to tigecycline and overexpressed AdeABC. AB211 had an Ala94 to Val substitution, conferred by a single single nucleotide polymorphism (SNP), compared with AB210 (Table 1.2) (Hornsey *et al.*, 2011; Hornsey *et al.*, 2010). It was postulated that AdeABC overexpression in the post-therapy isolate was due to the single nucleotide difference in *adeS*.

1.3.2.1.2 Other TCSs in Antibiotic and Biocide Resistance

PmrAB is Implicated in Colistin Resistance

PmrB is the histidine kinase and PmrA is the response regulator in a TCS associated with polymyxin E (colistin) and polymyxin B resistance (MIC of colistin > 2 µg/ml) in *A. baumannii*.

Table 1.4 Increased Expression of *adeABC* with Lack of Mutations in *adeRS*.

Strain/isolate	Phenotype/ genotype	*adeRS*	Source of strain	Reference
AYE	MDR The expression of *adeABC* was more than 2-fold higher than that in Cip 70-10, a susceptible reference strain	The mutations identified in *adeRS* by Marchand *et al.* were not identified in AYE.	Clinical isolate	Fournier *et al.*, 2006; Coyne *et al.*, 2010a
W7282	Tigecycline resistant (MIC = 8 μg/ml) Nine-fold higher expression of *adeABC* than a pre-therapy isolate (MIC = 0.5 μg/ml)	No difference in *adeRS* nucleotide sequence compared with a pre-therapy isolate	Clinical isolate. Post-therapy with tigecycline.	Hornsey *et al.*, 2010
Nine tigecycline-resistant isolates	MDR Expessed *adeB* to levels 57.6-fold higher than a tigecycline-susceptible control strain ATCC 15151	No mutations compared with ATCC 15151 No insertion of IS_{Aba1}	Clinical isolates, Taiwan	Sun *et al.*, 2010
A24D	Tigecycline-reistant (MIC = 24 μg/ml) Twenty five-fold increase in *adeB* expression compared with A24	Lack of mutations compared with A24	A24, tigecycline susceptible clinical isolate (MIC = 1 μg/ml) exposed to tigecycline for four days	Peleg *et al.*, 2007a
Various *A. baumannii* (2005-2007)	MDR, resistance to biocides 55% of isolates studied possessed *adeRSB* (most of those with *adeB* were in highest resistance group)	Point mutations in *adeRS* previously described were absent from isolates with *adeB*	Clinical isolates	Rajamohan *et al.*, 2010

MDR, multidrug resistant

pmrAB are in an operon with *pmrC*, which encodes a phosphoethanolamine transferase. PmrC adds phosphoethanolamine to lipid A, a constituent of LPS. LPS is the target of polymyxins and the modified LPS results in a weaker interaction between the antibiotic and the outer membrane and gives reduced susceptibility to the agent (Beceiro *et al.*, 2011). Beceiro *et al.* (2011) reported that Δ*pmrB* mutants had a decreased expression of *pmrC*, lack of addition of phosphoethanolamine to their LPS and were 100 times more susceptible to colistin compared with the parental strain. These data highlight the importance of PmrB and its regulation of *pmrC* in colistin resistance. Mutations in *pmrAB* have also been identified in colistin-resistant bacteria (Table 1.5). Adams, Nickel *et al.* (2009) reported that one colistin resistant derivative of *A. baumannii* AB0057 had a mutation in both *pmrA* and *pmrB*, and a colistin-resistant derivative of ATCC 17978 had two mutations in *pmrB*. Beceiro *et al.* (2011) reported amino acid substitutions in PmrB, but not PmrA or PmrC. However, Park *et al.* (2011) did not identify any amino acid substitutions in PmrAB in colistin resistant isolates, although mutants derived from susceptible isolates were found to have mutations in *pmrB*.

pmrA and *pmrB* expression is often elevated in polymyxin-resistant clinical isolates and polymyxin-resistant mutants (selected *in vitro*) compared with polymyxin-susceptible clinical isolates (Park *et al.*, 2011; Arroyo *et al.*, 2011; Beceiro *et al.*, 2011). Adams *et al.* (2009) reported that *pmrA* expression was 5-40 times higher in resistant strains compared with susceptible strains. However, an increased expression of *pmrAB* alone was not sufficient to result in polymyxin resistance. Arroyo *et al.* (2011) complemented Δ*pmrB* mutants with *pmrAB* DNA and reported that elevated expression of *pmrAB* in the absence of an increased expression of *pmrC* did not result in polymyxin resistance.

These reports all suggest that increased expression of *pmrA* and/ or *pmrB* and a resultant increased expression of *pmrC* are important in polymyxin resistance, but the role of amino

Table 1.5 Polymyxin Resistance Associated with Mutations in *pmrAB*.

Strain	Amino acid substitution	Phenotype/genotype	Source of Strain	Reference
RC64 (colistin resistant)	R134C and A227V (PmrB)	Colistin resistant Gain-of-function of PmrAB	Derived from ATCC 19606 (Colistin sensitive)	Lopez-Rojas *et al.*, 2011
MAC101	P102H (PmrA) A262P (PmrB)	Colistin resistant Elevated expression of PmrA (>40-fold) compared with AB0057	Selected from AB0057 (Colistin sensitive clinical isolate)	Adams *et al.*, 2009
MAC102	A227V (PmrB)	Colistin resistant Elevated expression of PmrA (5-fold) compared with AB0057	Selected from AB0057 (Colistin sensitive clinical isolate)	Adams *et al.*, 2009
MAC201	T13N and P233S (PmrB)	Colistin resistant Elevated expression of PmrA (>40-fold) compared with ATCC 17978	Selected from ATCC 17978 (Colistin sensitive clinical isolate)	Adams *et al.*, 2009
ACCA152	P233S (PmrB)	Colistin resistant	Clinical isolate	Adams *et al.*, 2009
In vitro selected mutants	In PmrB: T192I (most frequently found), I121F, A183T, A184V, P190S, Q228P	Colistin resistant Elevated expression of *pmrA* (12 to 63-fold) and *pmrB* (9 to 42-fold) No mutations in *pmrA* or *pmrC*	Selected from colistin-susceptible clinical isolates	Park *et al.*, 2011
Clinical isolates	S14L, M145K, P233S, L87F (Single substitutions in PmrB) F387Y and S403F (Double	Colistin-resistant Mutations identified compared with ATCC 17978 reference sequence.	UK, Spain, Saudi Arabia 2000-2009	Beceiro *et al.*, 2011

	substitution in PmrB)	Increased expression of *pmrA* (mean increase 12.4-fold) and *pmrB* (mean increase 6.8-fold) compared with susceptible clinical isolates. *pmrC* expression not significantly changed.		
In vitro selected mutants	A227V (ATCC 19606) N353Y (ABRIM)	Colistin-resistant Increased expression of *pmrA* and *pmrB* compared with parental strains	Selected from ATCC 19606 (colistin-susceptible type-strain) or ABRIM (colistin-susceptible clinical isolate)	Beceiro *et al.*, 2011
Ten clinical isolates	All isolates had different variations of PmrB; four had a substitution in the kinase domain of PmrB	Polymyxin B resistant Increased expression (26- to 292-fold) of *pmrC* compared with laboratory wild-type strain ATCC 17978	*A. baumannii* infections from five different countries 2001-2008	Arroyo *et al.*, 2011
Spontaneous resistant mutants	Five mutants had mutations in *pmrB*, most often in the predicted kinase domain One mutant had a mutation in the predicted receiver domain of *pmrA*.	Polymyxin B resistant (stable resistance; MIC 2-8 µg/ml) No mutation in *pmrC* in any mutant Increased expression (26- to 292-fold) of *pmrC* compared with laboratory wild-type strain ATCC 17978 Modified lipid A profile	Selected from ATCC 17978 (polymyxin B-sensitive; MIC 0.5 µg/ml) on polymyxin B	Arroyo *et al.*, 2011

acid changes in PmrAB are yet to be determined. In support of the involvement of amino acid changes in polymyxin resistance, Arroyo *et al.* (2011) reported that Δ*pmrB* mutants only reverted to the polymyxin-resistant phenotype when they were complemented with *pmrAB* from the resistant parental strain. Wild-type DNA, which did not contain mutations in *pmrAB*, did not lead to an increase in *pmrC* expression or resistance to polymyxin B. This study suggested that mutations have a dominant effect on the activity of *pmrCAB*, leading to constitutive expression of genes controlled by this operon. The exact regulatory mechanism of PmrAB in *A. baumannii* is unknown. One Δ*pmrB* clinical isolate has been reported to retain some *pmrB* expression and some polymyxin B resistance, suggesting that there may be multiple copies of *pmrB* in the isolate, a more complex regulatory mechanism involving PmrAB, or mechanisms of PmrAB-independent resistance (Arroyo *et al.*, 2011). In *Salmonella enterica* serovar Typhimurium, PmrA, PhoP and the TCS PreAB are known activators of *pmrCAB* (Merighi *et al.*, 2006). It is yet to be determined whether there are any TCSs in *A. baumannii*, which are involved in crosstalk and regulation of PmrAB. It is known that there are other genes, in addition to *pmrC*, which are annotated as phosphoethanolamine transferases in this species; these genes may play a role in polymyxin resistance (Arroyo *et al.*, 2011).

Environmental Stimuli

Adams and colleagues identified that environmental conditions such as acidity (pH 5.5) induced colistin resistance (MIC > 2 μg/ml) in wild-type, antibiotic susceptible strains and MICs increased from 1 to ≥64 μg/ml in such conditions. However, for a *pmrB* deletion mutant, the MIC was significantly reduced to 4 μg/ml, suggesting that PmrB is essential for colistin resistance. PmrA does not appear to be required for acid-induced resistance since

expression of this protein did not increase when pH was decreased from 7.7 to 5.5. Resistance occurred rapidly, suggesting that induced regulatory changes were responsible, rather than spontaneous resistance. Environmental $Fe^{3+}$and Mg^{2+} levels are also sensed by PmrAB in bacteria such as *Salmonella* and *P. aeruginosa* (Wosten *et al.*, 2000; McPhee *et al.*, 2003). Beceiro *et al.* (2011) reported that low Mg^{2+} levels resulted in a modest increase in *pmrC* expression and the addition of phosphoethanolamine to lipid A in *A. baumannii* ATCC 19606. This observation suggests that PmrAB may induce *pmrC* expression in response to Mg^{2+} in *A. baumannii* (Beceiro *et al.*, 2011).

1.3.2.2 TCSs in *Acinetobacter* Not Involved in Antibiotic or Biocide Resistance

BfmRS

BfmRS is important in morphology and biofilm formation in *A. baumannii* 19606 (Tomaras *et al.*, 2008). Inactivation of the histidine kinase (BfmS) resulted in a modest decrease in biofilm production and cell attachment, whilst inactivation of the response regulator (BfmR) resulted in biofilm deficiency, lack of cellular attachment and morphology changes. BfmR controls the expression of a chaperone-usher assembly system (*csu* operon) responsible for production of pili on the cell surface. The lack of expression of this system was responsible for the morphology changes. The less drastic effect when BfmS was inactivated suggests that a non-cognate histidine kinase may also activate BfmR or that the response regulator is active in its non-phosphorylated state. It is unknown whether the *csu* operon is regulated directly by BfmR and whether there are other target genes. Biofilm formation in bacteria is a complex process, and is often a result of many signalling pathways; the signals responsible for the activation of BfmR remain to be elucidated. Although BfmRS has a role in biofilm formation, it is important to note that the composition of the growth medium can affect the

ability of *A. baumannii* to form biofilms, independent of BfmRS. BfmRS has not been reported to be involved in antimicrobial resistance. However, biofilm formation is known to increase a bacterium's tolerance to antibiotics (Lewis, 2001). Thus, it is possible that this TCS may be linked to the MDR of *A. baumannii.* Clemmer *et al.* (2011) used transposon mutagenesis to identify genes required for motility in this species. One mutant with an 80% reduction in motility compared with the parent was found to have a transposon inserted in *bfmS.* To investigate whether the *csu* operon is essential for motility, *csuD* was disrupted. CsuD is an outer membrane usher protein, which is required for production of fimbriae. The *csuD* mutant did not exhibit altered motility, but was defective in biofilm formation. These results suggest that the reduced motility of the *bfmS* mutant was not due to altered regulation of the *csu* operon and that BfmRS has other downstream target genes which are important for motility. However, the *csu* operon appears to be important for biofilm formation and BfmR may be an important regulator in this process.

1.4 Methods to Study *Acinetobacter*

1.4.1 Genetic Manipulation in *A. baumannii*

The activity of bacterial genes can be inhibited by inserting an antibiotic resistance cassette (Datsenko and Wanner, 2000) or vector (Magnet *et al.*, 2001; Hornsey *et al.*, 2010) into the gene. Alternatively, the gene of interest can deleted, whereby the whole gene, or part of the gene, is removed from the chromosome by double recombination (Aranda *et al.*, 2010), using a vector that contains sequences flanking the target region. All of these methods have been used to genetically manipulate *A. baumannii* (Marchand *et al.*, 2004; Choi *et al.*, 2009; Roca *et al.*, 2009)

Gene Disruption: Gene disruption is used in this project to mean that a plasmid is inserted into the target gene, thereby inactivating the gene. The gene disruption method was used to inactivate *adeB, adeC, adeR and adeS* in the MDR *A. baumannii* clinical isolate, BM4454 (Marchand *et al.*, 2004; Magnet *et al.*, 2001). A DNA fragment internal to the target gene was amplified by polymerase chain reaction (PCR) and cloned into pUC18, a vector which confers ticarcillin resistance and is a suicide vector in *A. baumannii.* The vector was electrotransformed into BM4454 and mutants were selected on ticarcillin. This method involved a single recombination step between the homologous sequences in the gene and PCR fragment in the vector, which led to insertion of the vector into the target gene. Since only one recombination step is required, it is the least reliable of the discussed methods. Aranda *et al.,* (2010) investigated the stability of mutants created by this method. The mutants were unstable and could be reverted to wild-type by a second recombination step, although maintenance of selection pressure prevented this (Aranda *et al.*, 2010). Another disadvantage is that the same vector could not be used to create multiple deletions in the same strain since recombination between the vector inserted in the chromosome and the newly introduced vector would be very likely (Aranda *et al.*, 2010).

Gene Replacement: This method involves the introduction of a suicide vector containing a deleted or modified gene into the host (Aranda *et al.*, 2010). The vectors, pSSK10, pEX100T and pJQ200 have been used for this purpose in *A. baumannii* (Camarena *et al.*, 2010; Choi *et al.*, 2009; Roca *et al.*, 2009). Choi *et al.,* (2009) created an in-frame deletion of the *pga* locus. Sequences flanking the target region were amplified by PCR. The primers contained restriction sites, which were also present in the vector pSSK10. The fragments were digested and ligated sequentially into pSSK10, which had been digested with the same enzymes. The

plasmid was electroporated into *A. baumannii* and became incorporated into the chromosome. Double recombination steps were selected to identify mutants. *sacB*, which prevents growth of bacteria on sucrose was present on the vector and used to counter-select single recombinants. Aranda *et al.*(2010) used this method to produce stable mutants with a kanamycin cassette inserted in *oxyR*, *omp33* and *soxR*; no gene reversions were identified after 10 passages of these mutants in Luria Bertani (LB) broth. However, there was a lower efficiency of mutant selection (10^{-7}) than for the gene disruption method (10^{-5}) due to the second recombination step. This method is time consuming since it requires several subcloning and verification steps to introduce the inserts into the suicide vector and several phenotypic screenings to select single and double recombinants (Kim-Lee Chua, Unpublished). The phenotypic screenings alone take at least 4 days (Choi *et al.*, 2009).

Combination of gene disruption and gene replacement: Aranda *et al.*(2010) developed a quick method of inactivating chromosomal genes that does not require cloning steps, and can be confirmed by PCR. A linear PCR fragment carrying an antibiotic resistance cassette flanked by DNA homologous to the target gene was transformed into *Acinetobacter* and the target gene was replaced with the resistance cassette by double recombination. Advantages of this method are that no cloning steps are required, mutants are stable in the absence of antibiotic selective pressure and it could possibly be used to create multiple gene inactivations or deletions.

A major limitation of all the reported methods is that they have relied upon an antibiotic resistance cassette for selection of mutants. Since *A. baumannii* are resistant to most antibiotics available, there are few choices when choosing a suitable antibiotic to select mutants (Towner, 2009). The method developed by collaborator Kim Lee Chua.

(unpublished) to inactivate genes in this study is based on the gene replacement method (Hamad *et al.*, 2009), but relies upon tellurite resistance genes on the suicide vector, pMo130-Tel^{R} (Section 0; Figure 2.1; Figure 2.2). The MIC of tellurite for many *A. baumannii* strains is between 2 and 16 μg/ml (G. Richmond, unpublished data).

1.4.2 Virulence Models

Galleria mellonella, the larva of the greater wax moth, can be used to model *A. baumannii* infection and also to assess the effect of antimicrobial treatment upon infection (Peleg *et al.*, 2009). *G. mellonella* are a suitable organism for the study of human infection since they can be maintained at 37°C, a known inoculum of bacteria can be precisely administered and insects have a similar innate immune system to that found in mammals (Kavanagh and Reeves, 2004; Peleg *et al.*, 2009). The virulence of bacteria *P. aeruginosa* in *G. mellonella* has also been shown to correlate with their virulence in mammalian models (Jander *et al.*, 2000). A common end point of experiments using *G. mellonella* is the measurement of percentage survival at increasing time-points post-infection. The immune response can also be monitored, by measuring the level of haemocytes (phagocytes) present in the larva or by measuring the expression of antimicrobial peptides (Kavanagh and Reeves, 2004). Insect models of infection are suitable to identify any differences in virulence between mutant strains of bacteria. These models are less expensive than mammalian models and are more ethically acceptable. However, mammalian-specific disease processes will not be identified, thus mammalian models are required to study disease processes in the host (Kavanagh and Reeves, 2004).

Caenorhabditis elegans and mice have also been used to study virulence of *A. baumannii* (Smith *et al.*, 2007; Gaddy *et al.*, 2012). The *C. elegans* model has limitations compared with

the *G. mellonella* model; exposure of *C. elegans* to a temperature of 37°C for extended periods of time is lethal and the exact bacterial inoculum cannot be quantified, as in this model an unknown number of bacterial cells are ingested by the worm (Marsh and May, 2012).

In the mouse sepsis model, *A. baumannii* rapidly disseminates to different organ systems (McConnell *et al.*, 2011). This model may not be the best for simulating sepsis in humans, as sepsis in human infection is more progressive (McConnell *et al.*, 2012). Other mammalian models, such as a rat model of burn infection (Uygur *et al.*, 2009) and murine model of pneumonia (Montero *et al.*, 2004) result in dissemination of *A. baumannii* to other body sites, and may be more suitable for modelling human disseminated infection (McConnell *et al.*, 2012).

1.5 Background to the Project

1.5.1 Overview

The overall aim of the programme of work in which this project is part, is to investigate the role of TCSs in MDR and biocide resistance in *A. baumannii.*

The specific questions to be answered are:

a) how does *A. baumannii* respond to commonly used biocides (disinfectants)?

b) can these responses regulate pathways that lead to resistance to multiple antibiotics?

1.5.2. Hypotheses to be Tested

The main hypothesis is that the exposure of *A. baumannii* to biocides in the hospital environment predisposes the bacterium to develop resistance to multiple antibiotics. It is

hypothesised that two component regulatory systems are involved in the regulation of adaptive responses that allow survival of *A. baumannii* in the presence of these antimicrobials.

The hypotheses to be investigated are:

1. The deletion of *adeS* will result in down-regulation of AdeABC, decreased MICs of pump substrates and therefore a loss of MDR. Published results by two research teams have suggested that a Δ*adeS* mutant is more susceptible to agents such as aminoglycosides and fluoroquinolones than the wild type strain (Wong *et al.*, 2009; Marchand *et al.*, 2004).
2. The deletion of *pmrB* will result in increased susceptibility to polymyxin antibiotics (Beceiro *et al.*, 2011; Arroyo *et al.*, 2011).
3. Deletion of *adeS* and/or *pmrB* will affect the level of accumulation of antimicrobials.
4. Deletion of *adeS* will affect biocide susceptibility. This will affect growth of planktonic cells and formation of a biofilm in the presence of biocides.
5. Planktonic cells will be more susceptible to biocides than cells in a biofilm.
6. Deletion of *adeS* will affect biofilm formation.

1.5.3. Aims and Objectives

1. To interrogate the available genome sequences for TCSs.
2. To use the available genome sequences to identify a representative strain of *A. baumannii* for initial genetic manipulation experiments. (For this study, a representative strain was one which was MDR and had the largest number of core genes for its species, compared with other candidate strains (Section 3.2.2).

3. To use a gene deletion technique established for *Acinetobacter* (Kim Lee Chua, unpublished) to delete *adeS* and *pmrB* in a representative strain of *A. baumannii*.
4. To confirm and add to published data by determining the phenotype of AYEΔ*adeS* and AYE Δ*pmrB*.
5. To use the A. baumanii mutants in which *adeS* and *pmrB* have been deleted to investigate if AdeRS and PmrAB are involved in both antibiotic and biocide susceptibility.
6. To determine whether deletion of *adeS* or *pmrB* influence growth and biofilm formation in the presence and absence of biocides.

2. Materials and Methods

2.1. Bioinformatics

2.1.1. Comparison of *A. baumannii* Genomes

Sequence data were obtained from xBASE where possible (Chaudhuri *et al.*, 2008). BM4454 sequence data (Accession AF370885.1; (Marchand *et al.*, 2004)) and *adeR* sequence data for AB307-0294 were obtained from Genbank (Accession CP001172.1), since these data were not in the xBASE database.

Nucleotide and protein sequences of *adeR* and *adeS* were aligned using ClustalW2 (Larkin *et al.*, 2007). X-base (http://www.xbase.ac.uk) (Chaudhuri *et al.*, 2008) was used to align *adeS* and the *adeRS* and *adeABC* operons from six different strains (Table 2.1) of *A. baumannii* in order to determine the level of conservation of sequence.

2.1.2. Identification of TCS genes in AYE

At the start of this project, November 2010, AciBASE (an online database containing *Acinetobacter* genome sequence data, which aims to support research into the epidemiology and molecular evolution and biology of *Acinetobacter*) was used to interrogate the genome sequences of the six available genome sequenced *A. baumannii* strains to identify TCSs in this species. Any TCSs reported in the literature, but not found in AciBASE were added to these results.

xBASE (Chaudhuri *et al.*, 2008) was used to search for *pmrB* and *bfmS* in AYE. Where the gene was unannotated, the literature was searched to find a homologue in an alternative

Table 2.1 Strains Compared in Bioinformatic Analyses.

Strain of *A. baumannii*	Description	Reference
AYE	MDR clinical isolate	(Poirel et al., 2003)
AB0057	MDR clinical isolate	(Hujer et al., 2006)
AB307-024	Drug susceptible clinical isolate	(Adams et al., 2008)
ATCC 17978	Drug susceptible clinical isolate	(Smith et al., 2007)
ACICU	MDR clinical isolate	(Iacono et al., 2008)
BM4454	MDR clinical isolate	(Magnet et al., 2001)

strain and this nucleotide sequence was compared against the AYE genome in xBASE using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990) algorithm. The ClustalW facility in Genious Pro 4.6.5 (Biomatters_Ltd., 2005-2009) was used for alignments. To check for multiple copies of the gene(s) of interest, the known AYE gene sequence was compared against the AYE genome sequence using BLAST. (Altschul *et al.*, 1990). The number of DNA sequences within the genome that the gene sequence aligned to was taken to be the number of copies of that gene present.

2.2. Bacterial Strains, Growth, Storage and Identification

The fifteen strains of *E. coli* and three *A. baumannii* (Table 2.2) were stored on Protect™ beads (Technical Service Consultants Ltd, UK) at -80°C until required. *E. coli* and *A. baumannii* were grown on LB agar or in LB broth (Sigma-Aldrich, UK, L3022) supplemented with the appropriate antibiotic (Table 2.3). For biocide susceptibility assays (Section 2.5.4) Tryptic Soy Agar (TSA; Sigma-Aldrich, UK, 22091) and Tryptic Soy Broth (TSB; Sigma-Aldrich, UK, 22092) were used to maintain consistency with methods used by collaborators at the Health Protection Agency (HPA). To identify bacteria as *E. coli,* colonies were grown on MacConkey medium (Oxoid, UK, CM0007); *E. coli* grew as translucent colonies and produced red pigment. Colonies were Gram stained and observed microscopically to confirm they were Gram negative bacilli. The API20E identification system (BioMérieux UK Ltd., 20100) was used as a final check. All new strains were verified by the above methods.

To confirm isolates as *A. baumannii*, a multiplex PCR which amplifies *gyrB* was used (Table 2.4). This PCR can be used to distinguish between members of the *A. calcoaceticus-A. baumannii* complex. To confirm purity of cultures, *A. baumannii* were Gram stained and grown on MacConkey medium. *A. baumannii* from a plate were observed microscopically as

Table 2.2 Strains used in this Study.

Description	Phenotype	Reference
A. baumannii AYE, clinical isolate	MDR	(Fournier et al., 2006)
A. baumannii AYEΔ*adeRS*	MDR	This study
A. baumannii AYEΔ*pmrAB*	MDR	This study
E. coli DH5α + pMo130-Tel^R	Kan^R; Tel^R; not viable on sucrose	Kim Lee Chua (Unpublished)
E. coli DH5α + pMo130-Tel^R/*adeRS*UP	Kan^R; Tel^R; not viable on sucrose	This study
E. coli DH5α + pMo130-Tel^R/*adeRS*UPDOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli DH5α + pMo130-Tel^R/*pmrAB*UP	Kan^R; Tel^R; not viable on sucrose	This study
E. coli DH5α + pMo130-Tel^R/*pmrAB*UPDOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli DH5α + pMo130-Tel^R/*bfmRS*UP	Kan^R; Tel^R; not viable on sucrose	This study
E. coli DH5α + pMo130-Tel^R/*bfmRS*UPDOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli DH5α + pMo130-Tel^R/UP-*adeRS*-DOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli DH5α + pMo130-Tel^R/UP-*pmrAB*-DOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli S17-1	Amp^S, Tel^S	(Simon et al., 1983)
E. coli S17-1 + pMo130-Tel^R/*adeRS*UPDOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli S17-1 + pMo130-Tel^R/*pmrAB*UPDOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli S17-1 + pMo130-Tel^R/*bfmRS*UPDOWN	Kan^R; Tel^R; not viable on sucrose	This study
E. coli S17-1 + pMo130-Tel^R/UP-*adeRS*-DOWN	Kan^R; Tel^R; not viable on sucrose	This study

E. coli S17-1 + pMo130-Tel^{R}/UP-*pmrAB*-DOWN	Kan^{R}; Tel^{R}; not viable on sucrose	This study

MDR, Multidrug resistant

Table 2.3 Antibiotics used in this Study.

Agent	Solvent	Supplier
Amikacin disulphate salt[a]	SDW	Sigma-Aldrich, UK (A1774)
Ampicillin[a,b]	Sodium bicarbonate and SDW	Merck Chemicals, UK (171254)
Cefotaxime[a]	SDW	Sigma-Aldrich, UK (C7912)
Ceftazidime[a]	SDW	Sigma-Aldrich, UK (C3809)
Chloramphenicol[a]	70% V/V methanol	Sigma-Aldrich, UK (C0378)
Ciprofloxacin[a,c]	Drop of 100% V/V acetic acid to dissolve and SDW	Fluka Biochemika, Sigma-Aldrich, UK (17850)
Colistin sulphate salt[a]	SDW	Sigma-Aldrich, UK (C4461)
Erythromycin[a]	SDW + drop of acetic acid (100% V/V)	Sigma-Aldrich, UK (E5389)
Ethidium bromide[a]	SDW	Sigma-Aldrich, UK (E8751)
Gentamicin[a]	SDW	Sigma-Aldrich, UK (G1264)
Imipenem[a]	N/A	Biomeriéux, UK (513608)
Kanamycin[b]	SDW	Sigma-Aldrich, UK (60615)
Meropenem[a]	N/A	Biomeriéux, UK (513818)
Norfloxacin[c]	SDW + drop of acetic acid (100% V/V)	Sigma-Aldrich, UK (N9890)
Polymyxin B[a]	SDW	Sigma-Aldrich, UK (P1004)
Potassium tellurite hydrate[b]	SDW	Sigma-Aldrich, UK (P0677)
Tetracycline	SDW	Sigma-Aldrich, UK (T3383)
Tigecycline[a]	SDW	Pfizer, (PF-05208753)
Tobramycin	SDW	Sigma-Aldrich, UK (T4014)
Sulbactam[a]	SDW	Pfizer, (CP-045899)

SDW, sterile distilled water; [a]Minimum inhibitory concentration determined for AYE, AYEΔ*adeRS* and AYEΔ*pmrAB*; [b]Used during gene deletion; [c] Used in fluoroquinolone accumulation assays.

Table 2.4 *gyrB* Multiplex PCR to Distinguish Between *Acinetobacter* Species (Higgins *et al.*, 2010a; Higgins *et al.*, 2007).

Primer	*A. baumannii*	*A. nosocomialis*	*A. calcoaceticus*	*A. pittii*
sp4F	294bp amplimer	294bp amplimer		
sp4R	490bp amplimer			
sp2F				
D14			428bp amplimer	
D19				
D16				194bp amplimer
D8				

In this study, all seven primers were used in a PCR to identify the species of *Acinetobacter.* The primers (Higgins *et al.* 2010) are specific for the different species.

The PCR reagents were: 1.1 x Reddymix PCR master mix (22 µl; Thermo Fisher Scientific, PCR-300-610D), *gyrB* primer mix[a] (2 µl) and template DNA (lysate) or Ultrapure water (1 µl). The PCR parameters (30 cycles for stages 2-4) were: 94°C, 2 min; 94°C, 1 min; 59°C, 1 min; 72°C, 1 min; 72°C, 10 min.

[a] A *gyrB* primer mix was prepared for the PCR reaction (all seven primers were added to one eppendorf at a final concentration of 25 µM).

Gram negative coccobacilli, *A. baumannii* from a liquid culture were observed microscopically as a pleiomorphic population, comprising short coccobacilli and more elongated cells, resembling bacilli. On MacConkey agar, *A. baumannii* grew as opaque pink colonies and the medium turned orange.

To confirm that cells had reached the required stage of growth for experiments, culture (1 ml) was added to a cuvette and absorbance was measured in a spectrophotometer.

2.3. DNA Extraction, Purification, Quantification and Sequencing

Genomic DNA was extracted from *A. baumannii* AYE using the Wizard® Genomic DNA Purification Kit (Promega Corporation, UK, A1120). Plasmids (Table 2.5) were extracted using the GeneJET™ Plasmid Miniprep kit (Fermentas Life Sciences, UK, K0503). PCR amplimers and pMo130-Tel^{R} were purified using the QIAquick® PCR Purification Kit (QIAGEN, UK, 28104). All kits were used according to the manufacturer's instructions. Elution of purified PCR amplimers was in 30 µl sterile distilled water (SDW) to obtain a high DNA concentration for cloning. Elution of plasmids was in 50 µl SDW, since this was found to provide sufficient plasmid DNA for downstream manipulation. Where lysates were used as the template for PCRs, these were prepared by suspending a colony in SDW (100 µl) and heating the suspension (100°C, 10 min).

Electrophoresis of UP and DOWN fragments was in 1% agarose (100 V, 1 hr). To determine a size difference of 1 kb between plasmids, electrophoresis was in 0.7% agarose (80 V, 2 hr). Electrophoresed DNA was visualised using GeneSnap (Syngene, Synoptics Ltd, UK, product version 6.07) and if required, DNA concentrations were then determined using GeneTools (Syngene, Synoptics Ltd, UK, product version 3.07).

Table 2.5 Plasmids used in this Study.

Plasmid	Description	Antibiotic determinants	Reference
pMo130-Tel^R	Contains multiple cloning site	Tel^R; Amp^R	Kim-Lee Chua (Unpublished)
pMo130-Tel^R/*adeRS*UPDOWN	Constructed to delete *adeRS* in AYE	Tel^R; Amp^R	This study
pMo130-Tel^R/*pmrAB*UPDOWN	Constructed to delete *pmrAB* deletion in AYE	Tel^R; Amp^R	This study
pMo130-Tel^R/*bfmRS*UPDOWN	Constructed to delete *bfmRS* in AYE	Tel^R; Amp^R	This study
pMo130-Tel^R/UP-*adeRS*-DOWN	Constructed to complement AYEΔ*adeRS*	Tel^R; Amp^R	This study
pMo130-Tel^R/UP-*pmrAB*-DOWN	Constructed to complement AYEΔ*pmrAB*	Tel^R; Amp^R	This study

A diagram of pMo130-Tel^R is shown in Figure 2.2.

All DNA sequencing was carried out by the Functional Genomics, Proteomics and Metabolomics Facility, University of Birmingham.

2.4. Inactivation of Histidine Kinase Genes in *A. baumannii* AYE

The method used to inactivate histidine kinase genes in *A. baumannii* involved a double recombination step between chromosomal regions of DNA flanking the gene of interest and those same fragments present on a vector (Figure 2.1). The flanking fragments were amplified by PCR and cloned into the vector pMo130-Tel^{R}. All cloning steps were carried out in *E. coli* DH5α. *E. coli* S17-1 was used to conjugate pMo130-Tel^{R} into *A. baumannii* AYE. S17-1 was used as the donor since it is a mobilizing strain with chromosomally integrated transfer genes from a broad host range Inc-P-type plasmid, enabling the transfer of pMo130-Tel^{R} to *A. baumanii* AYE (Simon *et al.*, 1983). DH5α cannot be used for conjugation, as it does not have genes for vector mobilisation. Single recombinants were those which produced a yellow pigment when sprayed with pyrocatechol. These colonies were passaged in LB broth containing sucrose to induce loss of the vector. Deletion mutants were those passaged cells which remained white after spraying with pyrocatechol. These colonies had undergone a second recombination step and the gene of interest had been incorporated into the vector and removed from *A. baumanii* AYE when the vector was removed.

2.4.1. Construction of pMo130-Tel^{R}/UPDOWN

2.4.1.1. Primer design

Primers (Table 2.6) to amplify fragments (approximately 1kb in length) flanking the target gene were designed in Geneious Pro 4.6.5 (Biomatters_Ltd., 2005-2009) against DNA sequences (2000bp) immediately upstream and downstream of the target gene. All primers

Figure 2.1 Gene Deletion Method.

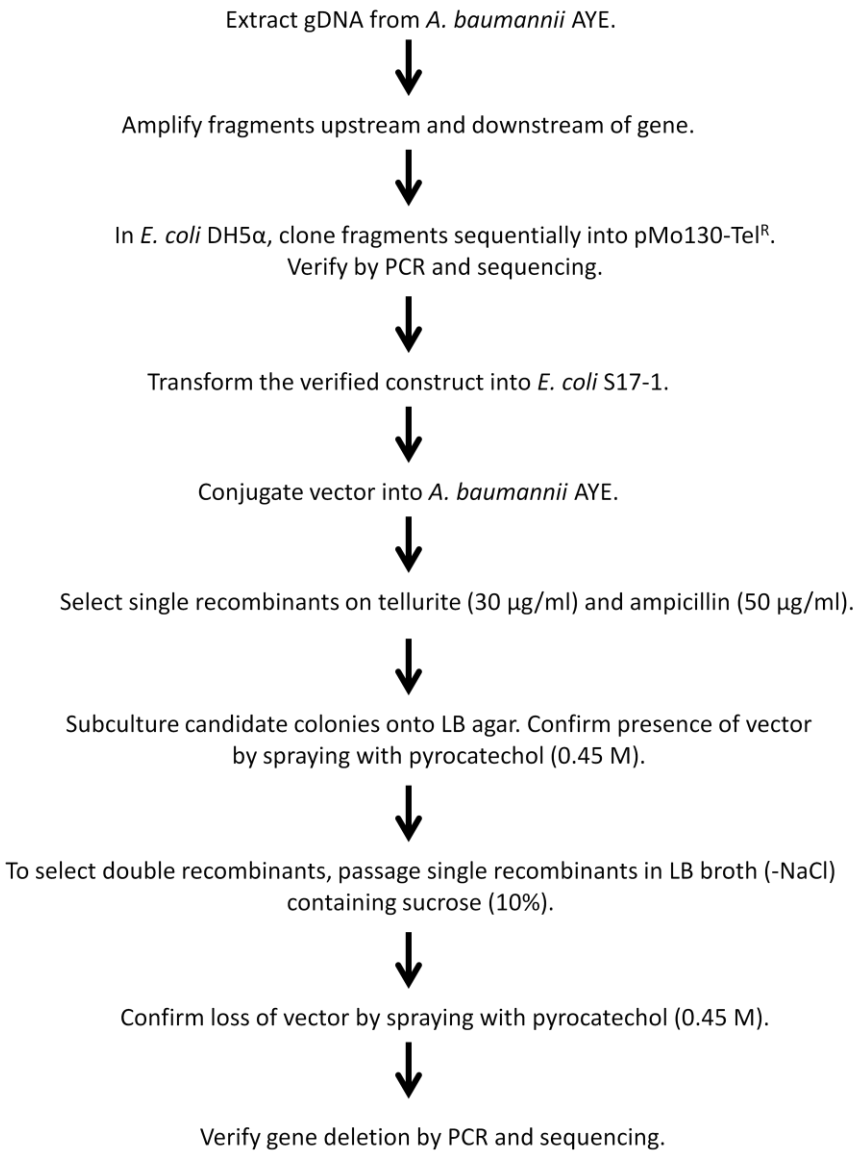


Table 2.6 Primers used in this Study

Description	Sequence
pMo130-Tel^{R}FW Check primer upstream of multiple cloning site in pMo130-Tel^{R}	CCA TCT ACT TCT TCG ACC C
pMo130-Tel^{R}RV Check primer downstream of multiple cloning site in pMo130-Tel^{R}	TCA CAG CTT GTC TGT AAG CG
adeSgeneFW *adeS* internal primer	GTAAGTTAGGAATTAGTAAGC
adeSgeneRV *adeS* internal primer	GCATGTACAACAGCAAGACC
pmrBgeneFW *pmrB* internal primer	TTCGCTTGGGAGCTTGCGGG
pmrBgeneRV *pmrB* internal primer	TCAGGGTCTATTCCTGCACCGC
UPFWadeS (Contains *Not*I recognition site)	GGG GCG GCC GCC CTC CGA CTT GCG GAC GGA T
UPRVadeS (Contains *Bam*HI recognition site)	GGG GGA TCC GGT TCG CTC TAG TGC ATC GC
DOWNFWadeS (Contains *Bam*HI recognition site)	GGG GGA TCC AAG CTG TAA CCG CAG CGC CA
DOWNRVadeS (Contains *Sph*I recognition site)	GGG GCA TGC AGG TGA GCA AGT CGG CCC TT
adeSRVcomp (Contains *Bam*HI recognition site)	GGG GGA TCC AGG TGA GCA AGT CGG CCC TT
UPFWpmrB (Contains *Not*I recognition site)	GGG GCG GCC GCC TTA TGC AAT CGC ACC GAG C
UPRVpmrB (Contains *Bam*HI recognition site)	GGG GGA TCC CAA ACG GTA GCC CAG TCC TC

DOWNFWpmrB (Contains *Bam*HI recognition site)	GGG GGA TCC CCT GCA CTT GCA TGA CCG CC
DOWNRVpmrB (Contains *Sph*I recognition site)	GGG GCA TGC GTG ATT GGT GGT GCA GCG GG
UPFWbfmS (Contains *Not*I recognition site)	GGG GCG GCC GCG AGA TAG CAT ACC AAA GCT G
UPRVbfmS (Contains *Bam*HI recognition site)	GGG GGA TCC ACG TCA ATT GAA CGG TCT TG
DOWNFWbfmS (Contains *Bam*HI recognition site)	GGG GGA TCC AGG TTT GCC GCT TCT CAG GC
DOWNRVbfmS (Contains *Sph*I recognition site)	GGG GCA TGC CGA CTC GGG TCC AGG TTC CC
UP_flankadeS (Used for colony PCR during complementation; Figure 2.3)	CCC TGC TCT AAC TTC ACT ACC
DN_flankadeS (Used for colony PCR during complementation; Figure 2.3)	GAT GTT TAT CCT GCC ACT GTA CG
UPFW_colonyadeS (Used for colony PCR during complementation; Figure 2.3)	CGA CTT GCG GAC GGA TTT C
DNRV_colonyadeS (Used for colony PCR during complementation; Figure 2.3)	GAT ACA AAA GGT GAG CAA GTC G

Recognition sites are highlighted

The position of primers pMo130-Tel^{R}FW and pMo130-Tel^{R}RV on pMo130-Tel^{R} is shown in (Figure 2.2).

were ordered from Invitrogen (Invitrogen Ltd., UK). On arrival, the primers were re-hydrated with Ultrapure water (Invitrogen Ltd., UK, 10977035) to a concentration of 100 µM and these stocks used to prepare working stock solutions (25 µM). All primers were stored at -20°C.

2.4.1.2. Cloning

The upstream fragment (UP fragment) of *adeRS, pmrAB* and *bfmRS* was amplified from *A. baumannii* genomic DNA using primers UPFWadeS and UPRVadeS (*adeRS*), UPFWpmrB and UPRVpmrB (*pmrAB*) and UPFWbfmS and UPRVbfmS (*bfmRS*) (Table 2.7, Reactions 4, 6 and 7). The downstream fragment (DOWN fragment) was amplified using primers DOWNFWadeS and DOWNRVadeS (*adeRS*), DOWNFWpmrB and DOWNRVpmrB (*pmrAB*) and DOWNFWbfmS and DOWNRVbfmS (*bfmRS*) (Table 2.7, Reactions 5, 8 and 9). These primers contain restriction sites for cloning the amplimers into pMo130-Tel^R (Table 2.6).

The identity of pMo130-Tel^R was confirmed by restriction digestion with *Bam*HI (Table 2.8, Digestion 1) and PCR (Table 2.7, Reaction 1) which amplified across the multiple cloning site (Figure 2.2). Digested vector was treated with calf intestinal alkaline phosphatase (CIAP) (Promega Corporation, UK, M1821). CIAP 10x reaction buffer (5µl) and CIAP (1µl; 1µg/µl) were added directly to the double-digested vector (20µl) and the reaction volume made up to 50 µl with Ultrapure water (Invitrogen). Ligation of the digested insert and CIAP-treated vector was carried out using Quick-Stick (QS) ligase (Bioline Ltd., UK, BIO-27027). QS Ligase (1µl) and QS buffer (5µl) were added to vector and insert DNA (vector: insert volume ratios, 1:1, 1:2, 1:3, 1:4 and 1:5) and the reaction volume made up to 20µl with Ultrapure water (Invitrogen). Incubation was at room temperature (30 min).

Table 2.7 PCR Parameters used in this Study.

PCR	Primer Forward	Primer Reverse	PCR Parameters (30 cycles)				
			Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
1[b]	pMo130-Tel^{R}FW	pMo130-Tel^{R}RV	95°C (5 min)	95°C (30 sec)	53°C (30 sec)	72°C (3 min)	72°C (10 min)
2[b]	adeSgeneFW	adeSgeneRV	95°C (5 min)	95°C (30 sec)	53°C (30 sec)	72°C (1 min)	72°C (10 min)
3[b]	pmrBgeneFW	pmrBgeneRV					
4[a]	UPFWadeS	UPRVadeS	95 °C (5 min)	95 °C (30 sec)	58°C (30 sec)	72°C (1 min)	72°C (10 min)
5[a]	DOWNFWadeS	DOWNRVadeS					
6[a]	UPFWpmrB	UPRVpmrB	95 °C (5 min)	95 °C (30 sec)	58°C (30 sec)	72 °C (75 sec)	72°C (10 min)
7[a]	UPFWbfmS	UPRVbfmS					
8[a]	DOWNFWpmrB	DOWNRVpmrB					
9[a]	DOWNFWbfmS	DOWNRVbfmS					
10[b]	pMo130-Tel^{R}FW	UPRVadeS	95°C (5 min)	95°C (30 sec)	53°C (30 sec)	72°C (3 min)	72°C (10 min)
11[b]	pMo130-Tel^{R}FW	UPRVpmrB					
12[b]	pMo130-Tel^{R}FW	UPRVbfmS					
13[b]	pMo130-Tel^{R}FW	DOWNRVadeS					
14[be]	pMo130-Tel^{R}FW	DOWNRVpmrB					

15[b]	pMo130-Tel^{R}FW	DOWNRVbfmS	95°C (5 min)	95°C (30 sec)	53°C (30 sec)	72°C (3 min)	72°C (10 min)
16[c]	pMo130-Tel^{R}FW	adeSRVcomp	94 °C (2min)	94 °C (10 min)	58 °C (30 sec)	68 °C (2 ½ min)	68 °C (7 min)
17[ce]	pMo130-Tel^{R}FW	DOWNRVpmrB					
18[c]	UPFWadeS	DOWNRVadeS	94 °C (2min)	94 °C (10 min)	58 °C (30 sec)	68 °C (2 ½ min)	68 °C (7 min)
19[c]	UPFWadeS	adeSRVcomp					
20[c]	UPFWpmrB	DOWNRVpmrB					
21[c]	UPFWbfmS	DOWNRVbfmS					
22[d]	UP_flankadeS	DNRV_colonyadeS	95°C (5 min)	95°C (30 sec)	55°C (30 sec)	72°C (3 ½ min)	72°C (10 min)
23[d]	UPFW_colonyadeS	DN_flankadeS					

The primers are listed in Table 2.6

[a] 1.1 x Reddymix PCR master mix (90 µl; Thermo Fisher Scientific, PCR-300-610D), forward primer (3 µl), reverse primer (3 µl) and template DNA or Ultrapure water (4 µl).

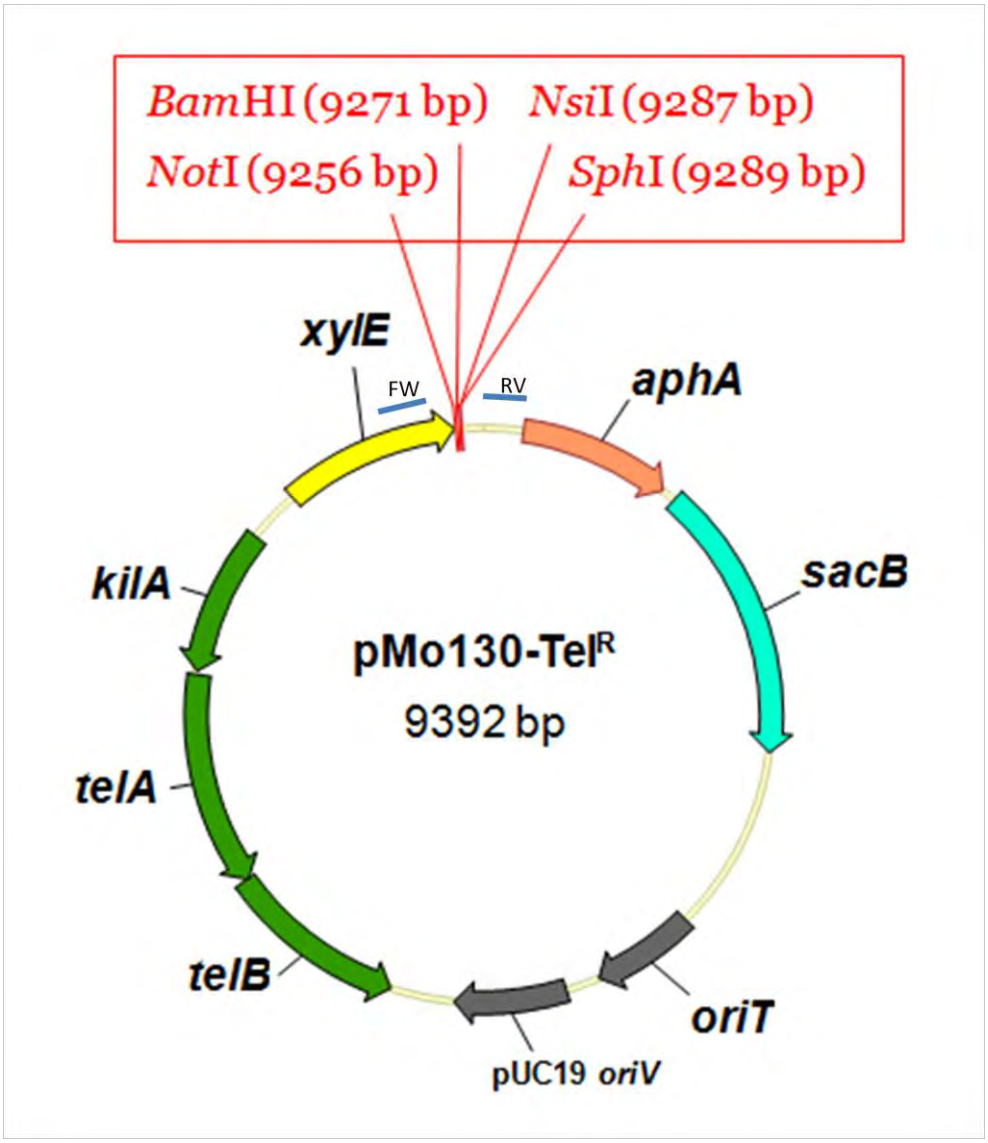
[b] 1.1 x Reddymix PCR master mix (22µl), forward primer (1µl), reverse primer (1µl), template DNA or Ultrapure water (1µl).

[c] 2.1 x Extensor Hi-Fidelity PCR master mix (11µl; Thermo Fisher Scientific, PCR-110-005G), Ultrapure water (11µl; Invitrogen), forward primer (1µl), reverse primer (1µl) and template DNA or Ultrapure water (1µl).

[d] GoTaq master mix (12.5 µl; Promega, M5122), Ultrapure water (8.5 µl), forward primer (10 µM, 1 µl), reverse primer (10 µM, 1 µl) and template DNA (lysate, 2 µl).

[e] The same primers were used for reactions 14 and 17. Reaction 14 was used to verify the deletion construct; reaction 17 was used to verify the complementation construct. Extensor was used for reaction 17 because a larger amplimer was expected, which could not be amplified with Reddymix PCR master mix.

Figure 2.2 Vector used in the Construction of pMo130-TelR/UPDOWN



The multiple cloning site, used for cloning of the UP and DOWN fragments, is highlighted with a red box.
XylE breaks down pyrocatechol to produce a yellow product and so is used to select single and double recombinants (Section 2.4.3). SacB confers sucrose toxicity and is a counter selectable marker used to select double recombinants (Section 2.4.3). *kilAtelAtelB* confers tellurite resistance; *aph* confers kanamycin resistance.
Primers pMo130-Tel^{R}FW (FW) and pMo130-Tel^{R}RV (RV) were used to verify the identity of pMo130-Tel^{R} (Table 2.6; Table 2.7).

The sequence of pMo130-Tel^{R} and the locations of primers pMo130-Tel^{R}FW (FW) and pMo130-Tel^{R}RV (RV) are shown in Appendix 2.

Table 2.8 Restriction Digestions used in this Study.

Digestion	Enzymes	Buffer and activity	Reaction		Incubation	Enzyme inactivation
1	*Bam*HI-HF (R3136)	NEBuffer4 100% activity	DNA *Bam*HI-HF NEBuffer4 SDW	5µl 1µl 2µl 2µl	1hr	Purification [QIAquick® PCR Purification Kit (QIAGEN, UK, 28104)].
2	*Not*I-HF (R3189)	NEBuffer4 100% activity	DNA *Not*I-HF NEBuffer4 BSA SDW	5µl 1µl 2µl 0.2 µl 1.8 µl	1hr	Purification [QIAquick® PCR Purification Kit (QIAGEN, UK, 28104)].
3	*Bam*HI-HF (R3136) *Not*I-HF (R3189)	NEBuffer4 Both enzymes 100% active	DNA *Bam*HI-HF *Not*I-HF NEBuffer4 BSA SDW	5µl 1µl 1µl 4µl 0.4µl 8.6µl	1hr	Purification [QIAquick® PCR Purification Kit (QIAGEN, UK, 28104)].
4	*Bam*HI-HF *Sph*I-HF (R3182)	NEBuffer4 Both enzymes 100% active	DNA *Bam*HI-HF *Sph*I-HF NEBuffer4 BSA SDW	5µl 1µl 1µl 4µl 0.4µl 8.6µl	50 min	Incubation (65°C, 20min) then purification [QIAquick® PCR Purification Kit (QIAGEN, UK, 28104)].
5	*Not*I-HF *Sph*I-HF	NEBuffer4 Both enzymes 100% active	DNA *Bam*HI-HF *Not*I-HF NEBuffer4 BSA SDW	5µl 1µl 1µl 4µl 0.4µl 8.6µl	50 min	Incubation (65°C, 20min).

All enzymes were ordered from New England Biolabs Ltd., UK (product codes in brackets).

2.4.1.3. Verification of Construct

Before verification, the constructs were transformed into electrocompetent *E. coli* DH5α (Bioline Ltd., UK, BIO-85028). DH5α were added to construct (3 µl and 5 µlof each ligation mixture) in a chilled 0.2 cm electroporation cuvette to a total volume of 50 µl. A negative control (no vector) and a positive control (3 µl pMo130-Tel^{R}) were also included. Electrotransformation was carried out at 1.5 kV (200 Ω, 25 µF; Gene Pulser II, Bio-Rad, UK) and cells were immediately recovered with pre-warmed (37°C) LB broth and transferred to a universal tube for incubation (2 hr, 37°C, 180 rpm; Multitron Infors, UK). Positive control cells (100 µl) were plated directly onto LB agar containing kanamycin (50 µg/ml). All other recovered cells were harvested, resuspended in LB broth (100 µl) and plated on LB agar containing kanamycin (50 µg/ml) to increase the selection frequency of transformants. The plates were incubated for up to 2 days (37^{o}C) and candidate pMo130-Tel^{R} transformants were subcultured onto LB agar containing kanamycin (50 µg/ml). Construct was extracted (Section 2.3) from the candidates and the presence of UP and DOWN fragments was checked by PCR using a forward primer specific for pMo130-Tel^{R} (pMo130-Tel^{R}FW) and a reverse primer specific for the insert (Table 2.7, Reactions 10-15).

A second check for the presence of insert DNA was carried out by digesting the construct and unmanipulated pMo130-Tel^{R} with *Bam*HI-HF (Table 2.8, digestion 1) and comparing their sizes after agarose gel electrophoresis.

The chosen candidate construct (pMo130-Tel^{R}/UPDOWN) was verified by DNA sequencing. A PCR amplimer obtained using primer pMo130-Tel^{R}FW and the reverse primer specific for the DOWN fragment (DOWNRV) was purified and sequenced forwards (primer pMo130-Tel^{R}FW) and backwards (primer DOWNRV). The following were mixed for sequencing: template (50

ng), primer (3.2 pmol), ddH_2O to a final volume of 10 µl. The sequence data were aligned with the source sequences.

2.4.2. Transfer of pMo130-Tel^R/UPDOWN into S17-1 by Transformation

LB broth (10 ml) was inoculated with a single colony of S17-1 and incubated overnight (37°C, 200 rpm; Multitron Infors, UK). The following day, fresh LB broth (100 ml) was inoculated with overnight culture (4 ml, 4% inoculum) and incubated (37°C, 200 rpm) until mid-logarithmic phase of growth ($OD_{600nm} \approx 0.6$). The pellet was sequentially washed to make the cells electrocompetent. This was done by resuspending in decreasing volumes of 15% ice cold sterile glycerol (50, 25, 10, 5 ml), harvesting the cells (15 min, 3000 x *g*, 4°C; Heraeus Megafuge 40R, Thermo Fisher Scientific, UK) before each resuspension. The competent cells were resuspended in ice cold glycerol (1 ml) and kept on ice. Transformation of S17-1 and selection and verification of transformants were carried out exactly as described in section 0.

2.4.3. Transfer of pMo130-Tel^R/UPDOWN into AYE by Conjugation and Selection of Deletion Mutants

LB broth (2 ml) containing kanamycin (50 µg/ml) and LB broth containing no antibiotic (2 ml) were inoculated with a colony of S17-1/pMo130-Tel^R/UPDOWN and AYE respectively and incubated overnight (37°C, 180 rpm; Multitron Infors, UK). Donor (0.2 ml) and recipient (0.2 ml) were added to LB broth (0.6 ml) in a micro capped centrifuge tube. The mixture was centrifuged (2 min, 12,066 x *g*, room temperature**;** MiniSpin® microcentrifuge, Scientific Laboratory Supplies, UK) and the supernatant discarded. The cells were washed twice in fresh LB broth (1 ml). The pellet was resuspended in fresh LB broth (30 µl) and placed onto the centre of a 0.45 µm sterile filter disc (Merck Millipore, HNWP02500) on an LB agar plate.

The plate was incubated for 6 hr (37°C). The filter disc was placed in a universal tube and sterile 0.9% NaCl (400 µl) was added. The NaCl solution was prepared by dissolving 3.6 g NaCl (Sigma-Aldrich, UK, S7653) in 500 ml water and passing the solution through a stericup (Millipore Corporation; Pro2439). The universal tube was vortexed to resuspend the bacterial cells and 100 µl aliquots were spread onto LB plates containing tellurite (10 µg/ml) and ampicillin (50 µg/ml). Donor and recipient cultures (100 µl) were also spread onto these selective plates to confirm that growth of each was inhibited by the concentrations of agent used. The plates were incubated (37°C, overnight) and transconjugants selected for subculture the next day.

pMo130-Tel^R contains *xylE,* which encodes catechol 2,3-dioxygenase. This enzyme breaks down pyrocatechol (catechol) to produce a yellow product, 2-hydroxymuconic semialdehyde (Zukowski *et al.*, 1983). Yellow colonies are single recombinants, which have pMo130-Tel^R/UPDOWN incorporated into their chromosome, and are therefore able to break down pyrocatechol. Any organisms which have not undergone recombination, or have undergone double recombination, would not be viable on these selective plates as pMo130-Tel^R is a suicide vector in *A. baumannii* (it is lost if not present on the chromosome). To select single recombinants, colonies were sprayed (100 ml aerosol bottle, Cole Parmer, UK, EW-06081-00) with pyrocatechol (0.45 M; prepared by dissolving 0.99 g in 20 ml SDW) (Sigma-Aldrich, UK, C9510) and the colour of the colonies observed after one minute. Two yellow colonies were cultured overnight (37°C, 180 rpm) in LB broth (2 ml) without NaCl, containing 10% sucrose. The gene product of *sacB* is levansucrase, which breaks down sucrose to produce levan, a toxic product (Pelicic *et al.*, 1996). Therefore, the presence of sucrose selects double recombinants. Serial dilutions (1/100) were prepared and 100 µl of the $1/10^{16}$ and $1/10^{18}$

dilutions spread onto LB plates (-NaCl) containing 10% sucrose. Each time the culture was plated out, a 1/100 dilution of most recent undiluted culture was prepared in fresh LB broth (-NaCl +10% sucrose) and incubated (4 hr, 37°C, 180 rpm). Serial dilutions were prepared, and the cells plated as above ($1/10^{16}$ and $1/10^{18}$ dilutions were chosen as they gave rise to many well isolated colonies after four hours of growth). Cells plated after each passage were sprayed with pyrocatechol and screened for white colonies. As the *xylE* gene is on the vector, white colonies comprised cells which had lost the vector, were unable to break down the pyrocatechol and so did not produce the yellow product. These cells were derived from the single recombinants and previously had the vector incorporated into their chromosome. They had therefore lost the vector through a second recombination event and were candidate deletion mutants.

2.4.4. Other methods used to transfer pMo130-Tel^R Constructs into AYE

2.4.4.1. Transformation

AYE competent cells were prepared exactly as described in section 2.4.2. Electrocompetent cells were added to construct (3 µl and 5 µl) in a chilled 0.2 cm electroporation cuvette to a total volume of 50 µl. A negative control (50 µl cells, no vector) and two positive controls (3 µl and 5 µl unmanipulated pMo130-Tel^R) were also included. Electrotransformation was carried out at 1.6, 1.8 and 2.0 kV (200 Ω, 25 µF; Gene Pulser II, Bio-Rad, UK) and cells were incubated, recovered and resuspended as described in Section 2.4.1.3. The cells were plated on LB agar containing tellurite (30 µg/ml) and incubated for up to two days (37°C). Candidate pMo130-Tel^R transformants were subcultured onto LB agar containing tellurite (30 µg/ml). Tel^R colonies were subcultured onto LB agar and sprayed with pyrocatechol to verify presence of the construct.

2.4.4.2. Patch Conjugation

A single colony of donor and recipient were streaked onto LB agar until growth was just visible (early logarithmic phase, approximately 2 hr). At this time, donor and recipient were mixed on LB agar on approximately 1/10 of an LB plate and incubated overnight (37°C). The aim of the mixing was to obtain a range of ratios of donor and recipient and increase conjugation frequency. The next day, all of the cells were resuspended in LB broth (10 ml), harvested (2200x g, 10 min, Heraeus Megafuge 40R, Thermo Fisher Scientific, UK) and resuspended in a smaller volume (500 µl) of LB broth. Aliquots (100 µl) were spread onto LB plates containing tellurite (30 µg/ml) and ampicillin (50 µg/ml) and incubated overnight (37^{o}C).

2.4.5 Verification of Gene Deletion

The expected sucrose^{R} Tel^{S} phenotype of candidate mutants was verified by subculturing the mutants on LB agar (-NaCl) containing 10% sucrose and on LB agar containing tellurite (30 µg/ml). Deletion of the target gene was verified by PCR (Table 2.7, Reactions 18, 20 and 21) and one verified mutant was selected for DNA sequencing. The amplimer obtained from this mutant was purified and sequenced forwards (forward primer used in the PCR) and backwards (reverse primer used in the PCR). The following were mixed for sequencing: template (50ng), primer (3.2 pmol) and ddH_2O to a final volume of 10 µl. The sequence data were aligned with the source sequences.

2.4. Complementation of deletion mutants

The aim of this method was to introduce the whole of the deleted region back onto the chromosome by double recombination.

A region of the wild-type AYE chromosome comprising the region to be complemented and the UP and DOWN fragments (Section 2.4.1.2; Figure 3.15; Figure 3.31) was amplified by PCR using primers UPFWadeS and adeSRVcomp (Table 2.6; Table 2.7, Reaction 19) to complement AYEΔ*adeRS* and primers UPFWpmrB and DOWNRVpmrB to complement AYEΔ*pmrAB* (Table 2.6; Table 2.7, Reaction 20).

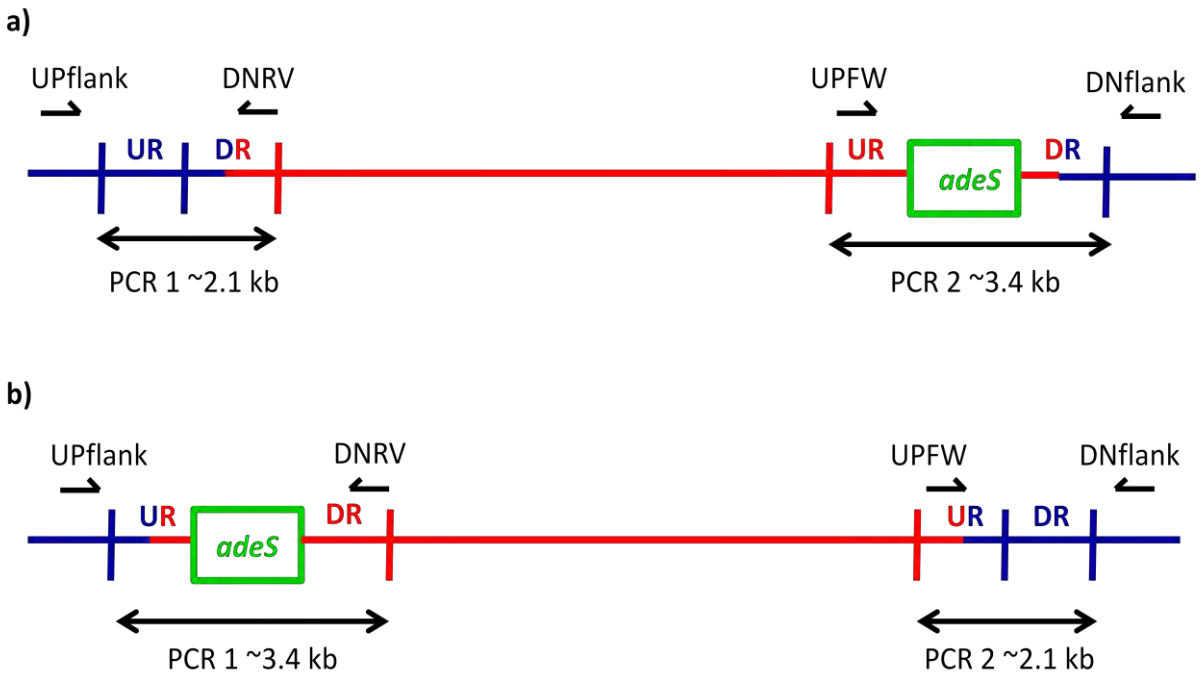
The amplified fragment was cloned into pMo130-Tel^R and the complementation construct was verified as described in sections 2.4.1.2 and 2.4.1.3. Introduction of the construct into S17-1 and AYE were carried out exactly as described in section 2.4.2 and 2.4.3. Presence of the construct in AYE was investigated by colony PCR (Table 2.6; Table 2.7, Reactions 22 and 23; Figure 2.3). Lysate was prepared from candidate single recombinants and this was used as the template DNA in the PCRs.

2.5. Determination of Phenotype

2.5.1. Growth Kinetics in the Absence of Antimicrobials

Overnight cultures of the strain of interest and control strain (approximately 10^9 colony forming units/ml (CFU/ml)) were diluted to approximately 10^4 CFU/ml. This was done by adding 10 µl of the overnight culture to fresh sterile broth (1 ml), and adding 10 µl of this dilution to fresh sterile broth (1 ml). This final suspension (10 µl) was added to fresh broth (90 µl) in a microtitre tray. Fresh broth (100 µl) was added to three of the wells as contamination controls. Growth at 37°C was determined by measuring absorbance at 600

Figure 2.3 Colony PCR to Investigate Complementation of AYEΔ*adeRS*.



The AYE chromosome is shown in blue; the pMo130-Tel^{R} complementation construct containing the UP-*adeRS*-DOWN fragment is shown in red. UPflank, UP_flankadeS; DNRV, DNRV_colonyadeS; UPFW, UPFW_colonyadeS; DNflank, DN_flankadeS (Table 2.6; Table 2.7).

a) and b) are two possible orientations of pMo130-Tel^{R}/UP-*adeRS*-DOWN in the AYE chromosome. AYEΔ*adeRS*/pMo130-Tel^{R}/UP-*adeRS*-DOWN single recombinants are expected to give rise to a 2.1 kb and 3.4 kb amplimer (one for each PCR, depending on the orientation of the construct). Wild-type AYE is expected to give rise to two amplimers of 3.4 kb (one for PCR 1 and one for PCR 2). AYEΔ*adeRS* is expected to give rise to two amplimers of 2.1 kb (one for PCR 1 and one for PCR 2). This colony PCR could therefore be used to investigate whether the complementation construct was present or absent in AYEΔ*adeRS*, and whether complementation (double recombination) had occurred.

nm every 10 min for 16 ½ hr. Growth kinetics were determined for three independent cultures, each tested in triplicate. Student's *t* test was used to compare the generation times and final optical density (600nm) of AYE with AYEΔ*adeRS* and AYEΔ*pmrAB*.

2.5.2 Growth Kinetics in the Presence of Biocides.

An MIC plate was set up (Section 2.5.4.1) and growth at room temperature (surface biocides, Table 2.9) or 37°C (antiseptics, Table 2.9) was determined by measuring absorbance at 600nm every 20 min for 24 hr in a plate reader (FLUOstar Omega, BMG Labtech, UK). Growth kinetics were determined for at least one culture and each culture was tested in duplicate.

2.5.3. Antibiotic Susceptibility

The agar doubling dilution method (BSAC, 2011) was used to determine the MIC of all antibiotics except imipenem and meropenem (Table 2.3). The agents were prepared on the day of use and the appropriate concentration and volume aliquoted into a universal tube to which molten iso-sensitest agar (42°C, 20 ml) (Oxoid, UK) was added using a peristaltic pump dispenser (Jencons Scientific Ltd., UK). The agar and antibiotic were poured into labelled tri-vented petri dishes, mixed gently and allowed to set. LB broth (10 ml) was inoculated with overnight culture (100 µl) to obtain a culture of approximately 10^7 CFU/ml. A 21 pin multipoint inoculator (AQS manufacturing, UK) was used to deliver 1 µl of this culture to the surface of the agar. Two antibiotic-free plates were included as positive growth controls; one was inoculated before all antibiotic plates and the other inoculated after all antibiotic plates. The MICs were determined after incubation for 18 hours. The MIC of an antibiotic was the

Table 2.9 Biocides used in this Study.

Biocide	Agent	Concentration/solvent	Supplier
Surface	Ethanol	100%	Hayman, UK (F200238)
	H_2O_2	3%	Sigma-Aldrich, UK (88597)
	H_2O_2	50%	Sigma-Aldrich, UK (516813)
	Peracetic acid	36-40% (W/V) in acetic acid	Sigma-Aldrich, UK (433241)
	Spor-Klenz	100% W.C.	Steris, UK (6525)
	T.B.Q	129 x W.C.	Steris, UK (634505)
Antiseptic	Chlorhexidine digluconate	20% (V/V) in H_2O	Sigma-Aldrich, UK (C9394)
	Benzalkonium chloride	50% (V/V) in H_2O	Sigma-Aldrich, UK (63249)
	Cetylpyridinium chloride	Solvent: SDW	Sigma-Aldrich, UK (C9002)

H_2O_2, Hydrogen peroxide (3% used unless a higher concentration was required); Cetylpyridinium chloride, Hexadecylpyridinium chloride monohydrate (synonym); W.C., working concentration.

concentration at which there was no growth compared to the controls (a haze of growth or one or two colonies were ignored). MICs were determined in triplicate on at least three separate occasions. The MICs of imipenem and meropenem were determined by E-test, according to the manufacturer's instructions. This was carried out with three independent biological cultures.

2.5.4. Biocide Susceptibility

2.5.4.1. Determination of MICs and MBCs of Biocides for Planktonic Cells

Biocide was diluted in TSB to obtain double the desired starting concentration. Cetylpyridinium chloride (powder) was first dissolved in SDW to obtain a stock solution (10,000 µg/ml) before dilution. An overnight culture of each strain was corrected to $OD_{600} \approx$ 0.02 and 100 µl was added to 100 µl of increasing concentrations of biocide in a 96 well plate. Fresh TSB was used as a blank control for each concentration of biocide.

To replicate the conditions in which each biocide is used, the plates containing the surface biocides (Table 2.9) were incubated at room temperature (24 hr, static) and those containing the antiseptics (Table 2.9) were incubated at 37°C (24 hr, static). After incubation, the OD_{600} was determined in a plate reader (FLUOstar Omega, BMG Labtech, UK) and compared with the OD_{600} value for the corresponding blank well. The MIC was the lowest concentration for which there was no growth of bacteria.

To determine the minimum bactericidal concentration (MBC), a loopful (≈100 µl) of the culture at the MIC level and the three next highest concentrations was streaked onto a TSA plate and incubated overnight (37°C). The MBC was determined as the lowest concentration for which there was no visible growth.

2.5.4.2. Determination of the Effect of Biocides on Biofilm Growth

This method is as essentially described in Wand *et al*. (2012). The biocide concentrations of interest, containing each test strain, were prepared as described previously (Section 2.5.4.1), except that a microtitre plate (Thermo Fisher Scientific, UK, DIS-976-220U), whose lid (Thermo Fisher Scientific, UK, DIS-972-050U) had pegs for biofilm formation was used. TSB was used as a blank control for biofilm staining. After incubation (24 hr, static, room temperature), the medium was removed and the wells washed twice with phosphate buffered saline (PBS). The plate (including the lid) was dried (1 hr, 80°C, static) in a heating block (ThermoStat plus, Eppendorf, UK). To stain the biofilm, crystal violet (0.2% V/V, 200 µl; Sigma Aldrich, UK, 94448) was added to each well and the plate (including the lid) was left for 30 min (room temperature, static). The biofilm was washed by immersing the plate and lid separately in a container of cold water until the water ran clear. The biofilm was dried and then destained by adding ethanol (100%) to the wells and replacing the lid (incubation for 45 min, static, room temperature). Biofilm formation was quantified by measuring the absorbance of the crystal violet staining at OD_{570} in a plate reader (FLUOstar Omega, BMG Labtech, UK). The MIC was the lowest concentration at which there was no growth of biofilm. The MIC was determined for three independent cultures, each tested four times.

2.5.4.3. Determination of MBCs of Biocides for Cells in Biofilm (Biofilm Protection Assay).

A biofilm protection assay was used to measure the ability of established biofilms to tolerate biocides.

The susceptibility of biofilms to biocides was determined as described previously (minimum biofilm eradication concentration in Olson *et al.*, 2002). An overnight culture of each test

organism was corrected to OD_{600} ≈ 0.01 and 200 µl added directly to four wells of a microtitre plate with a pegged lid. TSB was added to four wells as a blank control. The plate was incubated (24 hr, static, room temperature) and the lid transferred to a fresh 96 well plate containing appropriate concentrations of biocides. The plate was incubated (24 hr static, room temperature) and the pegs were placed in a fresh plate containing TSB with no biocide. The biofilms were disrupted from the pegs using a Titramax plate shaker (Heidolph, UK, 544-12200-00) for 10 min (1,000 rpm, room temperature) and the plate was then incubated (24 hr, static, room temperature). Viability of the biofilm in the presence of biocide was quantified by measuring the absorbance of each well at OD_{600}. The minimum biofim eradication concentration (MBEC) was the lowest concentration at which there was no growth (equivalent to the MBC, Section 2.5.4.1). The MBEC was determined for two independent cultures, each tested four times.

2.5.5. Bis-benzimide (Hoechst 33342) assay

Fresh pre-warmed LB broth (3 ml, 37°C) was inoculated with overnight culture (120 µl; 4% inoculm) in a universal tube and incubated (180 rpm, 37°C) until mid-logarithmic phase of growth (OD_{600} ~ 0.6; approximately 1 ½ hr). The cells were harvested by centrifugation (2200 x *g*, 10 min, room temperature; Heraeus Megafuge 40R, Thermo Fisher Scientific, UK) and the supernatant discarded. The cell pellet was resuspended in ice-cold PBS (3 ml, 0.1 M, pH 7.2, room temperature; Sigma-Aldrich, UK, P5119) and the absorbance measured at 600nm. The OD_{600} was adjusted to 0.4-0.5 using PBS. As a control and to measure the accumulation of bis-benzimide (Hoechst H33342) in the absence of efflux, wild-type AYE cells (overnight culture) were boiled (10 min) to kill the cells and 200 µl samples added to a black, flat bottomed, 96 well microtitre plate (Greiner, UK, 655076). PBS alone (200 µl) was also

included as a blank control for the wells containing cells suspended in PBS. Hoechst (20 µl, 25 µM) was added to wells containing the test and control strains (180 µl) to a final concentration of 2.5 µl. The test and control strains (200 µl) without Hoechst added were included as negative fluorescence controls. The fluorescence (excitation 350 nm, emission 460 nm) was measured in a fluorescent plate reader (FLUOstar Optima, BMG LabTech, Germany) for 110 min (37°C). The assay was carried out in triplicate on three separate occasions. Steady state fluorescence values were compared by Student's *t* test (two-tailed).

2.5.6. Determination of Cell Dry Weight

To investigate the amount of fluoroquinolone taken up per milligram of bacterial cells, the mass of AYE cells per millilitre of culture was determined for a range of optical densities. Sterilin universal tubes were placed in a desiccator and weighed daily until a stable mass was recorded. Isosensitest broth (15 ml) was inoculated with a single colony of *A. baumannii* AYE and incubated (overnight, 37°C, 180 rpm). Fresh pre-warmed isosensitest broth (300 ml, 37°C) was inoculated with overnight culture (12 ml) and incubated (37°C, 180 rpm; Multitron Infors, UK). Absorbance of the culture at 660 nm was monitored and samples (20 ml) were extracted at an OD_{660} of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.65, 0.7, 0.75, 0.8, 0.9 and 1.0. Each sample was placed in a labelled, pre-weighed universal tube and the cells were harvested immediately (2200 x *g*, 10 min; Heraeus Megafuge 40R). The supernatant was removed and the pellet was dried (60°C, overnight). The universal tubes were weighed and placed in a desiccator until a stable mass was recorded. The cell weight (mg/ml) was calculated and plotted against OD_{660}.

2.5.7. Fluoroquinolone Accumulation

Fluoroquinolone accumulation assays were carried out as described in Mortimer and Piddock (1991). Isosensitest broth (12 ml) was inoculated with a single colony of *A. baumannii* and incubated overnight (37°C, 180 rpm; Multitron Infors, UK). Fresh, pre-warmed isosensitest broth (250 ml, 37°C) was inoculated with overnight culture (10 ml) and incubated (37°C, 180 rpm; Multitron Infors, UK) until mid-logarithmic phase of growth (OD_{660} ~ 0.55). The exact OD_{660} was recorded and the cells were harvested immediately (4500 x *g,* 20 min, 4°C; Heraeus Megafuge 40R). To wash the cells, the cell pellets were resuspended in ice-cold sodium phosphate buffer (10 ml, 50 mM, pH 7.0; Appendix 1). The cells were harvested (4500 x *g*, 20 min, 4°C; Heraeus Megafuge 40R) and resuspended in ice-cold sodium phosphate buffer (50 mM, pH 7.0) to a final OD_{660} of 20 units. The cell suspensions were divided into two sterile McCartney tubes, each containing a magnetic flea. The cells were left to equilibriate to 37°C in a water bath on a magnetic stirrer (10 min). At time 0 min, a sample (500 µl) was taken from each McCartney tube and added to a micro capped centrifuge tube (on ice) containing ice-cold sterile sodium phosphate buffer (1 ml). Ciprofloxacin (Table 2.3; final concentration 10 µg/ml) was added to the remaining cells in McCartney tube 1 and ciprofloxacin plus efflux inhibitor (CCCP or PAβN; Table 2.10) were added to McCartney tube 2. Five minutes after addition of antibiotic ± inhibitor, duplicate samples (500 µl) were removed to micro capped centrifuge tubes (on ice) containing ice-cold sterile sodium phosphate buffer (1 ml). Immediately after removal of the final sample, the samples were centrifuged (8915 x *g*, 5 min, 4°C; MiniSpin® microcentrifuge) and returned to ice. The supernatants were discarded and the cells resuspended in sterile sodium phosphate buffer (1 ml). The cells were centrifuged (8915 x *g*, 5 min, 4°C; MiniSpin® microcentrifuge) to

remove any extracellular ciprofloxacin and lysed by adding 0.1 M glycine hydrochloride (pH 7.0; Appendix 1) and incubation overnight at room temperature, in the absence of light. The samples were centrifuged (8915 x *g*, 5 min, room temperature; MiniSpin® microcentrifuge) to remove cell debris and the supernatants transferred to fresh micro capped centrifuge tubes and centrifuged as before. The samples were diluted (1/100) with 0.1 M glycine hydrochloride (the level of ciprofloxacin in the undiluted supernatant exceeded the measurable range) and fluorescence (excitation 279 nm, emission 447 nm) was measured in a calibrated fluorescence spectrophotometer (LS 45 Fluorescence Spectrometer (230 V), Perkin Elmer, UK, L2250106). The concentration of ciprofloxacin (ng/mg dry cells) accumulated by the cell was calculated (Appendix 3).

Accumulation of norfloxacin (Table 2.3; final concentration 10 µg/ml) in the presence and absence of PAβN, CCCP and verapamil (Table 2.10) was determined exactly as for ciprofloxacin, except that fluorescence was measured at excitation wavelength 281 nm and emission wavelength 440 nm.

Experiments were carried out in duplicate for three independent biological cultures. A Student's *t* test was used to compare the level of accumulation of each fluoroquinolone at five minutes for each strain in the presence of efflux inhibitor with that same strain in the absence of the efflux inhibitor. The accumulation of norfloxacin at five minutes for the mutants in the absence of inhibitor was also compared with the accumulation for AYE in the absence of inhibitor.

Table 2.10 Efflux Inhibitors used in Fluoroquinolone Uptake Assays.

Efflux inhibitor	Supplier	Solvent	Final concentration in assay	Target
PAβN	Sigma-Aldrich, UK (P4157)	Sterile distilled water	50 µg/ml	RND efflux pumps (Yu et al., 2005)
CCCP	Sigma-Aldrich, UK (C2759)	70% DMSO	50 µM	All efflux pumps except ABC efflux pumps (Piddock, 2006)
Verapamil	Sigma-Aldrich, UK (V-4629)	Sterile distilled water	50 µg/ml	ABC efflux pumps (Zechini, 2009)

PAβN, phenylalanine-arginine beta-naphthylamide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DMSO, dimethyl sulphoxide; RND, resistance nodulation division; ABC, ATP-binding cassette.

2.5.8. Growth of *A. baumannii* in the Presence of Efflux Inhibitors

To ensure that the concentration of efflux inhibitor used in the accumulation assay did not have an effect on bacterial growth, viable counts of each strain were determined under the conditions of the accumulation assay. During a norfloxacin accumulation assay, additional samples (500 µl) were taken from the cultures at time 0 min, and time 5 min both in the presence and absence of efflux inhibitor. The samples were harvested and washed exactly as discussed in section 2.5.7. At the final step of the protocol, instead of resuspending in glycine hydrochloride, which lyses the cells, the cells were resuspended in sodium phosphate buffer. Each of the samples (20 µl) was added to isosensitest broth (180 µl) in a 96-well microtitre tray and serially diluted ten-fold each time to obtain a final dilution of $1/10^8$. Samples (20 µl) of the final three dilutions ($1/10^6$ to $1/10^8$) were inoculated in triplicate onto the surface of LB plates and the number of colonies counted after incubation (37^oC) for 16 hr. The number of colonies obtained after dilution were converted to CFU/ml, assuming that each colony was derived from a single bacterial cell. The experiment was carried out on three separate occasions. Student's *t* test was used to compare the growth of the cells in the absence of norfloxacin and efflux inhibitor with the growth of each of samples taken at time 5 min.

2.5.9. Virulence Experiments

As part of the collaborative research programme, virulence of all *A. baumannii* strains was investigated in *Galleria mellonella* by Matthew Wand, (HPA, Porton Down). *G. mellonella* were infected with 10^6 bacterial cells and the percentage survival of *Galleria* was recorded every 12 hours for five days. Experiments were carried out on three separate occasions, using ten larvae each time. At Birmingham, Graphpad Prism 5 (GraphPad_Prism) was used to generate a survival curve and perform a Log-rank test on the data.

3. Construction of Histidine Kinase Deletion Mutants

3.1. Background

adeS, *adeR* (Marchand *et al.*, 2004; Wong *et al.*, 2009), *pmrB* (Arroyo *et al.*, 2011; Beceiro *et al.*, 2011) and *pmrA* (Arroyo et al., 2011) have previously been inactivated in *A. baumannii* by inserting an antibiotic resistance cassette into the gene. *bfmS* has been inactivated by transposon mutagenesis (Clemmer *et al.*, 2011; Tomaras *et al.*, 2008), but there are no reports of inactivation or deletion of this histidine kinase. To date, genes have never been deleted without relying on susceptibility to an antibiotic. This is not desirable because the *A. baumannii* isolates, which cause infections in hospitals are often resistant to all antibiotics used for genetic manipulation. Therefore, representative strains of *A. baumannii* can often not be investigated, using the previously reported methods.

3.2. Aims and Hypotheses

It was hypothesised that the role of TCSs in MDR could be investigated in a MDR strain of *A. baumannii* using a method, which used tellurite to overcome the problem of antibiotic resistance. The initial aims were to identify and delete histidine kinase genes in a representative (MDR) strain of *A. baumannii*.

3.2.1. TCSs in *Acinetobacter*

Acibase was used to compile a list of TCSs in *A. baumannii.* The whole gene database was searched (search term, two-component) and an initial list made. The gene database was then filtered by "*Acinetobacter* core genome" and the list was manually searched to identify

additional TCS genes. These results were compared with those published by Adams *et al.* (2008; 2009) and all results were combined (Table 3.1). PubMed was searched to identify the predicted functions of the gene products and any potential role in MDR. Where information specific to *Acinetobacter* was not available, these predictions were based on reports in other bacteria. AdeRS, PmrAB and BfmRS were predicted to be important for antimicrobial resistance in *A. baumannii* and were selected for investigation (Table 3.1).

3.2.2. Choice of Strain

One consideration when choosing the strain in which to delete *adeS* was that genome data were available. The strains selected from xBASE for alignment of *adeS* and its flanking sequences were: AYE, AB0057, AB307-0294, ATCC 17978 and ACICU, since genome sequencing of these strains was complete. Strains whose genomes were not fully sequenced such as ATCC 19606 were ignored. Although the *A. baumannii* SDF genome has been fully sequenced, it was not included in the comparison with other strains. This is because the status of SDF as a human pathogen is undetermined and its genome is smaller than that of other *A. baumannii* (Adams *et al.*, 2008). This suggests that SDF is not representative of the *A. baumannii* species.

When choosing a representative strain of *A. baumannii*, the level of conservation of *adeS* and its surrounding genes between five different strains of *A. baumannii* were aligned in xBASE (Figure 3.1). BM4454 was included in any sequence analysis where possible, since *adeS* has already been insertionally inactivated in this strain. The sequences of *adeS* and AdeS in all six strains (including BM4454) were aligned to compare the level of identity and similarity between the gene and protein, respectively in different strains (Figure 3.2; Figure 3.3).

Table 3.1 Putative Two Component System Proteins in *Acinetobacter*.

Protein	Component	Predicted function	Predicted involvement in antibiotic or biocide resistance
QseB	RR	In *E. coli*, affects motility and virulence in response to quorum sensing and hormonal signals (Merighi *et al.*, 2009). Transcriptional regulation of *flhDC* (master regulator operon for flagella and motility genes) in *E. coli*	*qseDC E. coli* mutants were hypersensitive to toxic cations-caesium, cobalt, copper, nickel, ruthenium. This TCS may be involved in metal metabolism (Zhou *et al.*, 2003)
QseC	HK		
NtrB (NR(II))	HK	Nitrogen regulation (Li and Lu, 2007)	*ntrBC E. coli* mutants were hypersensitive to several aminoglycosides (Zhou *et al.*, 2003)
NtrC (NR(I))	RR		
RstB	HK	RstA promotes RpoS degradation and involved in biofilm formation in Salmonella. RstB acts on PhoQ to control expression of PhoP-regulated genes (Nam *et al.*, 2010; Cabeza *et al.*, 2007)	*rstAB E. coli* mutants were hypersensitive to ketoprofen, prinidol, troleandomycin. ((Zhou *et al.*, 2003))
RstA			
BfmS	HK	Controls biofilm formation and cellular morphology (Tomaras *et al.* 2008)	None found, but biofilms known to increase resistance to antibiotics and biocides (Lewis, 2001; Rajamohan *et al.*, 2009)
BfmR	RR		
PhoB	RR	PhoBR responds to environmental phosphate levels	None found for PhoBR but PhoPR is a TCS

PhoR	HK	(Baek *et al.*, 2007). Identified in *A. calcoaceticus* strain ADP1 (Elbahloul and Steinbuchel, 2006)	in M*ycobacterium tuberculosis. A phoP* mutant was more sensitive than the wild-type parent to cumene hydrogen peroxide (an organic peroxide), $CdCl_2$ (a superoxide generator and toxic heavy metal), and to the antibiotics vancomycin, and cloxacillin (Walters *et al.*, 2006)
EnvZ	HK	OmpR-EnvZ controls expression of genes such as *ompF* and *ompC* in *E. coli* in response to osmolarity (Zhou *et al.*, 2003)	*ompR-envZ E. coli* mutants showed greater resistance to several antibiotics (including cephalosporins and β-lactams), which may have been due to defects in porin synthesis. The mutants were hypersensitive to sodium dichromate and cobalt chloride (Zhou *et al.*, 2003)
OmpR	RR		
AgmR	RR	Alcohol metabolism. Glycerol metabolism activator in *P. putida* (Vrionis *et al.*, 2002)	None found
spoIIE family	RR	Sporulation protein. Activates σ^F in *Bacillus subtilis* sporulation. Promotes polar division of cells (Carniol *et al.*, 2005). *Acinetobacter* do not form spores.	None found
BvgS precursor	HK	Virulence sensor protein. Implicated in biofilm formation in *Bordetella* (Mishra *et al.*, 2005).	*Bordetella* in biofilm were more resistant to antibiotics than planktonic cells (Mishra *et al.*, 2005)

KdpD	HK	Osmotic sensor	*kdpDE E. coli* mutants were more resistant to novobiocin (targets topoisomerase), but more sensitive to hygromycinB (aminoglycoside) (Zhou *et al.*, 2003)
KdpE	RR	Transcriptional regulatory protein. (Kdp expressed in response to osmotic upshift) (Ballal *et al.*, 2007)	
WspR	RR	In *Pseudomonas spp.* involved in generation of c-di-GMP which stimulates biofilm formation and suppresses motility (Guvener and Harwood, 2007)	Biofilm formation is generally associated with increased antibiotic resistance (Fux *et al.*, 2005)
AdeR	RR	Transcription factor of *adeABC* operon	AdeABC has broad specificity (β-lactams, fluoroquinolones, tetracyclines, chloramphenicol and aminoglycosides etc). Best substrates: netilimicin and gentamicin (Magnet *et al.*, 2001; Nemec *et al.*, 2007a)
AdeS	HK	Stimulus unknown. Activates AdeR (Marchand *et al.*, 2004)	
PmrB	HK	*E. coli*: responds to polymyxins, pH, Fe^{3+} and Mg^{2+} concentrations (Moffatt *et al.*, 2010)	Colistin resistance (Adams *et al.*, 2009)
PmrA	RR		
CusS	HK	In *E. coli*, CusSR stimulates gene transcription in response to copper ions (Munson *et al.*, 2000)	Heavy metal resistance-target genes likely to be involved in copper resistance (Adams *et al.* 2008)
CusR	RR		
IrlR	RR	Unknown	None found
PetR	RR	Unknown	None found

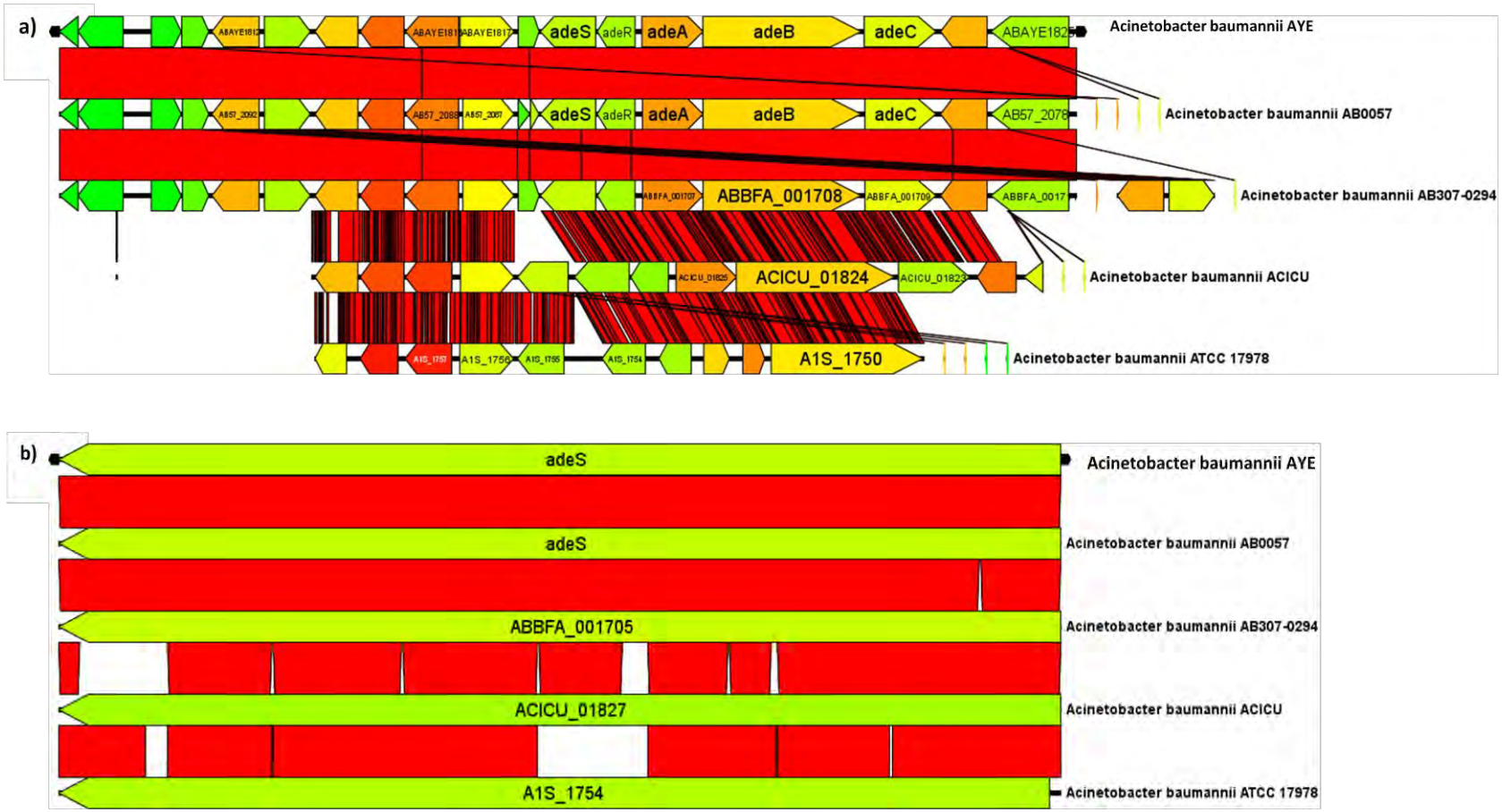
RsbU	RR	Serine phosphatase, responds to environmental stress in *B. subtilis* (Holtmann *et al.*, 2004)	Restoring *rsbU* in *rsbU* defective *S. aureus* enhanced teicoplanin resistance (Galbusera *et al.*, 2011)
BaeS	HK	In *E.* coli, involved in envelope stress response. Detected signals of envelope stress include indole, spheroblast formation and misfolded PapG (Raffa and Raivio, 2002)	BaeSR activates transcription of a MDR efflux pump gene cluster (*mdtABCD*) in *E. coli. baeR* overexpression has been implicated in resistance to novobiocin and deoxycholate (Baranova and Nikaido, 2002)
GGDEF domain-containing protein	RR	Synthesis of Bis-(3'-5')-cyclic di-GMP (c-di-GMP), a second messenger in bacteria. Involved in processes such as commitment to biofilm formation and surface attachment (from a motile state) (Zahringer *et al.*, 2011)	Unknown
Osmolarity RR	RR	Responds to changes in osmolarity	Unknown
Heavy metal sensor	HK	Responds to presence of heavy metals	Unknown
NarL family	RR	Responds to nitrate or nitrite levels in *E. coli* (Partridge *et al.*, 2008)	Unknown
LuxR family	RR	Involved in survival, replication, virulence and biofilm formation in Gram negative bacteria (Chen	Unknown

		and Xie, 2011)	
Fis family	RR	Unknown (But Fis regulates DNA transcription) (Browning *et al.*, 2010)	Unknown
ColR-like	RR	ColRS is involved in phenol tolerance in *Pseudomonas putida* (Putrins *et al.*, 2010)	ColR deficient mutant is sensitive to phenol (Putrins *et al.*, 2010)
ColS-like	HK		
PilL	Hybrid HK/RR	Encodes an outer membrane lipoprotein involved in thin pilus synthesis (Sakai and Komano, 2002)	None found
PilH	RR	Part of putative chemosensory system (Chp system encoded by*pilGHIJK-chpABC* gene cluster) PilG is involved in pilus extension and PilH is involved in pilus retraction (Bertrand *et al.*, 2010)	None found
PilG	RR		None found
PilR	RR	In *P. aeruginosa,* PilR induces the transcription of type IV pilus synthesis genes, involved in adhesion to host cells and twitching motility (Bertrand *et al.*, 2010). Postulated to respond to levels of pilin (Jelsbak and Kaiser, 2005)	None found

Initially found in Acibase; Search by core genome in Acibase; (Adams *et al.*, 2008); (Adams *et al.*, 2009)

c-di-GMP, cyclic diguanylate; MDR, Multi-drug Resistance

Figure 3.1 Alignment of *adeRS* and its surrounding genes in xBASE.



a) Alignment of *adeS* and its surrounding genes; b) Alignment of *adeS*.
Red regions (outlined by black lines) are those, which are conserved between immediately adjacent strains. White regions are those, which do not align and are therefore not present in two immediately adjacent strains.

Figure 3.2 Alignment of *adeS*.

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AYE             ATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTATTGTGAATTTA 60
AB0057          ATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTATTGTGAATTTA 60
AB307-0294      ATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTATTGTGAATTTA 60
ATCC            ------------------------------------------------------------
ACICU           ATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTATTGTAAATTTA 60
BM4454          ATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTATTGTAAATTTA 60


AYE             AGCGTTACGCTATTTTCTATAGTATTGGGTTATATCATTTATAACTATGCGATTGAAAAA 120
AB0057          AGCGTTACGCTATTTTCTATAGTATTGGGTTATATCATTTATAACTATGCGATTGAAAAA 120
AB307-0294      AGCGTTACGCTATTTTCTATAGTATTGGGTTATATCATTTATAACTATGCGATTGAAAAA 120
ATCC            ------------------------------------------------------------
ACICU           AGCGTTACGCTATTTTCTGTAGTACTGGGTTATGTCATTTATAACTATGCGATTGAAAAA 120
BM4454          AGCGTTACGCTATTTTCTGTAGTACTGGGTTATGTCATTTATAACTATGCGATTGAAAAA 120


AYE             GGCTGGATTAGCTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCATTTTGTAGAC 180
AB0057          GGCTGGATTAGCTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCATTTTGTAGAC 180
AB307-0294      GGCTGGATTAGCTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCATTTTGTAGAC 180
ATCC            ------------------------------------------------------------
ACICU           GGCTGGATTAGTTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCATTTTGTAGAC 180
BM4454          GGCTGGATTAGTTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCATTTTGTAGAC 180


AYE             TGGATCTGGTTAGCCACTGTTATCTTCTGTGGCTGTATTATTTCATTAGTGATTGGCATG 240
AB0057          TGGATCTGGTTAGCCACTGTTATCTTCTGTGGCTGTATTATTTCATTAGTGATTGGCATG 240
AB307-0294      TGGATCTGGTTAGCCACTGTTATCTTCTGTGGCTGTATTATTTCATTAGTGATTGGCATG 240
ATCC            ------------------------------------------------------------ATG 3
ACICU           TGGATTTGGTTAGCCACTGTTATCTTTTGCGGCTGTATTATTTCATTAGTGATTGGCATG 240
BM4454          TGGATTTGGTTAGCCACTGTTATCTTTTGCGGCTGTATTATTTCATTAGTGATTGGCATG 240
                                                                        ***

AYE             CGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGCAAAAAAAATT 300
AB0057          CGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGCAAAAAAAATT 300
AB307-0294      CGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGCCGAAGCAGCAAAAAAAATT 300
ATCC            CGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGCCGAAGCAGCAAAAAAAATT 63
ACICU           CGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGCCGAAGCAGCAAAAAAAATT 300
BM4454          CGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGCCGAAGCAGCAAAAAAAATT 300
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AYE             AGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCG 360
AB0057          AGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCG 360
AB307-0294      AGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCG 360
ATCC            AGTCACGGCGACCTCTCTGCTAGAGCTTACGATAACCGAATTCACTCCGCCGAAATGTCG 123
ACICU           AGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCG 360
BM4454          AGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCG 360
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AYE             GAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGTCAAAAATGCG 420
AB0057          GAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGTCAAAAATGCG 420
AB307-0294      GAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGTCAAAAATGCG 420
ATCC            GAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGTTAAAAATGCG 183
ACICU           GAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCTGTTAAAAATGCG 420
BM4454          GAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCTGTTAAAAATGCG 420
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AYE             CAGGTTTGGAATGCAGCTATCGCACATGAGTTAAGAACGCCTATAACGATATTACAAGGT 480
AB0057          CAGGTTTGGAATGCAGCTATCGCACATGAGTTAAGAACGCCTATAACGATATTACAAGGT 480
AB307-0294      CAGGTTTGGAATGCAGCTATCGCACATGAGTTAAGAACGCCTATAACGATATTACAAGGT 480
ATCC            CAGGTTTGGAATGCAGCCATCGCACATGAGTTAAGAACGCCTATAACGATATTACAAGGT 243
ACICU           CAGGTTTGGAATGCAGCCATCGCACATGAGTTAAGAACGCCTATAACGATATTACAAGGT 480
BM4454          CAGGTTTGGAATGCAGCCATCGCACATGAGTTAAGAACGCCTATAACGATATTACAAGGT 480
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AYE             CGTTTACAGGGAATTATTGATGGCGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTT 540
AB0057          CGTTTACAGGGAATTATTGATGGCGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTT 540
AB307-0294      CGTTTACAGGGAATTATTGATGGCGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTT 540
ATCC            CGTTTACAGGGAATTATTGATGGCGTTTTTAAACTTGATGAAGTTCTATTTAAAAGTCTT 303
ACICU           CGTTTACAAGGCATCATCGACGGTGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTT 540
BM4454          CGTTTACAAGGCATCATCGACGGTGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTT 540
                ******** ** ** ** ** ** ********** ********* *********** ***

AYE             TTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTA 600
AB0057          TTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTA 600
AB307-0294      TTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTA 600
ATCC            TTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTA 363
ACICU           TTAAATCAAGTTGAAGTTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTA 600
BM4454          TTAAATCAAGTTGAAGTTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTA 600
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AYE             GAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGTAGTTGAAAAA 660
AB0057          GAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGTAGTTGAAAAA 660
AB307-0294      GAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGTAGTTGAAAAA 660
ATCC            GAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTTAAGGCGGTAGTTGAAAAA 423
ACICU           GAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTTAAGGCGGTAGTTGAAAAA 660
BM4454          GAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTTAAGGCGGTAGTTGAAAAA 660
                ************************************** ******************

AYE             GTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTA 720
AB0057          GTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTA 720
AB307-0294      GTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTA 720
ATCC            GTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTA 483
ACICU           GTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTA 720
BM4454          GTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTA 720
                ************************************************************

AYE             ACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATT 780
AB0057          ACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATT 780
AB307-0294      ACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATT 780
ATCC            ACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATT 543
ACICU           ACTTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATT 780
BM4454          ACTTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATT 780
                ** ********************************************************

AYE             GATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGAAGTGGTTGCA 840
AB0057          GATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGAAGTGGTTGCA 840
AB307-0294      GATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGAAGTGGTTGCA 840
ATCC            GATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAATTTCCTCGGAAGTGGTATCA 603
ACICU           GATAATGCTATACGCTATTCACATGCAGGCAAACTTAAAATTTCCTCGGAAGTGGTATCA 840
BM4454          GATAATGCTATACGCTATTCACATGCAGGCAAACTTAAAATTTCCTCGGAAGTGGTATCA 840
                ******** ** ********* ******************* ** ** ********  **

AYE             GACAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTTCGGGAC 900
AB0057          GACAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTTCGGGAC 900
AB307-0294      GACAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTTCGGGAC 900
ATCC            CAAAACTGGATATTAAAAATTGAGGATGAAGGCCCTGGCATTGCAACCGAGTTCCAAGAC 663
ACICU           CAAAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTCCAAGAC 900
BM4454          CAAAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTCCAAGAC 900
                 * ******************************** ***************** *  ***

AYE             GATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGCACA 960
AB0057          GATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGCACA 960
AB307-0294      GATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGCACA 960
ATCC            GATTTATATAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGCACA 723
ACICU           GATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGCACA 960
BM4454          GATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGCACA 960
                ******* ************************************************

AYE             GGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGGCACTGAAAGGCACTATTCAATAT 1020
AB0057          GGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGGCACTGAAAGGCACTATTCAATAT 1020
AB307-0294      GGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGGCACTGAAAGGCACTATTCAATAT 1020
ATCC            GGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGGCACTGAAAGGTACTATTCAATAT 783
ACICU           GGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGGCACTGAAAGGCACTATTCAATAT 1020
BM4454          GGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGGCACTGAAAGGCACTATTCAATAT 1020
                ********************************************** ************

AYE             AGCAATCAAGGCTCGAAAAGTGTTTTCACCATAAAAATTTCTATGGGTCATGAAGAGATG 1080
AB0057          AGCAATCAAGGCTCGAAAAGTGTTTTCACCATAAAAATTTCTATGGGTCATGAAGAGATG 1080
AB307-0294      AGCAATCAAGGCTCGAAAAGTGTTTTCACCATAAAAATTTCTATGGGTCATGAAGAGATG 1080
ATCC            AGCAACCAAGGCTCGAAAAGTGTTTTCACCATAAAAATTTCTATGGGTCATGAAGAAATA 843
ACICU           AGCAATCAAGGCTCGAAAAGTATTTTCACCATAAAAATTTCTATGAATAACTAA------ 1074
BM4454          AGCAATCAAGGCTCGAAAAGTATTTTCACCATAAAAATTTCTATGAATAACTAA------ 1074
                ***** *************** ***********************  * *  **

AYE             GGGTAA 1086
AB0057          GGGTAA 1086
AB307-0294      GGGTAA 1086
ATCC            GGGTAA 849
ACICU           ------
BM4454          ------
```

The identity between *adeS* in all strains was calculated for the aligned regions of the gene (extra bases (-) were not included).

adeS and its surrounding regions were most highly conserved between AYE, AB0057 and AB307-0294 (Figure 3.1). There was 95% identity between the aligned region of *adeS* in all strains investigated (Figure 3.2) although it is important to note that ATCC 17978 had a shorter *adeS* than the other strains, which gave rise to a shorter AdeS protein. There was 99% similarity between the aligned region of AdeS (Figure 3.3) in all strains. Another difference identified for ATCC 17978 is that none of the analysed region of the genome aligned with *adeC* of other strains (Figure 3.1).

The sequences of *adeR* and AdeR in the six strains were also compared (Figure 3.4; Figure 3.5) to investigate the level of identity and similarity between the second component of AdeRS. The aligned regions of *adeR* shared 97% identity and the AdeR proteins were 97% similar in all strains. However, ATCC 17978 and BM4454 both produced a smaller AdeR protein than AYE, AB0057, ACICU and AB307-0294 due to a shorter gene.

It was decided that research into the role of TCSs in adaptive responses in *A. baumannii* would be carried out in strain AYE. This was because AYE had the highest number of BLAST hits with other sequenced *A. baumannii* genomes (personal correspondence, Jacqueline Chan, University of Birmingham) and AYE is a multidrug-resistant clinical strain (Poirel *et al.*, 2003). Therefore, the involvement of TCSs and their downstream targets in MDR can be investigated in a strain which has been implicated in nosocomial infection. Moreover, the genome of AYE has been sequenced and thoroughly analysed. Several publications have compared the genome of this strain with those of other isolates (antibiotic resistant, susceptible and environmental isolates) to identify virulence and antibiotic resistance determinants (Adams *et al.*, 2010; Adams *et al.*, 2008; Coyne *et al.*, 2010a; Fournier *et al.*, 2006; Vallenet *et al.*, 2008). AB307-0294 and ATCC 17978 were not investigated, since these

Figure 3.3 Alignment of AdeS.

```
AYE             MKSKLGISKQLFIALTIVNLSVTLFSIVLGYIIYNYAIEKGWISLSSFQQEDWTSFHFVD 60
AB0057          MKSKLGISKQLFIALTIVNLSVTLFSIVLGYIIYNYAIEKGWISLSSFQQEDWTSFHFVD 60
AB307-0294      MKSKLGISKQLFIALTIVNLSVTLFSIVLGYIIYNYAIEKGWISLSSFQQEDWTSFHFVD 60
ATCC            ------------------------------------------------------------
ACICU           MKSKLGISKQLFIALTIVNLSVTLFSVVLGYVIYNYAIEKGWISLSSFQQEDWTSFHFVD 60
BM4454          MKSKLGISKQLFIALTIVNLSVTLFSVVLGYVIYNYAIEKGWISLSSFQQEDWTSFHFVD 60


AYE             WIWLATVIFCGCIISLVIGMRLAKRFIVPINFLVEAAKKISHGDLSARAYDNRIHSAEMS 120
AB0057          WIWLATVIFCGCIISLVIGMRLAKRFIVPINFLVEAAKKISHGDLSARAYDNRIHSAEMS 120
AB307-0294      WIWLATVIFCGCIISLVIGMRLAKRFIVPINFLAEAAKKISHGDLSARAYDNRIHSAEMS 120
ATCC            -------------------MRLAKRFIVPINFLAEAAKKISHGDLSARAYDNRIHSAEMS 41
ACICU           WIWLATVIFCGCIISLVIGMRLAKRFIVPINFLAEAAKKISHGDLSARAYDNRIHSAEMS 120
BM4454          WIWLATVIFCGCIISLVIGMRLAKRFIVPINFLAEAAKKISHGDLSARAYDNRIHSAEMS 120
                          **************.************************


AYE             ELLYNFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDGVFKPDEVLFKSL 180
AB0057          ELLYNFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDGVFKPDEVLFKSL 180
AB307-0294      ELLYNFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDGVFKPDEVLFKSL 180
ATCC            ELLYNFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDGVFKLDEVLFKSL 101
ACICU           ELLYNFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDGVFKPDEVLFKSL 180
BM4454          ELLYNFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDGVFKPDEVLFKSL 180
                ************************************************ ********


AYE             LNQVEGLSHLVEDLRTLSLVENQQLRLNYELFDLKAVVEKVLKAFEDRLDQAKLVPELDL 240
AB0057          LNQVEGLSHLVEDLRTLSLVENQQLRLNYELFDLKAVVEKVLKAFEDRLDQAKLVPELDL 240
AB307-0294      LNQVEGLSHLVEDLRTLSLVENQQLRLNYELFDLKAVVEKVLKAFEDRLDQAKLVPELDL 240
ATCC            LNQVEGLSHLVEDLRTLSLVENQQLRLNYELFDFKAVVEKVLKAFEDRLDQAKLVPELDL 161
ACICU           LNQVEVLSHLVEDLRTLSLVENQQLRLNYELFDFKAVVEKVLKAFEDRLDQAKLVPELDL 240
BM4454          LNQVEVLSHLVEDLRTLSLVENQQLRLNYELFDFKAVVEKVLKAFEDRLDQAKLVPELDL 240
                ***** ***************************:************************


AYE             TSTPVYCDRRRIEQVLIALIDNAIRYSNAGKLKISSEVVADNWILKIEDEGPGIATEFRD 300
AB0057          TSTPVYCDRRRIEQVLIALIDNAIRYSNAGKLKISSEVVADNWILKIEDEGPGIATEFRD 300
AB307-0294      TSTPVYCDRRRIEQVLIALIDNAIRYSNAGKLKISSEVVADNWILKIEDEGPGIATEFRD 300
ATCC            TSTPVYCDRRRIEQVLIALIDNAIRYSNAGKLKISSEVVSQNWILKIEDEGPGIATEFQD 221
ACICU           TSTPVYCDRRRIEQVLIALIDNAIRYSHAGKLKISSEVVSQNWILKIEDEGPGIATEFQD 300
BM4454          TSTPVYCDRRRIEQVLIALIDNAIRYSHAGKLKISSEVVSQNWILKIEDEGPGIATEFQD 300
                **************************:***********::*****************:*


AYE             DLFKPFFRLEESRNKEFGGTGLGLAVVHAIIVALKGTIQYSNQGSKSVFTIKISMGHEEM 360
AB0057          DLFKPFFRLEESRNKEFGGTGLGLAVVHAIIVALKGTIQYSNQGSKSVFTIKISMGHEEM 360
AB307-0294      DLFKPFFRLEESRNKEFGGTGLGLAVVHAIIVALKGTIQYSNQGSKSVFTIKISMGHEEM 360
ATCC            DLYKPFFRLEESRNKEFGGTGLGLAVVHAIIVALKGTIQYSNQGSKSVFTIKISMGHEEI 281
ACICU           DLFKPFFRLEESRNKEFGGTGLGLAVVHAIIVALKGTIQYSNQGSKSIFTIKISMNN--- 357
BM4454          DLFKPFFRLEESRNKEFGGTGLGLAVVHAIIVALKGTIQYSNQGSKSIFTIKISMNN--- 357
                **:*********************************************:*******.:


AYE             G 361
AB0057          G 361
AB307-0294      G 361
ATCC            G 282
ACICU           -
BM4454          -
```

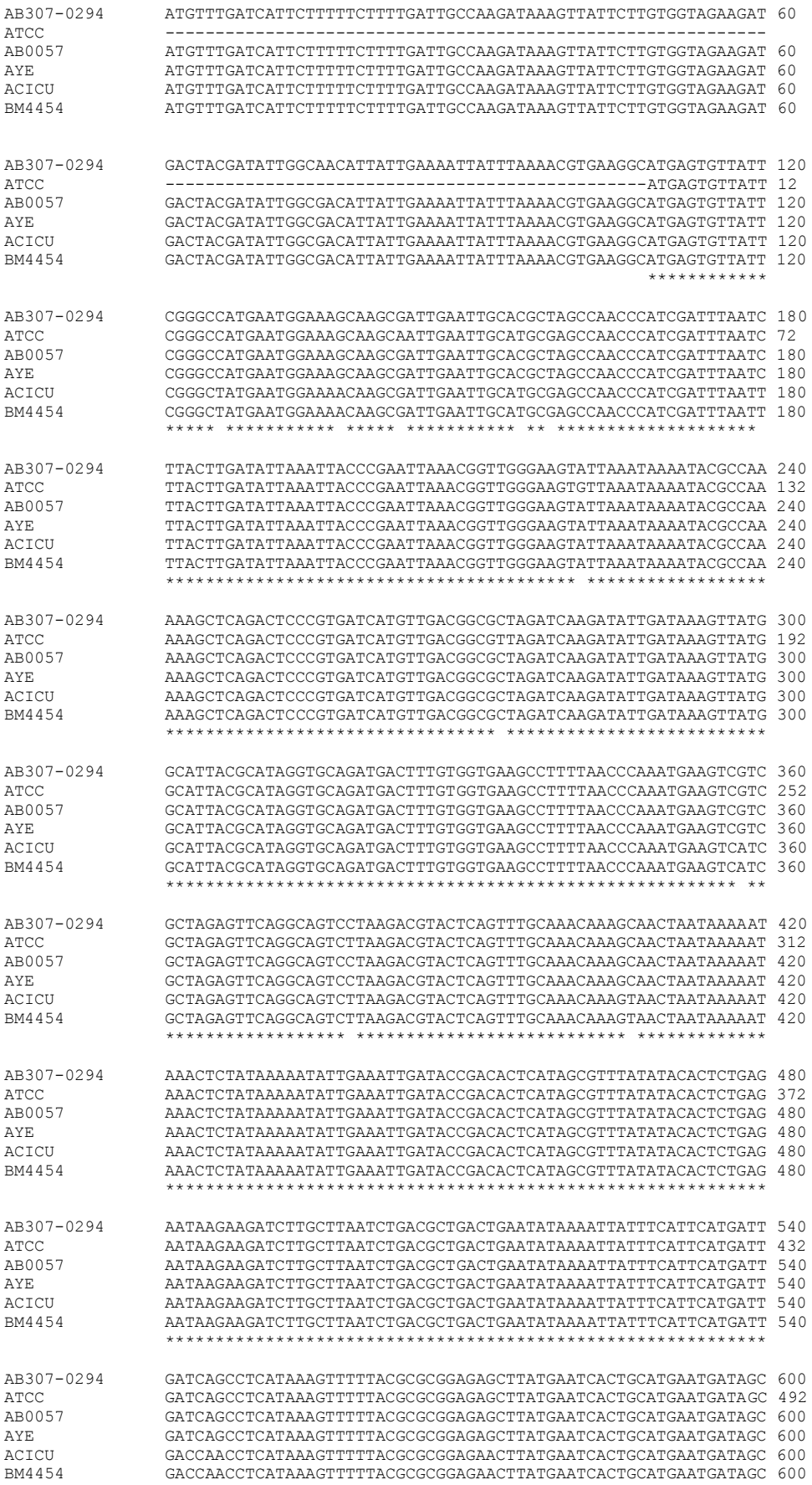
Similarity was calculated for the aligned regions of AdeS (extra residues (-) were not included). The following residues were considered to be similar:

* identical residue

: strongly similar residue (scoring >0.5 in the Gonnet PAM matrix)

. weakly similar residue (scoring <0.5 in the Gonnet PAM matrix)

Figure 3.4 Aligment of *adeR*.



```
                ** ** ************************ **************************

AB307-0294      GATGCACTAGAGCGAACCGTAGATAGCCATGTGAGTAAGCTGAGAAAAAAACTAGAAGAA 660
ATCC            GATGCACTAGAGCGAACCGTAGATAGCCATGTAAGTAAGCTGAGAAAAAAACTAGAAGAA 552
AB0057          GATGCACTAGAGCGAACCGTAGATAGCCATGTGAGTAAGCTGAGAAAAAAACTAGAAGAA 660
AYE             GATGCACTAGAGCGAACCGTAGATAGCCATGTGAGTAAGCTGAGAAAAAAACTAGAAGAA 660
ACICU           GATGCACTAGAGCGAACCGTAGATAGCCATGTAAGTAAGCTGAGAAAAAAACTAGAAGAA 660
BM4454          GATGCACTAGAGCGAACCGTAGATAGCCATGTAAGTAAGCTGAGAAAAAAACTAAAAGAA 660
                ****************************** ******************** *****

AB307-0294      -CAAGGCATATTTCAAATGTTAATTAATGTGCGTGGCGTGGGATATAGACTAGATAATCC 719
ATCC            -CAAGGCATATTTCAAATGTTAATTAATGTGCGTGGCGTGGGATATAGGCTAGATAATCC 611
AB0057          -CAAGGCATATTTCAAATGTTAATTAATGTGCGTGGCGTGGGATATAGACTAGATAATCC 719
AYE             -CAAGGCATATTTCAAATGTTAATTAATGTGCGTGGCGTGGGATATAGACTAGATAATCC 719
ACICU           -CAAGGCATATTTCAAATGTTAATTAATGTGCGTGGCGTGGGATATAGGCTAGATAATCC 719
BM4454          ACAAGGCATATTTCAAATGTTAATTAA-------------------------------- 687
                 *************************

AB307-0294      CCTAGCTGTAAAAGATGATGCCTAA 744
ATCC            CCTAGCTGTAAAAGATGACGCCTAA 636
AB0057          CCTAGCTGTAAAAGATGATGCCTAA 744
AYE             CCTAGCTGTAAAAGATGATGCCTAA 744
ACICU           CCTAGCTGTAAAAGATGACGCCTAA 744
BM4454          -------------------------
```

The identity between *adeR* in all strains was calculated for the aligned regions of the gene (extra bases (-) were not included).

Figure 3.5 Alignment of AdeR.

```
AB307-0294      MFDHSFSFDCQDKVILVVEDDYDIGNIIENYLKREGMSVIRAMNGKQAIELHASQPIDLI 60
ATCC            ---------------------------------MSVIRAMNGKQAIELHASQPIDLI 24
AB0057          MFDHSFSFDCQDKVILVVEDDYDIGDIIENYLKREGMSVIRAMNGKQAIELHASQPIDLI 60
AYE             MFDHSFSFDCQDKVILVVEDDYDIGDIIENYLKREGMSVIRAMNGKQAIELHASQPIDLI 60
ACICU           MFDHSFSFDCQDKVILVVEDDYDIGDIIENYLKREGMSVIRAMNGKQAIELHASQPIDLI 60
BM4454          MFDHSFSFDCQDKVILVVEDDYDIGDIIENYLKREGMSVIRAMNGKQAIELHASQPIDLI 60
                                              ************************

AB307-0294      LLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDIDKVMALRIGADDFVVKPFNPNEVV 120
ATCC            LLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDIDKVMALRIGADDFVVKPFNPNEVV 84
AB0057          LLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDIDKVMALRIGADDFVVKPFNPNEVV 120
AYE             LLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDIDKVMALRIGADDFVVKPFNPNEVV 120
ACICU           LLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDIDKVMALRIGADDFVVKPFNPNEVI 120
BM4454          LLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDIDKVMALRIGADDFVVKPFNPNEVI 120
                ************************************************************:

AB307-0294      ARVQAVLRRTQFANKATNKNKLYKNIEIDTDTHSVYIHSENKKILLNLTLTEYKIISFMI 180
ATCC            ARVQAVLRRTQFANKATNKNKLYKNIEIDTDTHSVYIHSENKKILLNLTLTEYKIISFMI 144
AB0057          ARVQAVLRRTQFANKATNKNKLYKNIEIDTDTHSVYIHSENKKILLNLTLTEYKIISFMI 180
AYE             ARVQAVLRRTQFANKATNKNKLYKNIEIDTDTHSVYIHSENKKILLNLTLTEYKIISFMI 180
ACICU           ARVQAVLRRTQFANKVTNKNKLYKNIEIDTDTHSVYIHSENKKILLNLTLTEYKIISFMI 180
BM4454          ARVQAVLRRTQFANKVTNKNKLYKNIEIDTDTHSVYIHSENKKILLNLTLTEYKIISFMI 180
                ***************.********************************************

AB307-0294      DQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRKKLEEQGIFQMLINVRGVGYRLDNP 240
ATCC            DQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRKKLEEQGIFQMLINVRGVGYRLDNP 204
AB0057          DQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRKKLEEQGIFQMLINVRGVGYRLDNP 240
AYE             DQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRKKLEEQGIFQMLINVRGVGYRLDNP 240
ACICU           DQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRKKLEEQGIFQMLINVRGVGYRLDNP 240
BM4454          DQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRKKLKET---RHISNVN--------- 228
                ************************************:*    : : **.

AB307-0294      LAVKDDA 247
ATCC            LAVKDDA 211
AB0057          LAVKDDA 247
AYE             LAVKDDA 247
ACICU           LAVKDDA 247
BM4454          -------
```

Similarity was calculated for the aligned regions of AdeR (extra residues (-) were not included). The following residues were considered to be similar:

* identical residue

: strongly similar residue (scoring >0.5 in the Gonnet PAM matrix)

. weakly similar residue (scoring <0.5 in the Gonnet PAM matrix)

are antibiotic-susceptible strains (Adams *et al.*, 2008). Moreover, the genomic region around *adeS* was most different for ATCC 17978 than for the other five strains. However, a disadvantage of AYE is that despite numerous descriptions of AYE in the literature, there are no reports of gene manipulation in this strain. The literature was reviewed to compile a list of researchers who have deleted or inactivated a gene in *A. baumannii* and the strains they chose; commonly used type strains were ATCC 19606 and ATCC 17978. To gather information for choosing an alternative strain if AYE proved difficult to manipulate, seven of the main authors were contacted and asked for their views and opinions on the strains used for genetic manipulation in *A. baumannii* (Table 3.2).

3.3. Gene Deletion and Complementation

To confirm the identity of pMo130-Tel^R, the vector was digested with Bam*HI* and a region across the multiple cloning site was amplified (Section 2.4.1.2). The linearised plasmid was of the correct size (Figure 3.6a) and an amplimer of the expected size was obtained (Figure 3.6b), confirming that the correct plasmid had been extracted.

Unmanipulated pMo130-Tel^R was transformed into DH5α (Section 2.4.1.3) to determine whether this vector could be used as a positive control for transformation of the ligated constructs into DH5α. After approximately 20 hr incubation, a bacterial lawn was present on each plate containing DH5α transformed with unmanipulated pMo130-Tel^R. To confirm that the correct vector had been introduced, a cell lysate was prepared from one isolated transformant and used in a PCR to confirm the vector identity (Section 2.4.1.2, Table 2.7, Reaction 1). An

Table 3.2 Investigation into an Alternative Strain to AYE for Genetic Manipulation.

Question	Consensus response
1. Why has AYE never been used for genetic manipulation despite many descriptions of this strain in the literature?	AYE is MDR and is therefore difficult to manipulate using common techniques. It is not possible to use common resistance cassettes to select mutants.
2. Why did they choose their particular strains for gene inactivation or deletion?	ATCC 19606 and ATCC 17978 were commonly used because there were previous reports of using these strains in the literature and they are susceptible to many antibiotics. Where a type strain was not used, the organism was a clinical isolate and was used because its phenotype was important to the study. The clinical isolates investigated by Hornsey *et al.* (2010) and Heritier *et al.* (2005) were susceptible to kanamycin. Therefore, this antibiotic could be used to select inactivation mutants to investigate resistance to other classes of antibiotic. Likewise, Wong *et al.* (2009) were able to use ticarcillin and rifampicin.
3. Did they have any recommendations or advice as to which standard strain of Acinetobacter would be best for making gene deletions?	All of those asked suggested either that ATCC 19606 or ATCC 17978 was most suitable since reported gene manipulation has been carried out in these strains and genome data are available (although sequencing of ATCC 19606 is incomplete (Chaudhuri *et al.*, 2008)). Common resistance markers such as kanamycin and rifampicin can also be used to inactivate the genes, since ATCC 19606 and ATCC 17978 are susceptible to these antibiotics. The consensus was that the most important consideration when choosing a strain is that it is susceptible to commonly used resistance markers; all of the methods relied upon an antibiotic to select mutants.

amplimer of the correct size (253bp) was obtained for both the vector pMo130-Tel^R and the transformant confirming that pMo130-Tel^R could be transformed into DH5α with high efficiency and it was included as the positive control for transformation of all constructs created in this study.

To confirm that DH5α and S17-1 did not already contain a vector, plasmid extraction was carried out for DH5α and S17-1 before transformation. Plasmid was not obtained from either strain, confirming that any plasmids identified in the candidate transformants were likely to be pMo130-Tel^R.

3.3.1. *adeRS*

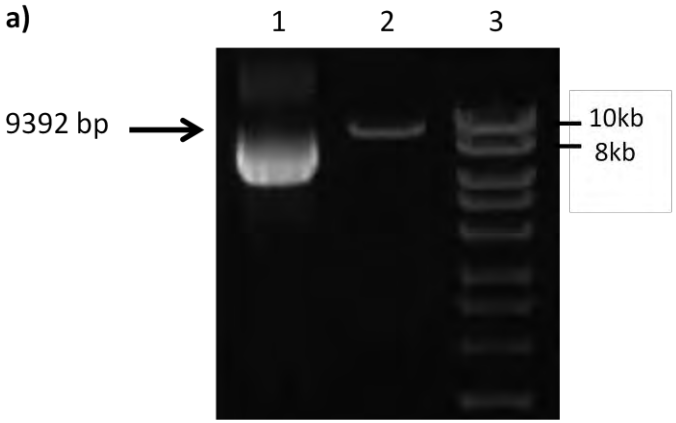
3.3.1.1. Deletion of *adeRS*

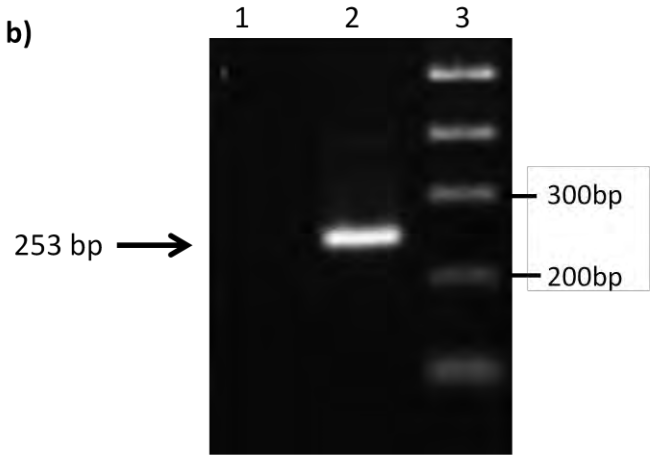
3.3.1.1.1. Construction of pMo130-Tel^R/*adeRS*UPDOWN

The *adeRS*UP and *adeRS*DOWN fragments were amplified by PCR from AYE genomic DNA and verified by electrophoresis (Figure 3.7).

pMo130-Tel^R and the *adeRS*UP fragment were each digested with *Bam*HI and *Not*I in double digests (Section 2.4.1.2; Table 2.8, Digestion 3) and the vector was treated with alkaline phosphatase to prevent re-ligation. The insert and vector were then purified and electrophoresed to confirm that the DNA had not been lost at any stage (Figure 3.7). Fifteen bp DNA was removed from pMo130-Tel^R in the double digest, and 13 bp DNA was removed from the UP fragment. Therefore, the double digestion could not be confirmed by electrophoresis.

Figure 3.6 Verification of pMo130-TelR identity.





Lane	DNA	Expected size	Actual size
1	pMo130-Tel^{R} undigested	---	---
2	pMo130-Tel^{R} + *Bam*HI	9392bp	~9392bp
3	Hyperladder™ I (Bioline)	---	---

Digestion 1, Table 2.8

Lane	DNA	Expected size	Actual size
1	Contamination control	No product	No product
2	pMo130-Tel^{R}	253bp	~253bp
3	Hyperladder™ I (Bioline)	---	---

Reaction 1, Table 2.7

However, the digestion was assumed to have worked, since the vector had been linearised (Figure 3.8). The vector and insert were ligated and transformed into DH5α.

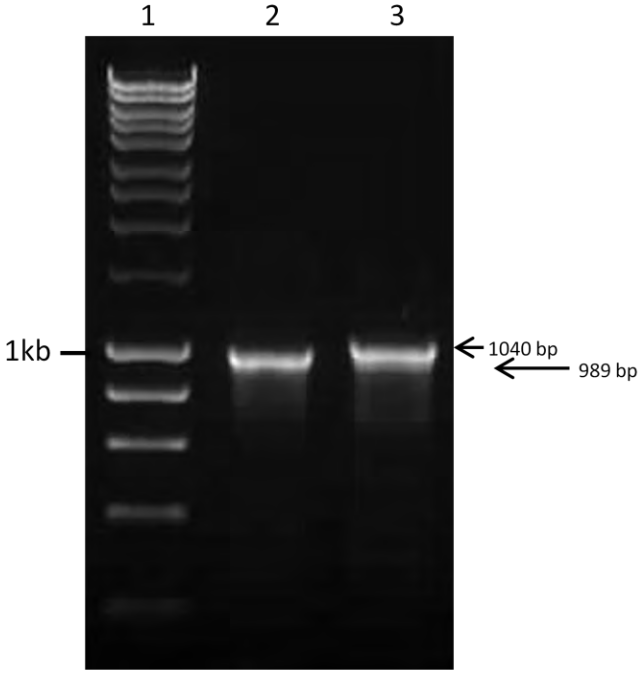
Approximately 100 colonies were obtained for DH5α transformed with pMo130-Tel^R/UP and three of these were selected for verification. Plasmid extraction and verification by PCR confirmed that the correct vector had been introduced and that the UP fragment had been ligated into each (Figure 3.9). The construct extracted from candidate 1 was selected for further verification. It was digested with *Bam*HI and compared with *Bam*HI-digested pMo130-Tel^R. pMo130-Tel^R/UP was approximately 1kb larger than pMo130-Tel^R, confirming that ligation of the UP fragment into S17-1 was successful (Figure 3.10). This construct was used for cloning of the DOWN fragment.

The DOWN fragment and pMo130-Tel^R/*adeRS*UP were digested with *Bam*HI and *Sph*I and electrophoresed to confirm that DNA was still present (Figure 3.11). The digested vector and insert were ligated and transformed into DH5α. Approximately 100 candidate transformants were obtained and three large colonies were selected for verification. Plasmid was extracted from each candidate and verified by PCR (Figure 3.12) and DNA sequencing (Figure 3.13).

3.3.1.1.2. Introduction of pMo130-Tel^R/*adeRS*UPDOWN into AYE and Verification of Deletion

pMo130-Tel^R was transformed into S17-1 and plasmid was extracted from one candidate transformant. The identity of the plasmid was verified by PCR (Table 2.7, PCR 13). An amplimer of the correct size (2186 bp) was obtained confirming that S17-1 contained pMo130-Tel^R/*adeRS*UPDOWN. pMo130-Tel^R/*adeRS*UPDOWN was then transferred from S17-1/pMo130-

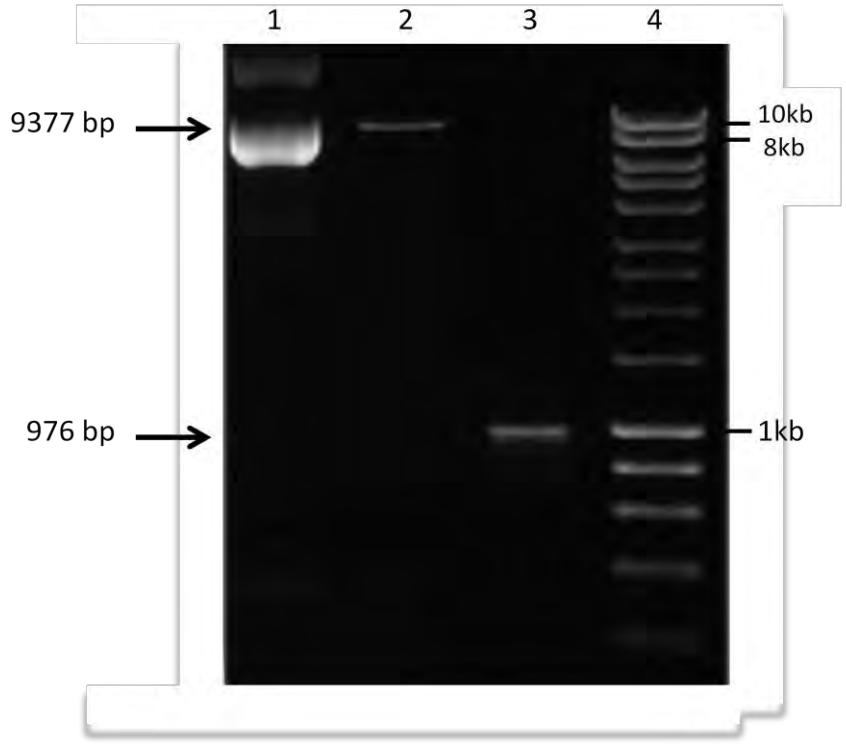
Figure 3.7 Verification of *adeRS* UP and DOWN fragments.



Lane	PCR[a]	DNA	Expected size	Actual size
1	---	Hyperladder™ I (Bioline)	---	---
2	2	Purified UP fragment	989bp	~989bp
3	3	Purified DOWN fragment	1040bp	~1040bp

[a] Table 2.7

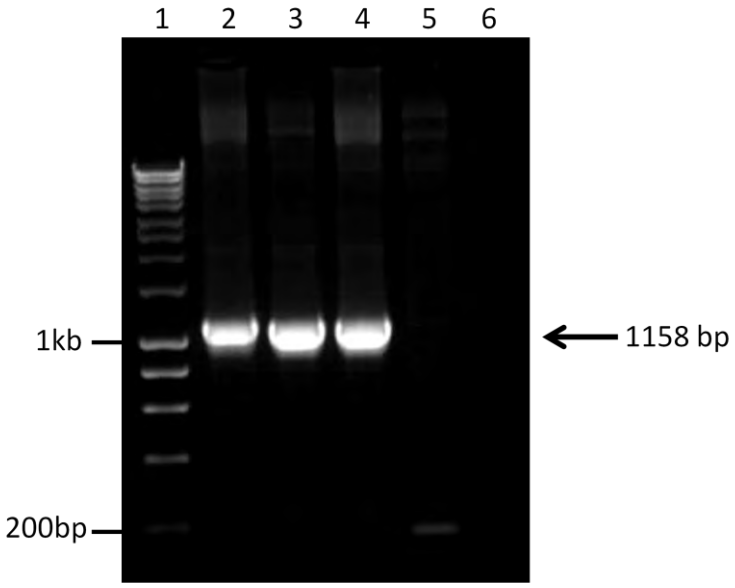
Figure 3.8 Digestion of pMo130-TelR and *adeRS*UP fragment.



Lane	DNA	Expected size	Actual size
1	pMo130-Tel^{R} Undigested	---	---
2	pMo130-Tel^{R} + *Bam*HI + *Not*I	9377bp	~ 9377bp
3	UP fragment + *Bam*HI + *Not*I	976bp	~ 976bp
4	Hyperladder™ I (Bioline)	---	---

Digestion 3, Table 2.8

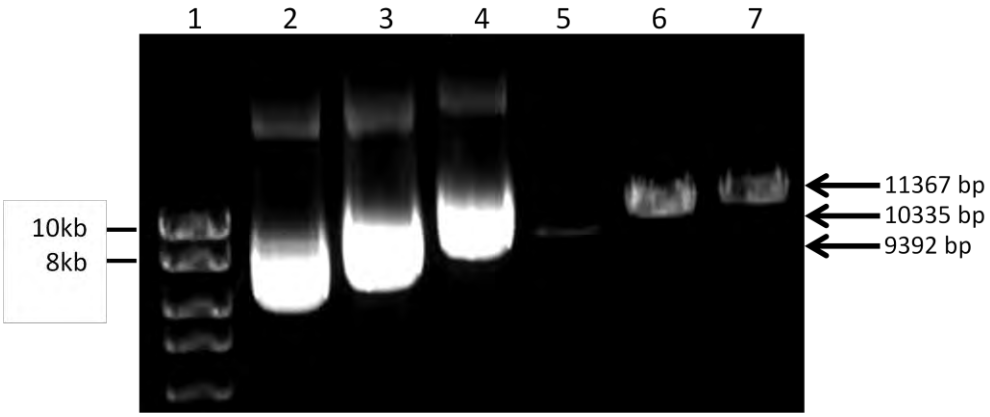
Figure 3.9 Verification of pMo130-TelR/*adeRS*UP by PCR.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ I (Bioline)	---	---
2	pMo130-Tel^{R}/UP Transformant 1	1158bp	1158bp
3	pMo130-Tel^{R}/UP Transformant 2	1158bp	1158bp
4	pMo130-Tel^{R}/UP Transformant 3	1158bp	1158bp
5	pMo130-Tel^{R} (Negative control)	No product	200bp
6	Contamination control	No amplimer	No amplimer

PCR 10, Table 2.7

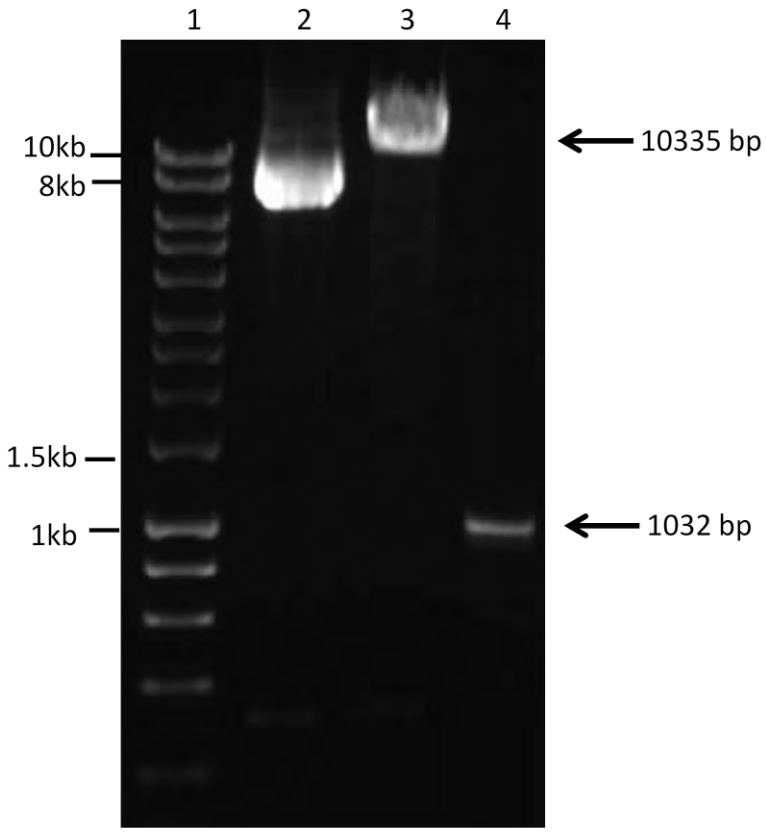
Figure 3.10 Verification of pMo130-TelR/*adeRS*UP and pMo130-TelR/*adeRS*UPDOWN by Restriction Digestion.



Lane	DNA	Expected size	Actual size
1	pMo130-Tel^{R} Undigested	---	---
2	pMo130-Tel^{R}/UP Undigested	---	---
3	pMo130-Tel^{R}/UPDOWN Undigested	---	---
4	pMo130-Tel^{R} + *Bam*HI	9392bp	~9392bp
5	pMo130-Tel^{R}/UP + *Bam*HI	10335bp	~10335bp
6	pMo130-Tel^{R}/UPDOWN + *Bam*HI	11367bp	~11367bp
7	Hyperladder™ I (Bioline)	---	---

Digestion 1, Table 2.8

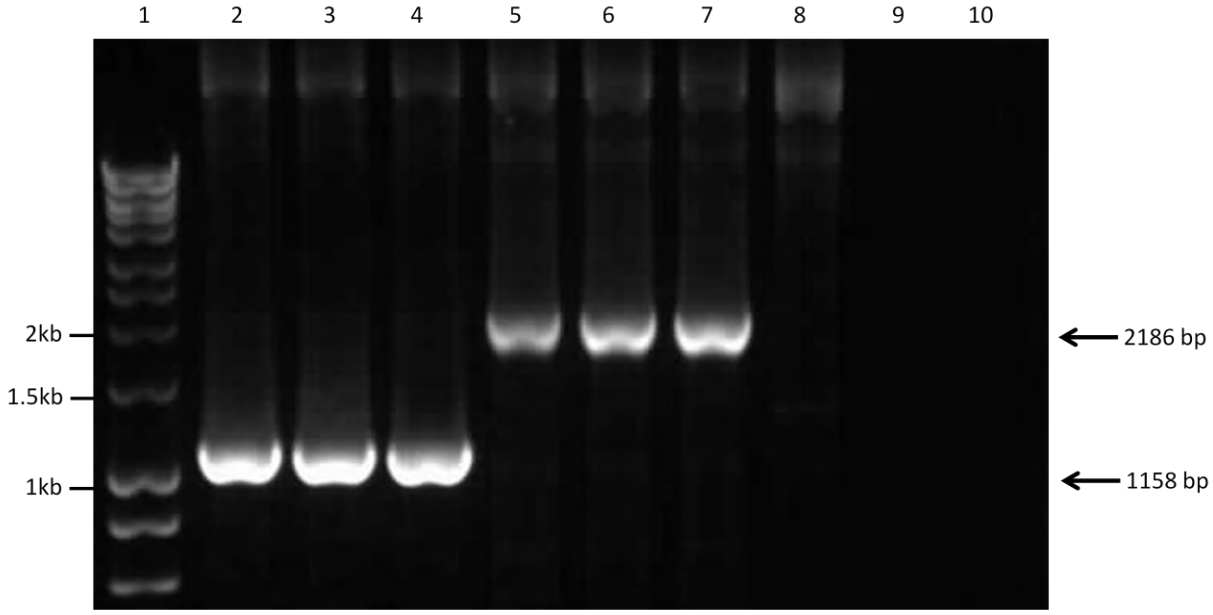
Figure 3.11 Digestion of pMo130-TelR/*adeRS*UP and *adeRS*DOWN fragment.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ I (Bioline)	---	---
2	pMo130-Tel^{R}/UP Undigested	---	---
3	pMo130-Tel^{R}/UP + *Bam*HI + *Sph*I	10335bp	10335bp
4	Insert + *Bam*HI + *Sph*I	1032bp	1032bp
5	Hyperladder™ I (Bioline)	---	---

Digestion 4, Table 2.8

Figure 3.12 Verification of pMo130-TelR/*adeRS*UPDOWN by PCR.



Lane	PCR[a]	DNA	Expected size	Actual size
1	---	Hyperladder™ I (Bioline)	---	---
2	10	pMo130-Tel^R/UPDOWN Transformant 1	1158bp	1158bp
3	10	pMo130-Tel^R/UPDOWN Transformant 2	1158bp	1158bp
4	10	pMo130-Tel^R/UPDOWN Transformant 3	1158bp	1158bp
5	13	pMo130-Tel^R/UPDOWN Transformant 1	2186bp	2186bp
6	13	pMo130-Tel^R/UPDOWN Transformant 2	2186bp	2186bp
7	13	pMo130-Tel^R/UPDOWN Transformant 3	2186bp	2186bp
8	10	pMo130-Tel^R/UP (negative control)	No amplimer	No amplimer
9	10	---	No amplimer	No amplimer
10	13	---	No amplimer	No amplimer

[a] PCR number, Table 2.7

Figure 3.13 Verification of pMo130-TelR/adeSUPDOWN by DNA Sequencing.

a) Forward sequencing

```
pMoadeSUPDN Expected      1 CCATCTACTTCTTCGACCCGTCCGGTAACCGCAACGAAGTGTTCTGCGGGGGAGATTACA   60
                                                  G++++  AGTGTTCTGCGGGGGAGATTACA
pMoadeSUPDN FW            1 ----------------------------TGGGSMMMMSAGTGTTCTGCGGGGGAGATTACA   33

pMoadeSUPDN Expected     61 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT  120
                            ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT
pMoadeSUPDN FW           34 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT   93

pMoadeSUPDN Expected    121 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGACCTGAGCGGCCGC  180
                            ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCT     GAGCGGCCGC
pMoadeSUPDN FW           94 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCT-----GAGCGGCCGC  148

pMoadeSUPDN Expected    181 CCTCCGACTTGCGGACGGATTTCCGCCGTACGGAACGCATGTACTCGTGCAGGAAGATTT  240
                            CCTCCGACTTGCGGACGGATTTCCGCCGTACGGAACGCATGTACTCGTGCAGGAAGATTT
pMoadeSUPDN FW          149 CCTCCGACTTGCGGACGGATTTCCGCCGTACGGAACGCATGTACTCGTGCAGGAAGATTT  208

pMoadeSUPDN Expected    241 TCACTAAAATTTACCGACTGCGGTTGAATGCTTAATACACTGACTTTAGCCGGTGGTGGC  300
                            TCACTAAAATTTACCGACTGCGGTTGAATGCTTAATACACTGACTTTAGCCGGTGGTGGC
pMoadeSUPDN FW          209 TCACTAAAATTTACCGACTGCGGTTGAATGCTTAATACACTGACTTTAGCCGGTGGTGGC  268

pMoadeSUPDN Expected    301 TCAGCTTGAGCGACTTCTTTTGAATCACACCCCTGTAATATCAGCCCAATAGATAAAAAT  360
                            TCAGCTTGA+CGACTTCTTTTGAATCACACCCCTGTAATATCAGCCCAATA+ATAAAAAT
pMoadeSUPDN FW          269 TCAGCTTGARCGACTTCTTTTGAATCACACCCCTGTAATATCAGCCCAATAKATAAAAAT  328

pMoadeSUPDN Expected    361 AAAGGAAGTAAAAGATGCTTTTGCATACTGTCCAAACCTAGTGACTTTTTGATGTTCGTA  420
                            AAAGGAA+TAAAAGATGCTTTTGCATACTGTCCAAACCTA+TGACTTTTTGATGTTCGTA
pMoadeSUPDN FW          329 AAAGGAARTAAAAGATGCTTTTGCATACTGTCCAAACCTARTGACTTTTTGATGTTCGTA  388

pMoadeSUPDN Expected    421 TTATTTTTGATGAGTGTGTAGGGATAATCACTAAAGTGTGGAGTAAGTGTGGAGAAATAC  480
                            TTATTTTTGATGA+TGTGTAGGGATAA+C++TA+AGTGTG+AGTAA+TGTGGAGAAATAC
pMoadeSUPDN FW          389 TTATTTTTGATGASTGTGTAGGGATAAWCWYTAMAGTGTGRAGTAARTGTGGAGAAATAC  448

pMoadeSUPDN Expected    481 GGATAATTTAGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATTTTCTATGTTTGAT  540
                            GGATAATTTA+CGTATGATGA+TTGAA+CACTTTC+A+A+CCAGATTTTCTATGTTTGAT
pMoadeSUPDN FW          449 GGATAATTTARCGTATGATGARTTGAARCACTTTCYAWAKCCAGATTTTCTATGTTTGAT  508

pMoadeSUPDN Expected    541 CATTCTTTTTCTTTTGATTGCCAAGATAAAGTTATTCTTGTGGTAGAAGATGACTACGAT  600
                            CATTCTTTTTCTTTTGATTGCCAA+ATAA GTTATTCTTGTGGTAGAAGA GACTAC A
pMoadeSUPDN FW          509 CATTCTTTTTCTTTTGATTGCCAAKATAACGTTATTCTTGTGGTAGAAGAGGACTACCAC  568

pMoadeSUPDN Expected    601 ATTGGCGACATTATTGAAAATTATTTAAAACGTGAAGGCATGAGTGTTATTCGGGCCATG  660
                             TTGGCGAC TTATTGAAAATTATTT A+ACGTG AGGCATGAGTGTTATTC  GCC T
pMoadeSUPDN FW          569 CTTGGCGACCTTATTGAAAATTATTTGARACGTGTAGGCATGAGTGTTATTCACGCCTTC  628

pMoadeSUPDN Expected    661 AATGGAAAGCAAGCGATTGAATTGCACGCTAGCCAACCCATCGATTTAATCTTACTTGAT  720
                              TG A AG AAG GA  G A TG  +GCTA+CCAA+ CATC  TTT ATC  AC TG T
pMoadeSUPDN FW          629 TCTGYACAGAAAGAGAGAGTAATGTTYGCTAKCCAAMACATCTTTTTCATCACACCTGCT  688

pMoadeSUPDN Expected    721 ATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAATAAAATACGCCAAAAAGCTCAG  780
                            AT+AAA    C+ A TT A CG +   GAA  ATT A TAAA TA +    A+  CT
pMoadeSUPDN FW          689 ATKAAAAACACMAACTTGATCGYYC--GAACGATTTATTAAATTATKWTTTAMT-CTACC  745

pMoadeSUPDN Expected    781 ACTCCCGTGATCATGTTGACGGCGCTAGATCAAGATATTGATAAAGTTATGGCATTACGC  840
                              TCCC  G  CA G +GA  G G     T  A+ TA TGA+  +        AT  + C
pMoadeSUPDN FW          746 CTTCCCCCGCCCAAGAKGAGAGTGAGTTGTATAS-TASTGAYSCRCCCCATCWATCCMTC  804

pMoadeSUPDN Expected    841 ATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGTCGTCGCTAGAGTT  900
                            A        G   A++ T +  T A     TT         +G   TC   G TA  +TT
pMoadeSUPDN FW          805 ACGCCCCGCGCCTASWCTTKTATAATATTATTCTCTGGGGCKGGKCTCTATGGTAATKTT  864

pMoadeSUPDN Expected    901 CAGGCAGTCCTAAGACGTACTCAGTTTGCAAACAAAGCAACTAATAAAAATAAACTCTAT  960
                              G     CC A  +  T CT   TTT C   C A   A  T      A  + +  C A
pMoadeSUPDN FW          865 TYGTT--CCCAACTMTTTTCTTTATTTTCCGGCSATATATTTTGSCGSAGGMGRGCCSAA  922
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pMoadeSUPDN Expected    961 AAAAATATTGAAATTGATACCGACACTCATAGCGTTTATATACACTCTGAGAATAAGAAG 1020
                             A  A ATT A A+  A    +A AC  ATAG G +      + +TCTG
pMoadeSUPDN FW          923 TAGTAAATTAATAWRAACSGWKA-ACCTATAGAGMK------MGMTCTGCTGGCCGCGTC  975

pMoadeSUPDN Expected   1021 ATCTTGCTTAATCTGACGCTGACTGAATATAAAATTATTTCATTCATGATTGATCAGCCT 1080
                             +   T+++  AT         + T+  T T     ++   C + +  G +       CC+
pMoadeSUPDN FW          976 RCTATRMWCTATT--------MTTRTGTGTCCYCCYMCCSCYKCMGCGGWGATGAGACCK 1027

pMoadeSUPDN Expected   1081 CATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGCATGAATGATAGCGATGC-ACT 1139
                             C    A++ TTT + ++C +A   +    GA  C+C  C+++A  GATA+ G +G  A+
pMoadeSUPDN FW         1028 CGAGKARWATTTTMTMKCCSACGAYAC-CGACCCRCCTCMKRAG-GATARAGCYGAGASG 1085

pMoadeSUPDN Expected   1140 AGAGCGAACCGGATCCAAGCTGTAACCGCAGCGCCAATTAAGGCTATACCGGTTTCATAA 1199
                             A A   AA +  +T CA G
pMoadeSUPDN FW         1086 ACAATCAATSATRTACATG---------------------------------------- 1104
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b) Reverse sequencing

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pMoadeSUPDN Expected    841 ATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGTCGTCGCTAGAGTT  900
                                      T A   +  GGT   + CT TT     A AT  + T GTC+CT  +  T
pMoadeSUPDN RV            1 ----------CTYAWCCKACGGTCG-STCTGTTGCGATAGATAGM-TGGTCKCTCCRAGT   48

pMoadeSUPDN Expected    901 CAGGCAGTCCTAAGACGTACTCAGTTTGCAAACAAAGCAACTAATAAAAATAAACTCTAT  960
                             CA G          +CGT CTCAGT T C++      + A TAATA +  T A  +  ++
pMoadeSUPDN RV           49 CATG---------AMCGT-CTCAGTCTACRM--GGCAYTATTAATAKWCGTGAGTKA-RK   95

pMoadeSUPDN Expected    961 AAAAATATTGAAATTGATACCGACACTCATAGCGTTTATATACACTCTGAGAATAAGAAG 1020
                             A  A +TTG+A T G  +CC+ C  T++   CGTTTA+   C  TC++  + TA G A+
pMoadeSUPDN RV           96 TAYGAGWTTGWAGTCGTCMCCRTCYATSMGGCCGTTTAWCCYCTWTCKRGAMGTAGGGAK  155

pMoadeSUPDN Expected   1021 A--TCTTGCTTAATCTGACGCTGACTGAATATAAAATTAT-TTCATTCATGATTGATCAG 1077
                                +C TGCT++ TC G C + G  TG   A A A TTA+ TTCA+T + + +++++CA
pMoadeSUPDN RV          156 GRTYCGTGCTKWKTCCG-CTSCGYRTGGTSAAAGAGTTAYATTCAYTGMARSWKKWKCAT  214

pMoadeSUPDN Expected   1078 CCTCATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGCATGAATGATAGCGATGCA 1137
                             C T+A+A  GTT++ A+G+++GGA+  +T + GA TC+C +CA GAAT+ATAG G T+CA
pMoadeSUPDN RV          215 CATMAYAC-GTTYKGAMGSRYGGASGTSTGWAGACTCWCCKCAMGAATRATAGTG-TKCA  272

pMoadeSUPDN Expected   1138 CTAGAGCGAACCGGATCCAAGCTGTAACCGCAGCGCCAATTAAGGCTATACCGGT-TTCA 1196
                              TAG GC  ++ GG  C  AGCT+ A CCGC GC  CAATTAAGG TA +C++GT TTC+
pMoadeSUPDN RV          273 -TAGGGC--RSAGGGGCTCAGCTRCA-CCGCTGCCWCAATTAAGGTTAAMCSKGTCTTCW  328

pMoadeSUPDN Expected   1197 TAAATAATATCTATAACAAATTCGAGCACTCCCTCCGACAAAAAATCTAATGAGCCTGAA 1256
                             TA+AT ATAT TATAACAAATTCGA+C+CTC+CT+CG+C+AAAA+T TAA++AGCCTGAA
pMoadeSUPDN RV          329 TAWATTATATWTATAACAAATTCGARCWCTCSCTSCGMCWAAAAMTWTAAKKAGCCTGAA  388

pMoadeSUPDN Expected   1257 AATTTAAGAAATTTAGCTATGGCAACCACTATAACTCCCAATAAAGCGCCAGCCC-ATAA 1315
                              ATT+AAGAAA+TTAGCTATGGCAACC+CTATAACTCCCA+TAAAGCGCCAGCCC ATA+
pMoadeSUPDN RV          389 CATTWAAGAAAWTTAGCTATGGCAACCMCTATAACTCCCAMTAAAGCGCCAGCCCCATAM  448

pMoadeSUPDN Expected   1316 GAATATTTTATGAATATTGATAGGTGCCGTGACTTGATCGATTTGATCGGGGTACTTTCG 1375
                             GAATATTTTATGAATATTGATAGGTGCCGTGAC TGATCGA+TTGATC+GGGTACTTTCG
pMoadeSUPDN RV          449 GAATATTTTATGAATATTGATAGGTGCCGTGACGTGATCGAWTTGATCKGGGTACTTTCG  508

pMoadeSUPDN Expected   1376 ATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTT-----AAATAATAATTTA 1430
                             ATAGTACTCAA+TTCCTC+ CT+TTACTTCAGAG T GATTT     AAATAATAAT+ A
pMoadeSUPDN RV          509 ATAGTACTCAAYTTCCTCWCCTSTTACTTCAGAGCTGGATTTGATTTAAATAATAATWAA  568

pMoadeSUPDN Expected   1431 ATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCGTAATAAG 1490
                             ATAA+T GCT GTCT GATCAAGCTTT T TTTTTCTTCATAG++GTGATGCGTAATAAG
pMoadeSUPDN RV          569 ATAARTCGCTCGTCTCGATCAAGCTTTTTTTTTTTCTTCATAGSWGTGATGCGTAATAAG  628

pMoadeSUPDN Expected   1491 TATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAGAGCTTAT 1550
                             TATT TAATGT+TAATGTTACTCACTTTT CT+TAGGGAGTAACTGT++TAAGAGCT+AT
pMoadeSUPDN RV          629 TATTATAATGTWTAATGTTACTCACTTTT-CTYTAGGGAGTAACTGTWWTAAGAGCTYAT  687
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pMoadeSUPDN Expected   1551 CGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGACCACTCT 1610
                            CGCTACC+TAACTCATTC+TTTCTTCT+TAGCTTGT+GTGGTGTT++ACCCG+CCACTCT
pMoadeSUPDN RV          688 CGCTACCWTAACTCATTCWTTTCTTCTSTAGCTTGTWGTGGTGTTYYACCCGMCCACTCT  747

pMoadeSUPDN Expected   1611 TTATAAGCCCGTGTAAATGGACTGTGCTCGGCATAACCTAACAGTAAAGCTATTTCTACA 1670
                            TTATAAGCCCGTGTAAATGGA+TGTGCTCGGCATAACCTAACAGTAAAGCTATTTCTACA
pMoadeSUPDN RV          748 TTATAAGCCCGTGTAAATGGAYTGTGCTCGGCATAACCTAACAGTAAAGCTATTTCTACA  807

pMoadeSUPDN Expected   1671 ATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGTATTTAAT 1730
                            ATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGTATTTAAT
pMoadeSUPDN RV          808 ATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGTATTTAAT  867

pMoadeSUPDN Expected   1731 TCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACGTGAATAA 1790
                            TCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACGTGAATAA
pMoadeSUPDN RV          868 TCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACGTGAATAA  927

pMoadeSUPDN Expected   1791 TTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTAAGCAATC 1850
                            TTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTAAGCAATC
pMoadeSUPDN RV          928 TTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTAAGCAATC  987

pMoadeSUPDN Expected   1851 TGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAACTTGTCA 1910
                            TGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAACTTGTCA
pMoadeSUPDN RV          988 TGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAACTTGTCA 1047

pMoadeSUPDN Expected   1911 GCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAGACTTAAG 1970
                            GCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAGACTTAAG
pMoadeSUPDN RV         1048 GCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAGACTTAAG 1107

pMoadeSUPDN Expected   1971 ATTTCCTTATCAAAACGCATCAGTGCCACAGGCTGTTCAAAAAGCACAGGACAACCAAAA 2030
                            ATTTCCTTATCAAAACGCATCAGTGCCACAGGCTGTTCAAAAAGCACAGGACAACCAAAA
pMoadeSUPDN RV         1108 ATTTCCTTATCAAAACGCATCAGTGCCACAGGCTGTTCAAAAAGCACAGGACAACCAAAA 1167

pMoadeSUPDN Expected   2031 TACTCCTCATAAGGCTGAACATTTTCAGGCCGTTCATGAATAAAATGTATTTCTTTGAGT 2090
                            TACTCCTCATAAGGCTGAACATTTTCAGGCCGTTCATGAATAAAATGTATTTCTTTGAGT
pMoadeSUPDN RV         1168 TACTCCTCATAAGGCTGAACATTTTCAGGCCGTTCATGAATAAAATGTATTTCTTTGAGT 1227

pMoadeSUPDN Expected   2091 CTTTCTTTTCCTCTAATGAGACTACGGCAAAATTGAACTATAACGGTACGGCCAGTTTCG 2150
                            CTTTCTTTTCCTCTAATGAGACTACGGCAAAAT GAACTATAACGGTACG CCAGT +C
pMoadeSUPDN RV         1228 CTTTCTTTTCCTCTAATGAGACTACGGCAAAAT-GAACTATAACGGTACG-CCAGTCKCC 1285

pMoadeSUPDN Expected   2151 TCCGATAAAGGGCCGACTTGCTCACCTGCATGCCCC 2186

pMoadeSUPDN RV         1286 CWT---------------------------------- 1288
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The region of pMo130-Tel^{R}/adeSUPDOWN comprising the UP and DOWN fragments was amplified by PCR (2816bp amplimer) and sequenced. The result was aligned with the expected sequence.

a) pMoUPDN FW was generated using primer pMo130-Tel^{R}FW (Table 2.6) , which is specific for pMo130-Tel^{R}.

b) pMoUPDN RV was generated using primer DOWNRVadeS (Table 2.6), which is specific for the DOWN fragment. This sequence was reverse complemented before alignment.

The first 1150bp of the sequence corresponds to 174bp of the vector and the entire UP fragment (a). The remainder of the sequence corresponds to the DOWN fragment (b).

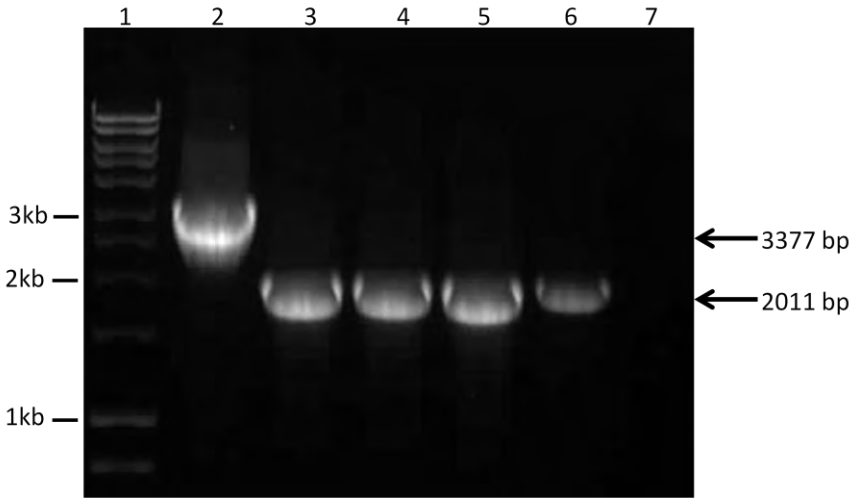
Tel^R to AYE by conjugation (section 2.4.3). After 24hrs, approximately 60 candidate transconjugants were obtained.

Sixteen candidates were subcultured onto LB plates. The colonies of two of these were light yellow in colour and turned the media yellow; the rest exhibited normal *A. baumannii* growth. All candidates were sprayed with pyrocatechol and yellow colonies selected. The two yellow subcultures turned a deeper shade of yellow; the white colonies remained white. The yellow colonies had undergone single recombination. To select double recombinants, a single colony of each yellow subculture was cultured in LB broth (-NaCl) containing 10% sucrose. Serial dilutions were plated to obtain single colonies (Section 2.4.3). After five passages in LB supplemented with sucrose, white colonies were obtained. Four, well isolated white colonies were selected for verification. All candidates were inhibited on LB plates containing tellurite (30 g/ml), suggesting that they no longer contained the suicide vector pMo130-Tel^R. The deletion in the four candidates was verified by PCR using AYE wild-type genomic DNA as a negative control (Figure 3.14). The PCR amplimer obtained for candidate 4 (Figure 3.14, Lane 6) was sequenced and the results confirmed that *adeRS* had been deleted (Figure 3.15; Figure 3.16). It was initially planned that just *adeS* would be deleted. However, *adeS,* 126 bp of *adeR* and 98 bp of a gene encoding a hypothetical protein (ABAYE1818) were deleted because the primers, which were designed to delete the whole of *adeS*, also flanked part of *adeR* and ABAYE1818 (Figure 3.15).

3.3.1.2. Complementation of *adeRS* Gene Deletion

The fragment containing the deleted region (including *adeS* and 126 bp of *adeR*) and the UP and DOWN fragments used to delete *adeRS* were amplified by PCR (Figure 3.17; Section 2.4.5) This amplimer and pMo130-Tel^R were digested with *Not*I and *Bam*HI

Figure 3.14 Verification of *adeRS* Gene Deletion in AYE by PCR.



Lane	Template	Expected size	Actual size
1	Hyperladder™ I (Bioline)	---	---
2	AYE (wild-type) genomic DNA	3377bp	3377bp
3	Candidate mutant 1	2011bp	2011bp
4	Candidate mutant 2	2011bp	2011bp
5	Candidate mutant 3	2011bp	2011bp
6	Candidate mutant 4	2011bp	2011bp
7	---	No amplimer	No amplimer

PCR number 18, Table 2.7

Figure 3.15 Region Around *adeS* in AYE and AYEΔ*adeRS* (expected sequences) and Primers used to Delete and Complement *adeRS*.

Key for the following DNA sequences:

Deleted region

adeR
-35: ttcaca
-10: tatata
Shine dalgano: agccaga

adeS
-10: tataa
-35: ttgctt
Shine dalgarno: aggga

ABAYE1818 Gene encoding a hypothetical protein (xBASE)

UP primers.
UPFWadeS (Contains *Not*I recognition site)
GGGGCGGCCGCCCTCCGACTTGCGGACGGAT

UPRVadeS (Contains *Bam*HI recognition site)
GGGGGATCCGGTTCGCTCTAGTGCATCGC

DOWN primers

DOWNFWadeS (Contains *Bam*HI recognition site)
GGGGGATCCAAGCTGTAACCGCAGCGCCA

DOWNRVadeS (Contains *Sph*I recognition site)
GGGGCATGCAGGTGAGCAAGTCGGCCCTT

UPFWadeS was also used to amplify the UP-*adeRS*-DOWN insert (from wild-type AYE) for the complementation construct (Section 2.4). The reverse primer (adeSRVcomp, Table 2.6) contained the same chromosome-homologous sequence as DOWNRVadeS, but contained a *Bam*HI restriction site, instead of *Sph*I. This was because there was an *Sph*I restriction site within *adeS*.

a) Expected Sequence for Wild-type AYE.

ggccagcaatgctttcatttgagcgacatcggctagagcctgacgatactgagcttgagc
attacttacttcttgcttactaaccgcattacttggtaatagctgctcataacgttctaa
ctgaactttgagtcttgccacctcagcttcagctttattgagagaagctctattgctatt
tacatcggcctcaaaagtctcggaattaatttatataaagcttgccctgctctaacttc
actaccttgtttaaatagaacctttcaataatacctccgacttgcggacggattccgc
cgtacggaacgcatgtactcgtgcaggaagattttcactaaaatttaccgactgcggttg
aatgcttaatacactgactttagccggtggtggctcagcttgagcgacttcttttgaatc
acacccctgtaatatcagcccaatagataaaaataaaggaagtaaaagatgcttttgcat
actgtccaaacctagtgacttttgatgttcgtattatttttgatgagtgtgtagggata
atcactaaagtgtggagtaagtgtggagaaatacggataatttagcgtatgatgagttga
agcactttctatagccagattttctatgtttgatcattctttttcttttgattgccaaga
TAAAGTTATTCTTGTGGTAGAAGATGACTACGATATTGGCGACATTATTGAAAATTATTT
AAAACGTGAAGGCATGAGTGTTATTCGGGCCATGAATGGAAAGCAAGCGATTGAATTGCA
CGCTAGCCAACCCATCGATTTAATCTTACTTGATATTAAATTACCCGAATTAAACGGTTG
GGAAGTATTAAATAAAATACGCCAAAAAGCTCAGACTCCCGTGATCATGTTGACGGCGCT
AGATCAAGATATTGATAAAGTTATGGCATTACGCATAGGTGCAGATGACTTTGTGGTGAA
GCCTTTTAACCCAAATGAAGTCGTCGCTAGAGTTCAGGCAGTCCTAAGACGTACTCAGTT
TGCAAACAAAGCAACTAATAAAAATAAACTCTATAAAAATATTGAAATTGATACCGACAC
TCATAGCGTTTATATACACTCTGAGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGA
ATATAAAATTATTTCATTCATGATTGATCAGCCTCATAAAGTTTTTACGCGCGGAGAGCT
TATGAATCACTGCATGAATGATAGCGATGCACTAGAGCGAACC***GTAGATAGCCATGTGAG***
TAAGCTGAGAAAAAAACTAGAAGAACAAGGCATATTTCAAATGTTAATTAATGTGCGTGG
CGTGGGATATAGACTAGATAATCCCCTAGCTGTAAAAGATGATGCCTAA*ataatattaaa*
aaatagct*agggaatattttATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTG*
CCTTAACTATTGTGAATTTAAGCGTTACGCTATTTTCTATAGTATTGGGTTATATCATTT
ATAACTATGCGATTGAAAAAGGCTGGATTAGCTTAAGCTCATTTCAACAAGAAGATTGGA
CCAGTTTTCATTTTGTAGACTGGATCTGGTTAGCCACTGTTATCTTCTGTGGCTGTATTA
TTTCATTAGTGATTGGCATGCGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAG
TCGAAGCAGCAAAAAAAATTAGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAA
TTCACTCCGCCGAAATGTCGGAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAG
AGGTTTCCGTCAAAAATGCGCAGGTTTGGAATGCAGCTATCGCACATGAGTTAAGAACGC
CTATAACGATATTACAAGGTCGTTTACAGGGAATTATTGATGGCGTTTTTAAACCTGATG
AAGTCCTATTTAAAAGCCTTTTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACT
TACGGACTTTAAGCTTAGTAGAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACT
TGAAGGCGGTAGTTGAAAAAGTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGC
TAGTACCAGAACTTGACCTAACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGC
AAGTTTTAATTGCTTTAATTGATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAA
TCTCTTCAGAAGTGGTTGCAGACAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCA
TTGCAACCGAGTTTCGGGACGATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGA
ATAAAGAATTTGGCGGCACAGGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGGCAC
TGAAAGGCACTATTCAATATAGCAATCAAGGCTCGAAAAGTGTTTTCACCATAAAAATTT
CTATGGGTCATGAAGAGATGGGGTAA*ttcgctaaattaaaaaatcttagagttaaagtgc*
ccctcactctcttttattcttctacgaatttcttctcgccattttgtggcattttcctg
ttgtttgtttaataggacacctaacatataagctgtaaccgcagcgccaattaaggctat
accggtttcataaataatatctataacaaattcgagcactccctccgacaaaaaatctaa
tgagcctgaaaatttaagaaatttagctatggcaaccactataactcccaataaagcgcc
agcccataagaatattttatgaatattgataggtgccgtgacttgatcgatttgatcggg
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aatctgtttacgaacttgatctgtaatttcatcaatatgtggcaggcttgccagtaactt
gtcagcatgtttttctagtatggcaactaaggcggcgtcggagttttaagcggtagact
taagatttccttatcaaaacgcatcagtgccacaggctgttcaaaaagcacaggacaacc

aaaatactcctcataaggctgaacattttcaggccgttcatgaataaaatgtatttcttt
gagtctttctttttcctctaatgagactacggcaaaattgaactataacggtacggccagt
ttcgtccgataaagggccgacttgctcacctttgtatcccatgaaagctctacatattc
agtataaatacgtacagtggcaggataaacatcatagacaagccgctgatatcgctcaaa
cctttctaaaaccgccccagattttcacaggcaagaaagactgaccctaaaacacctag
atgctgagctgtaatggtttgacccacatgtaaaccaatgaaagggtcatttaaataatc
tttggcaataatgagaagactttcccaatgatcgatatctacgccttctaaatgatttga
atctgctttaggccacggttcacccaatacctttctgggtcatgtcctagttttccag

b) Expected Sequence for AYEΔ*adeRS*.

cctccgacttgcggacggattccgc
cgtacggaacgcatgtactcgtgcaggaagattttcactaaaatttaccgactgcggttg
aatgcttaatacactgactttagccggtggtggctcagcttgagcgacttcttttgaatc
acacccctgtaatatcagcccaatagataaaaataaaggaagtaaaagatgcttttgcat
actgtccaaacctagtgacttttgatgttcgtattatttttgatgagtgtgtagggata
atcactaaagtgtggagtaagtgtggagaaatacggataatttagcgtatgatgagttga
agcactttctatagccagattttctatgtttgatcattctttttctttgattgccaaga
TAAAGTTATTCTTGTGGTAGAAGATGACTACGATATTGGCGACATTATTGAAAATTATTT
AAAACGTGAAGGCATGAGTGTTATTCGGGCCATGAATGGAAAGCAAGCGATTGAATTGCA
CGCTAGCCAACCCATCGATTTAATCTTACTTGATATTAAATTACCCGAATTAAACGGTTG
GGAAGTATTAAATAAAATACGCCAAAAAGCTCAGACTCCCGTGATCATGTTGACGGCGCT
AGATCAAGATATTGATAAAGTTATGGCATTACGCATAGGTGCAGATGACTTTGTGGTGAA
GCCTTTTAACCCAAATGAAGTCGTCGCTAGAGTTCAGGCAGTCCTAAGACGTACTCAGTT
TGCAAACAAAGCAACTAATAAAAATAAACTCTATAAAAATATTGAAATTGATACCGACAC
TCATAGCGTTTATATACACTCTGAGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGA
ATATAAAATTATTTCATTCATGATTGATCAGCCTCATAAAGTTTTTACGCGCGGAGAGCT
TATGAATCACTGCATGAATGATAGCGATGCACTAGAGCGAACC***GGATCC***
aagctgtaaccgcagcgccaattaaggctat
accggtttcataaataatatctataacaaattcgagcactccctccgacaaaaaatctaa
tgagcctgaaaatttaagaaatttagctatggcaaccactataactcccaataaagcgcc
agcccataagaatattttatgaatattgataggtgccgtgacttgatcgatttgatcggg
gtactttcgatagtactcaatttcctcatctgttacttcagaggtagatttaaataataa
tttaataaatagcttgtcttgatcaagctttgtatttttcttcatagctgtgatgcgtaa
taagtattctaatgtttaatgttactcacttttttctttagggagtaactgttttaagagc
ttatcgctaccttaactcattcatttcttctctagcttgttgtggtgttttacccgacca
ctctttataagcccgtgtaaatggactgtgctcggcataacctaacagtaaagctatttc
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taattctttccgaaaattcgtacctgcctcggttaaacggcgttgcaaggttctacgtga
ataatttaatcgctctgcaagctgttcaatagttggttcgccttgatgaagtaagtaagc
aatctgtttacgaacttgatctgtaatttcatcaatatgtggcaggcttgccagtaactt
gtcagcatgtttttctagtatggcaactaaggcggcgtcggagttttaagcggtagact
taagatttccttatcaaaacgcatcagtgccacaggctgttcaaaaagcacaggacaacc
aaaatactcctcataaggctgaacattttcaggccgttcatgaataaaatgtatttcttt
gagtctttctttttcctctaatgagactacggcaaaattgaactataacggtacggccagt
ttcgtccgataaagggccgacttgctcacct

It was expected that a *Bam*HI site (***GGATCC***) would be introduced in AYEΔ*adeRS*.

Figure 3.16 Verification of *adeRS* Gene Deletion in AYE by DNA Sequencing.

a) Forward Sequencing

```
adeS mutant expected      1 GGGGCGGCCGCCCTCCGACTTGCGGACGGATTTCCGCC--GTACGGAACGCATGTACTCG   58
                                                      TTC GCC  GTACGGAACGCATGTACTCG
adeS mutant FW            1 --------------------------------TTCGGCCCGGTACGGAACGCATGTACTCG   29

adeS mutant expected     59 TGCAGGAAGATTTTCACTAAAATTTACCGACTGCGGTTGAATGCTTAATACACTGACTTT  118
                            TGCAGGAAGA+TTTCACTAAAATTTACCGACTG+GGTTGAATG+TTAATACACTGACTTT
adeS mutant FW           30 TGCAGGAAGAWTTTCACTAAAATTTACCGACTGSGGTTGAATGSTTAATACACTGACTTT   89

adeS mutant expected    119 AGCCGGTGGTGGCTCAGCTTGAGCGACTTCTTTTGAATCACACCCCTGTAATATCAGCCC  178
                            AGCCGGTGG+GGCTCAGCTTGAGCGACTTCTTTTGAATCACACCCCTG+AATATCAGCCC
adeS mutant FW           90 AGCCGGTGGKGGCTCAGCTTGAGCGACTTCTTTTGAATCACACCCCTGKAATATCAGCCC  149

adeS mutant expected    179 AATAGATAAAAATAAAGGAAGTAAAAGATGCTTTTGCATACTGTCCAAACCTAGTGACTT  238
                            AATA+ATAAAAATAAAGGAAGTAAAAGATGCTTTTGCATACTGTCCAAACCTAGTGACTT
adeS mutant FW          150 AATARATAAAAATAAAGGAAGTAAAAGATGCTTTTGCATACTGTCCAAACCTAGTGACTT  209

adeS mutant expected    239 TTTGATGTTCGTATTATTTTTGATGAGTGTGTAGGGATAATCACTAAAGTGTGGAGTAAG  298
                            TTTGATGTTCGTATTATTTTTGATGA+TGTGTAGGGATAATCACTAAAGTGTGGA+TAAG
adeS mutant FW          210 TTTGATGTTCGTATTATTTTTGATGARTGTGTAGGGATAATCACTAAAGTGTGGARTAAG  269

adeS mutant expected    299 TGTGGAGAAATACGGATAATTTAGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATT  358
                            TGTGGA+AAATACGGA+AATTTAGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATT
adeS mutant FW          270 TGTGGARAAATACGGAWAATTTAGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATT  329

adeS mutant expected    359 TTCTATGTTTGATCATTCTTTTTCTTTTGATTGCCAAGATAAAGTTATTCTTGTGGTAGA  418
                            TTCTATGTTTGA+CATTCTTTTTCTTTTGATTGCCAAGA+AAAGTTATTCTTG+GGTA+A
adeS mutant FW          330 TTCTATGTTTGAWCATTCTTTTTCTTTTGATTGCCAAGAWAAAGTTATTCTTGKGGTARA  389

adeS mutant expected    419 AGATGACTACGATATTGGCGACATTATTGAAAATTATTTAAAACGTGAAGGCATGAGTGT  478
                            AGA+GACTACGATATTGGCGACATTATTGAAAATTATTTAAAACGTGAAGGCATGAGTGT
adeS mutant FW          390 AGAWGACTACGATATTGGCGACATTATTGAAAATTATTTAAAACGTGAAGGCATGAGTGT  449

adeS mutant expected    479 TATTCGGGCCATGAATGGAAAGCAAGCGATTGAATTGCACGCTAGCCAACCCATCGATTT  538
                            TATTCGGGCCATGAATGGAAAGCAAGCGATTGAATTGCACGCTA+CCAACCCATCG+TTT
adeS mutant FW          450 TATTCGGGCCATGAATGGAAAGCAAGCGATTGAATTGCACGCTARCCAACCCATCGRTTT  509

adeS mutant expected    539 AATCTTACTTGATATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAATAAAATACG  598
                            AATCTTACTTGATATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAATAAAATACG
adeS mutant FW          510 AATCTTACTTGATATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAATAAAATACG  569

adeS mutant expected    599 CCAAAAAGCTCAGACTCCCGTGATCATGTTGACGGCGCTAGATCAAGATATTGATAAAGT  658
                            CCAAAAAGCTCAGA+TCCCGTGATCATGTTGA+GGCGCTAGATCAAGATATTGATAAAGT
adeS mutant FW          570 CCAAAAAGCTCAGAMTCCCGTGATCATGTTGASGGCGCTAGATCAAGATATTGATAAAGT  629

adeS mutant expected    659 TATGGCATTACGCATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGT  718
                            TATGGCATTACGCATAGGTGCAGA+GACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGT
adeS mutant FW          630 TATGGCATTACGCATAGGTGCAGAWGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGT  689

adeS mutant expected    719 CGTCGCTAGAGTTCAGGCAGTCCTAAGACGTACTCAGTTTGCAAACAAAGCAACTAATAA  778
                            CGTCGCTAGAGTTCAGG+AGTCCTAAGACGTACTCAGTTTG+AAACAAAGCAACTAATAA
adeS mutant FW          690 CGTCGCTAGAGTTCAGGSAGTCCTAAGACGTACTCAGTTTGYAAACAAAGCAACTAATAA  749

adeS mutant expected    779 AAATAAACTCTATAAAAATATTGAAATTGATACCGACACTCATAGCGTTTATATACACTC  838
                            AAATAAACTCTATAAAAATATTGAAATTGATACCGACACTCATAGCGTTTATATACACTC
adeS mutant FW          750 AAATAAACTCTATAAAAATATTGAAATTGATACCGACACTCATAGCGTTTATATACACTC  809

adeS mutant expected    839 TGAGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGAATATAAAATTATTTCATTCAT  898
                            TGAGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGAA+ATAAAATTATTTCATTCAT
adeS mutant FW          810 TGAGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGAAKATAAAATTATTTCATTCAT  869

adeS mutant expected    899 GATTGATCAGCCTCATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGCATGAATGA  958
                            GATTGATC++CCTCATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGC+TGAATGA
adeS mutant FW          870 GATTGATCRSCCTCATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGCRTGAATGA  929
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```
adeS mutant expected    959 TAGCGATGCACTAGAGCGAACCGGATCCAAGCTGTAACCGCAGCGCCAATTAAGGCTATA 1018
                            TAGCGATGCACTAGA+CGAACCGGATCCAAGCTGTAACCGCAGCGCCAAT AAGGCTA+A
adeS mutant FW          930 TAGCGATGCACTAGAKCGAACCGGATCCAAGCTGTAACCGCAGCGCCAAT-AAGGCTAWA  988

adeS mutant expected   1019 CCGGTTTCATAAATAATATCTATAACAAATTCGAGCACTCCCTCCGACAAAAAATCTAAT 1078
                            CCGGTT CATAA+TAA+AT+TATAACAA TTCGAGCACTCCCTCCGAC+AAA+  CTAAT
adeS mutant FW          989 CCGGTT-CATAARTAAYATSTATAACAA-TTCGAGCACTCCCTCCGACRAAAW--CTAAT 1044

adeS mutant expected   1079 GAGCCTGAAAATTTAAGAAATTTAGCTATGGCAACCACTATAACTCCCAATAAAGCGCCA 1138
                            GAGC    AAATT+A+ AAATTTAGCTATGGCA+C++ TA+A    CC A +AAGC+C A
adeS mutant FW         1045 GAGCT--GAAATTYAR-AAATTTAGCTATGGCARCMM-TAYA--CTCCGAAWAAGCKC-A 1097

adeS mutant expected   1139 GCCCATAAGAATATTTTATGAATATTGATAGGTGCCGTGACTTGATCGATTTGATCGGGG 1198
                            GCCCATA++AA+ TTTTA+GAATAT GATAGG+GC GTGACT G+TCGATT GATCGGG
adeS mutant FW         1098 GCCCATAMRAAW-TTTTAWGAATAT-GATAGGKGC-GTGACT-GRTCGATT-GATCGGG- 1151

adeS mutant expected   1199 TACTTTCGATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTTAAATAATAAT 1258
                            TAC+T CGA+ GTACTCA T  +++C    G TACT    A GTAGAT    A +A A T
adeS mutant FW         1152 TACKT-CGAW-GTACTCAGTCCYMWC----GGTACTC--AAAGTAGAT---GACWAGATT 1200

adeS mutant expected   1259 TTAATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCGTAAT 1318
                            T AA +A T GC TG    GA   AGCT       TTTCT ++     G+  +GCGTAA
adeS mutant FW         1201 TCAAARACTGGCCTG----GAC--AGCT----GACTTTCTCMRC----GKTGWGCGTAAA 1246

adeS mutant expected   1319 AAGTATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAGAGCT 1378
                            A+GTAT C AA G +TAA GTT C+C C
adeS mutant FW         1247 ARGTATCC-AAAGGWTAAGGTTKCWCCCAC------------------------------ 1275

adeS mutant expected   1379 TATCGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGACCAC 1438

adeS mutant FW              ------------------------------------------------------------
```

b) Reverse Sequencing

```
adeS mutant expected    661 TGGCATTACGCATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGTCG  720

adeS RV Sequenced           ------------------------------------------------------------

adeS mutant expected    721 TCGCTAGAGTTCAGGCAGTCCTAAGACGTACTCAGTTTGCAAACAAAGCAACTAATAAAA  780
                                  GAGTT  GG AG C T A    TA T AG  T CA    A G A   A T   A
adeS RV Sequenced         1 ---TGCGAGTTGTGGAAGCCTTTACCAATAATGAGAGTTCACCTTACGTA--CAGTTGCA   55

adeS mutant expected    781 ATAAACTCTATAAAAATATTGAAATTGATACCGACACTCATAGCGTTTATATACACTCTG  840
                                A T TA   A  TAT  AAATTGAT C    ACTCATAGCGT   TAT   CTC
adeS RV Sequenced        56 CAGCATTATACTTACTTAT-AAAATTGATGCG---ACTCATAGCGT---TAT---CTC--  103

adeS mutant expected    841 AGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGAATATAAAATTATTTCATTCATGA  900
                              AATAAGA GATCT    TAATCTGACG TGACTGAAT TAAA    TTTCAT CATGA
adeS RV Sequenced       104 --AATAAGA-GATCTG--CTAATCTGACG-TGACTGAAT-TAAA----TTTCAT-CATGA  151

adeS mutant expected    901 TTGATCAGCCTCATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGCATGAATGATA  960
                            T GATCAGCCTCATAAAGTTTT ACGCGCG AGAGCT ATGAATCACTGCATGAATGATA
adeS RV Sequenced       152 T-GATCAGCCTCATAAAGTTTT-ACGCGCG-AGAGCT-ATGAATCACTGCATGAATGATA  207

adeS mutant expected    961 GCGATGCACTAGAGCGAACCGGATCCAAGCTGTAACCGCAGCGCCAATTAAGGCTATACC 1020
                            GCGATGCACTAGAGCGAAC GGATCCAAGCTGTAACCGCAGCGCCAATTAAGGCTATACC
adeS RV Sequenced       208 GCGATGCACTAGAGCGAAC-GGATCCAAGCTGTAACCGCAGCGCCAATTAAGGCTATACC  266

adeS mutant expected   1021 GGTTTCATAAATAATATCTATAACAAATTCGAGCACTCCCTCCGACAAAAAATCTAATGA 1080
                            GGTTTCATAAATAATATCTATAACAAATTCGAGCACTCC TCCGACAAAAA TCTAATGA
adeS RV Sequenced       267 GGTTTCATAAATAATATCTATAACAAATTCGAGCACTCC-TCCGACAAAAA-TCTAATGA  324

adeS mutant expected   1081 GCCTGAAAATTTAAGAAATTTAGCTATGGCAACCACTATAACTCCCAATAAAGCGCCAGC 1140
                            GCCTGAAAATT AAGAAATT AGCTATGGCAACCACTATAACTCCCAATAAAGCGCCAGC
adeS RV Sequenced       325 GCCTGAAAATT-AAGAAATT-AGCTATGGCAACCACTATAACTCCCAATAAAGCGCCAGC  382

adeS mutant expected   1141 CCATAAGAATATTTTATGAATATTGATAGGTGCCGTGACTTGATCGATTTGATCGGGGTA 1200
                            CCATAAGAATATTTTATGAATAT GATAGGTGCCGTGACTTGATCGATTTGATCGGGGTA
adeS RV Sequenced       383 CCATAAGAATATTTTATGAATAT-GATAGGTGCCGTGACTTGATCGATTTGATCGGGGTA  441

adeS mutant expected   1201 CTTTCGATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTTAAATAATAATTT 1260
                            CTTTCGATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTTAAATAATAATTT
adeS RV Sequenced       442 CTTTCGATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTTAAATAATAATTT  501

adeS mutant expected   1261 AATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCGTAATAA 1320
                            AATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCGTAATAA
adeS RV Sequenced       502 AATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCGTAATAA  561

adeS mutant expected   1321 GTATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAGAGCTTA 1380
                            GTATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAGAGCTTA
adeS RV Sequenced       562 GTATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAGAGCTTA  621

adeS mutant expected   1381 TCGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGACCACTC 1440
                            TCGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGACCACTC
adeS RV Sequenced       622 TCGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGACCACTC  681

adeS mutant expected   1441 TTTATAAGCCCGTGTAAATGGACTGTGCTCGGCATAACCTAACAGTAAAGCTATTTCTAC 1500
                            TTTATAAGCCCGTGTAAATGGACTGTGCTCGGCATAACCTAACAGTAAAGCTATTTCTAC
adeS RV Sequenced       682 TTTATAAGCCCGTGTAAATGGACTGTGCTCGGCATAACCTAACAGTAAAGCTATTTCTAC  741

adeS mutant expected   1501 AATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGTATTTAA 1560
                            AATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGTATTTAA
adeS RV Sequenced       742 AATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGTATTTAA  801

adeS mutant expected   1561 TTCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACGTGAATA 1620
                            TTCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACGTGAATA
adeS RV Sequenced       802 TTCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACGTGAATA  861

adeS mutant expected   1621 ATTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTAAGCAAT 1680
                            ATTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTAAGCAAT
```

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adeS RV Sequenced       862 ATTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTAAGCAAT  921

adeS mutant expected   1681 CTGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAACTTGTC 1740
                             CTGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAACTTGTC
adeS RV Sequenced       922 CTGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAACTTGTC  981

adeS mutant expected   1741 AGCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAGACTTAA 1800
                             AGCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAGACTTAA
adeS RV Sequenced       982 AGCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAGACTTAA 1041

adeS mutant expected   1801 GATTTCCTTATCAAAACGCATCAGTGCCACAGGCTGTTCAAAAAGCACAGGACAACCAAA 1860
                             GATTTCCTTATCAAAACGCATCAGTGCCACAGGCTGTTCAAAAAGCACAGGACAACCAAA
adeS RV Sequenced      1042 GATTTCCTTATCAAAACGCATCAGTGCCACAGGCTGTTCAAAAAGCACAGGACAACCAAA 1101

adeS mutant expected   1861 ATACTCCTCATAAGGCTGAACATTTTCAGGCCGTTCATGAATAAAATGTATTTCTTTGAG 1920
                             ATACTCCTCATAAGGCTGAACATTTTCAGGCCGTTCATGAATAAAATGTATTTCTTTGAG
adeS RV Sequenced      1102 ATACTCCTCATAAGGCTGAACATTTTCAGGCCGTTCATGAATAAAATGTATTTCTTTGAG 1161

adeS mutant expected   1921 TCTTTCTTTTCCTCTAATGAGACTACGGCAAAATTGAACTATAACGGTACGGCCAGTTTC 1980
                             TCTT CTTTTCCTCTAATGAGACTACGGCAAAATTGAACTATAACGGTACGGCCAGTT
adeS RV Sequenced      1162 TCTT-CTTTTCCTCTAATGAGACTACGGCAAAATTGAACTATAACGGTACGGCCAGTT-- 1218

adeS mutant expected   1981 GTCCGATAAAGGGCCGACTTGCTCACCTGCATGCCCC 2017
                               CCGA
adeS RV Sequenced      1219 -ACCGAA----------------------------- 1224
```

The region of the AYEΔ*adeRS* genome flanking *adeS* (UP and DOWN fragments) was amplified by a single PCR (2017 bp amplimer) and sequenced. The result was aligned with the expected sequence (Figure 3.15b).

a) *adeS* FW Sequenced was generated using primer UPFWadeS (Table 2.6); b) *adeS* RV Sequenced was generated using primer DOWNRVadeS (Table 2.6). This sequence was reverse complemented before alignment.

The first 980bp of the sequence corresponds to the UP fragment and 987-2017 bp corresponds to the DOWN fragment. A *Bam*HI site has been introduced at 981-986 bp in the mutant (highlighted).

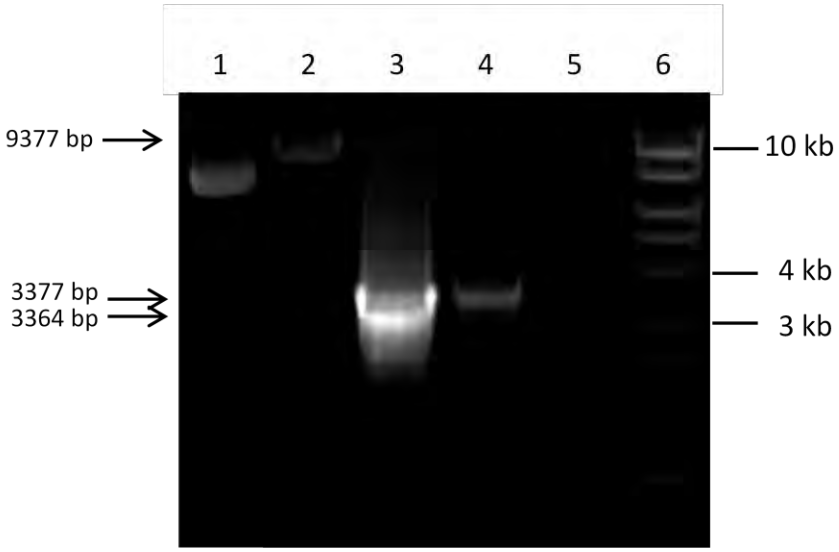
(Table 2.8, Digestion 3; Figure 3.17) and ligated. The ligation mixture was transformed into DH5α and three candidate transformants were obtained, which were all confirmed by PCR to harbour the correct construct (Figure 3.18). The construct from candidate 1 was further verified by digestion and DNA sequencing. Digestion with *Not*I (Figure 3.19) confirmed that the construct contained an insert. DNA sequencing confirmed that the insert contained the UP and DOWN fragments, *adeS* and the deleted 126 bp of *adeR* (Figure 3.20). The verified construct was transformed into S17-1 and four candidate transformants were obtained. Plasmids were extracted from each transformant and their identities were verified by PCR (Figure 3.21).

Despite 12 attempts at conjugation and modifications to the method (detailed below), no AYEΔ*adeRS*/pMo130-Tel^{R}/UP-*adeRS*-DOWN single recombinants were obtained and AYEΔ*adeRS* was not complemented. It is important to note that for all of the following experiments, both the donor and recipient were inhibited by the selective plates and that AYE/pMo130-Tel^{R}/*adeRS*UPDOWN (AYE with the *adeRS* deletion construct in the chromosome) was used as a positive control for pyrocatechol exposure.

Variation in the ratio of donor to recipient:

Initially, two conjugations between S17-1/pMo130-Tel^{R}/UP-*adeRS*-DOWN and AYEΔ*adeRS* were carried out, one according to the original method (Section 2.4.3) and the second using double the volume of donor and recipient (same ratio) to increase the chance of obtaining transconjugants. A total of 22 colonies were obtained in these two experiments (12 for experiment 1 and 10 for experiment 2), which remained white when exposed to pyrocatechol,

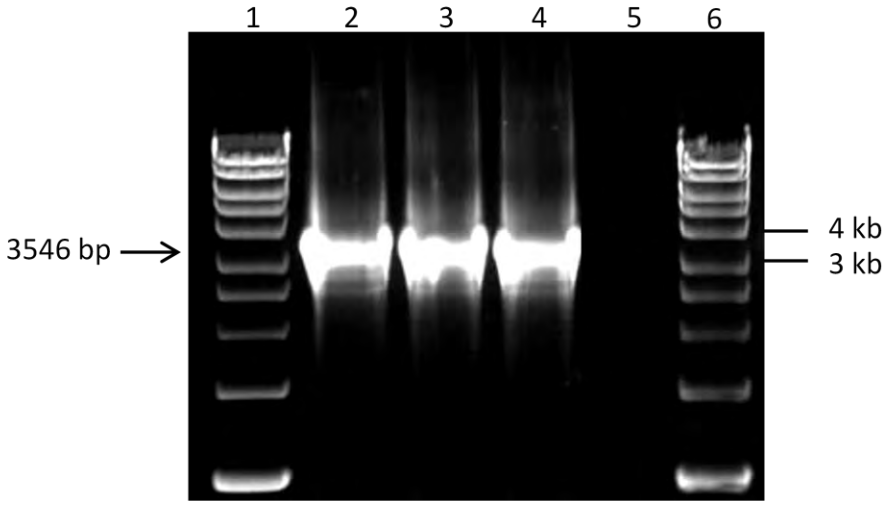
Figure 3.17 Digestion of pMo130-TelR and UP-*adeS*-DOWN



Lane	DNA	Expected size	Actual size
1	pMo130-Tel^{R} undigested	---	---
2	pMo130-Tel^{R} + *Not*I + *Bam*HI	9377bp	~ 9377bp
3	UP-*adeRS*-DOWN undigested	3377 bp	~ 3377 bp
4	UP-*adeRS*-DOWN + *Not*I + *Bam*HI	3364 bp	~ 3364 bp
5	--- (Contamination control for UP-*adeRS*-DOWN amplification)	No amplimer	No amplimer
6	Hyperladder™ 1 (Bioline)	---	---

Lanes 3 and 5, PCR 19, Table 2.7; Lanes 2 and 4, Digestion 3, Table 2.8.

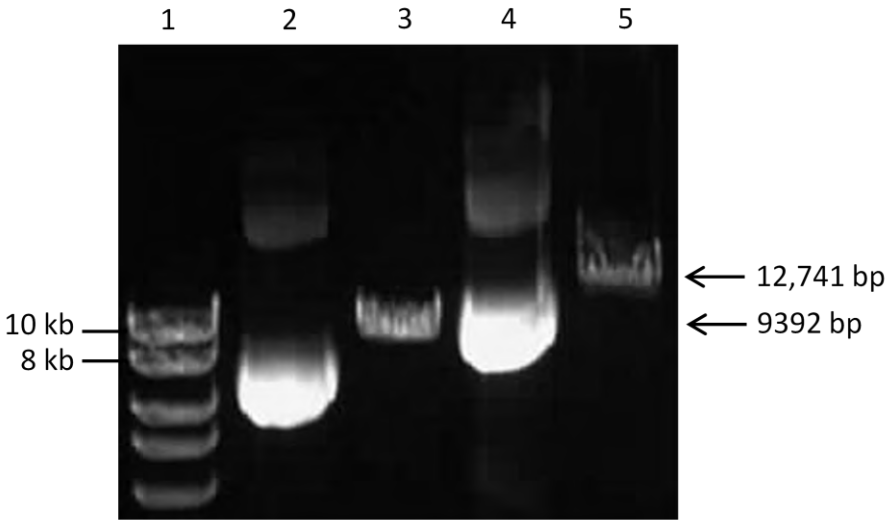
Figure 3.18 Verification of *adeRS* Complementation Construct by PCR.



Lane	DNA	Expected size	Actual size
1, 6	Hyperladder™ 1 (Bioline)	---	---
2	Candidate transformant 1	3546 bp	~ 3546 bp
3	Candidate transformant 3	3546 bp	~3546 bp
4	Candidate transformant 4	3546 bp	~ 3546 bp
5	---	No amplimer	No amplimer

PCR 16, Table 2.7

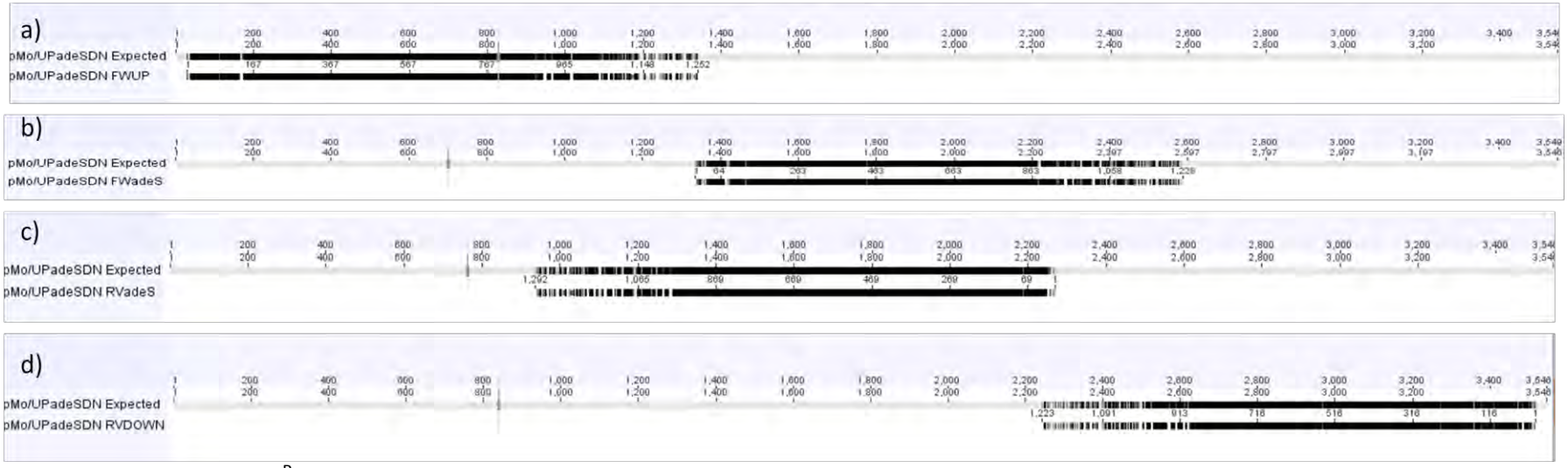
Figure 3.19 Verification of *adeRS* Complementation Construct by Restriction Digestion.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ 1 (Bioline)	---	---
2	pMo130-Tel^{R} undigested	---	---
3	pMo130-Tel^{R} + *Not*I	9392 bp	~ 9392 bp
4	PMo130-Tel^{R}/UP-*adeRS*-DOWN undigested	---	---
5	PMo130-Tel^{R}/UP-*adeRS*-DOWN + *Not*I	12,741 bp	~ 12,741 bp

Digestion 2, Table 2.8

Figure 3.20 Verification of *adeRS* Complementation Construct by DNA Sequencing.



A region of pMo130-Tel^{R} containing *the* UP-*adeRS*-DOWN complementation fragment was amplified and sequenced (the whole amlimer is shown above in four independent sequencing reactions a-d). The result was aligned with the expected sequence.
a) pMo/UPadeSDN FWUP was generated using primer pMo130-Tel^{R} FW, which is specific for pMo130-Tel^{R}.
b) pMo/UPadeSDN FWadeS was generated using primer adeSgeneFW, which binds inside *adeS*.
c) pMo/UPadeSDN RVadeS was generated using primer adeSgeneRV, which binds inside *adeS.* This sequence was reverse complemented before alignment.
d) pMo/UPadeSDN RVDOWN was generated using primer DOWNRVadeS, which is specific for the DOWN fragment. This sequence was reverse complemented before alignment.
The first 1149 bp of the sequence corresponds to 174 bp of the vector and the entire UP fragment (including 126bp of *adeR*); 1307-2392 bp corresponds to *adeS*; 2516-3546 bp corresponds to the DOWN fragment.

The sequences of the above figure (a-d) are shown below.

a) Forward sequencing of *adeRS* complementation construct (1).

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pMo/UPadeSDN Expected      1 CCATCTACTTCTTCGACCCGTCCGGTAACCGCAACGAAGTGTTCTGCGGGGGAGATTACA   60
                                                    GCA+ + AGTGTTCTGCGGGGGAGATTACA
pMo/UPadeSDN FWUP          1 ----------------------------TTGCAWASGAGTGTTCTGCGGGGGAGATTACA   32

pMo/UPadeSDN Expected     61 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT  120
                              ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGG+GATCTTTT
pMo/UPadeSDN FWUP         33 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGSGATCTTTT   92

pMo/UPadeSDN Expected    121 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGACCTGAGCGGCCGC  180
                              ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGA     GCGGCCGC
pMo/UPadeSDN FWUP         93 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGA-----GCGGCCGC  147

pMo/UPadeSDN Expected    181 CCTCCGACTTGCGGACGGATTTCCGCCGTACGGAACGCATGTACTCGTGCAGGAAGATTT  240
                              CCTCCGACTTGCGGACGGATTTCCGCCGTACGGAACGCATGTACTCGTGCAGGAAGATTT
pMo/UPadeSDN FWUP        148 CCTCCGACTTGCGGACGGATTTCCGCCGTACGGAACGCATGTACTCGTGCAGGAAGATTT  207

pMo/UPadeSDN Expected    241 TCACTAAAATTTACCGACTGCGGTTGAATGCTTAATACACTGACTTTAGCCGGTGGTGGC  300
                              TCACTAAAATTTACCGACTGCGGTTGAATGCTTAATACACTGACTTTAGCCGGTGGTGGC
pMo/UPadeSDN FWUP        208 TCACTAAAATTTACCGACTGCGGTTGAATGCTTAATACACTGACTTTAGCCGGTGGTGGC  267

pMo/UPadeSDN Expected    301 TCAGCTTGAGCGACTTCTTTTGAATCACACCCCTGTAATATCAGCCCAATAGATAAAAAT  360
                              TCAGCTTGAGCGACTTCTTTTGAATCACACCCCTGTAATATCAGCCCAATAGATAAAAAT
pMo/UPadeSDN FWUP        268 TCAGCTTGAGCGACTTCTTTTGAATCACACCCCTGTAATATCAGCCCAATAGATAAAAAT  327

pMo/UPadeSDN Expected    361 AAAGGAAGTAAAAGATGCTTTTGCATACTGTCCAAACCTAGTGACTTTTTGATGTTCGTA  420
                              AAAGGAAGTAAAAGATGCTTTTGCATACTGTCCAAACCTAGTGACTTTTTGATGTTCGTA
pMo/UPadeSDN FWUP        328 AAAGGAAGTAAAAGATGCTTTTGCATACTGTCCAAACCTAGTGACTTTTTGATGTTCGTA  387

pMo/UPadeSDN Expected    421 TTATTTTTGATGAGTGTGTAGGGATAATCACTAAAGTGTGGAGTAAGTGTGGAGAAATAC  480
                              TTATTTTTGATGAGTGTGTAGGGATAATCACTAAAGTGTGGAGTAAGTGTGGAGAAATAC
pMo/UPadeSDN FWUP        388 TTATTTTTGATGAGTGTGTAGGGATAATCACTAAAGTGTGGAGTAAGTGTGGAGAAATAC  447

pMo/UPadeSDN Expected    481 GGATAATTTAGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATTTTCTATGTTTGAT  540
                              GGATAATTTAGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATTTTCTATGTTTGAT
pMo/UPadeSDN FWUP        448 GGATAATTTAGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATTTTCTATGTTTGAT  507

pMo/UPadeSDN Expected    541 CATTCTTTTTCTTTTGATTGCCAAGATAAAGTTATTCTTGTGGTAGAAGATGACTACGAT  600
                              CATTCTTTTTCTTTTGATTGCCAAGATAAAGTTATTCTTGTGGTAGAAGATGACTACGAT
pMo/UPadeSDN FWUP        508 CATTCTTTTTCTTTTGATTGCCAAGATAAAGTTATTCTTGTGGTAGAAGATGACTACGAT  567

pMo/UPadeSDN Expected    601 ATTGGCGACATTATTGAAAATTATTTAAAACGTGAAGGCATGAGTGTTATTCGGGCCATG  660
                              ATTGGCGACATTATTGAAAATTATTTAAAACGTGAAGGCATGAGTGTTATTCGGGCCATG
pMo/UPadeSDN FWUP        568 ATTGGCGACATTATTGAAAATTATTTAAAACGTGAAGGCATGAGTGTTATTCGGGCCATG  627

pMo/UPadeSDN Expected    661 AATGGAAAGCAAGCGATTGAATTGCACGCTAGCCAACCCATCGATTTAATCTTACTTGAT  720
                              AATGGAAAGCAAGCGATTGAATTGCACGCTAGCCAACCCATCGATTTAATCTTACTTGAT
pMo/UPadeSDN FWUP        628 AATGGAAAGCAAGCGATTGAATTGCACGCTAGCCAACCCATCGATTTAATCTTACTTGAT  687

pMo/UPadeSDN Expected    721 ATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAATAAAATACGCCAAAAAGCTCAG  780
                              ATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAATAAAATACGCCAAAAAGCTCAG
pMo/UPadeSDN FWUP        688 ATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAATAAAATACGCCAAAAAGCTCAG  747

pMo/UPadeSDN Expected    781 ACTCCCGTGATCATGTTGACGGCGCTAGATCAAGATATTGATAAAGTTATGGCATTACGC  840
                              ACTCCCGTGATCATGTTGACGGCGCTAGATCAAGATATTGATAAAGTTATGGCATTACGC
pMo/UPadeSDN FWUP        748 ACTCCCGTGATCATGTTGACGGCGCTAGATCAAGATATTGATAAAGTTATGGCATTACGC  807

pMo/UPadeSDN Expected    841 ATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGTCGTCGCTAGAGTT  900
                              ATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGTCGTCGCTAGAGTT
pMo/UPadeSDN FWUP        808 ATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCAAATGAAGTCGTCGCTAGAGTT  867

pMo/UPadeSDN Expected    901 CAGGCAGTCCTAAGACGTACTCAGTTTGCAAACAAAGCAACTAATAAAAATAAACTCTAT  960
                              CAGGCAGTCCTAAGACGTACTCAG TTGCAAACAAAGCAACTAATAAAA TAAACTCTAT
pMo/UPadeSDN FWUP        868 CAGGCAGTCCTAAGACGTACTCAGCTTGCAAACAAAGCAACTAATAAAA-TAAACTCTAT  926

pMo/UPadeSDN Expected    961 AAAAATATTGAAATTGATACCGACACTCATAGCGTTTATATACACTCTGAGAATAAGAAG 1020
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                              AAAA TATTGAAATTGATACCGACACTCATAGCGTTTATATACACTCTGAGAATA   AG
pMo/UPadeSDN FWUP        927 AAAA-TATTGAAATTGATACCGACACTCATAGCGTTTATATACACTCTGAGAATAG--AG  983

pMo/UPadeSDN Expected   1021 ATCTTGCTTAATCTGACGCTGACTGAATATAAAATTATTTCATTCATGATTGATCAGCCT 1080
                              ATCTTGCTTAATCTGACGCTGACTGAATATAAAATTATTTCATTCATGATTGATCAGCCT
pMo/UPadeSDN FWUP         984 ATCTTGCTTAATCTGACGCTGACTGAATATAAAATTATTTCATTCATGATTGATCAGCCT 1043

pMo/UPadeSDN Expected   1081 CATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGCATGAATGATAGCGATGCACTA 1140
                              CATAAAGTTTT AC+C+CGGAGAGCTTATGA TCACTGCATGA TGATAGCGATGC+CTA
pMo/UPadeSDN FWUP        1044 CATAAAGTTTT-ACSCSCGGAGAGCTTATGA-TCACTGCATGA-TGATAGCGATGCMCTA 1100

pMo/UPadeSDN Expected   1141 GAGCGAACCGTAGATAGCCATGTGAGTAAGCTGAGAAAAAAACTAGAAGAACAAGGCATA 1200
                              +AGCGA CCGTA+A+ GCCATG+GAGTA GCTG   AAAAAACTAGA   A        +
pMo/UPadeSDN FWUP        1101 RAGCGA-CCGTARAW-GCCATGKGAGTA-GCTG---AAAAAACTAGAGACA------GCW 1148

pMo/UPadeSDN Expected   1201 TTTCAAATGTTAATTAATGTGCGTGGCGTGGGATATAGACTAGATAATCCCCTAGCTGTA 1260
                               TTCA  T       AAT TG G G+ ++GG         TA++T+A+  CCT++CTGTA
pMo/UPadeSDN FWUP        1149 ATTCATGT-------AATATGTGCGKGSKGGA--------TARMTRAWATCCTWRCTGTA 1193

pMo/UPadeSDN Expected   1261 AAAGATGATGCCTAAATAATATTAAAAAATAGCTAGGGAATATTTTATGAAAAGTAAGTT 1320
                              AA    +ATGCCTA        TA AAAAT GCTAG  A TA         AAGTA GTT
pMo/UPadeSDN FWUP        1194 AAGA--KATGCCTAG-------TATAAAAT-GCTAGAAATTA--------GAAGTA-GTT 1234

pMo/UPadeSDN Expected   1321 AGGAATTAGTAAGCAACTTTTTATTGCCTTAACTATTGTGAATTTAAGCGTTACGCTATT 1380
                              AGGA T+++  AGC +CTTT
pMo/UPadeSDN FWUP        1235 AGGA-TWWK--AGCCMCTTTG---------------------------------------- 1252
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b) Forward sequencing of *adeRS* complementation construct (2).

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pMo/UPadeSDN Expected   1321 AGGAATTAGTAAGCAACTTTTTATTGCCTTAACTATTGTGAATTTAAGCGTTACGCTATT 1380
                                        C+ TT A TGCCTT A++ TT TG   TT AGCGTTACGCTATT
pMo/UPadeSDN FWadeS        1 ---------------CCYATTAAATGCCTTTAMW-TTTTGTGATTTAGCGTTACGCTATT   44

pMo/UPadeSDN Expected   1381 TTCTATAGTATTGGGTTATATCATTTATAACTATGCGATTGAAAAAGGCTGGATTAGCTT 1440
                              TTCTATAGTATTGGGTTATATCATTTATAACTATG GA  G  AAAGGCTGGATTAGCTT
pMo/UPadeSDN FWadeS       45 TTCTATAGTATTGGGTTATATCATTTATAACTATG-GAAAGGGAAAGGCTGGATTAGCTT  103

pMo/UPadeSDN Expected   1441 AAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCATTTTGTAGACTGGATCTGGTTAGC 1500
                              AAGCTCATTTCAACAAGAAGATTG+A+CAGTTTTCATTTTGTAGACTGGATCTGGTTAGC
pMo/UPadeSDN FWadeS      104 AAGCTCATTTCAACAAGAAGATTGRAMCAGTTTTCATTTTGTAGACTGGATCTGGTTAGC  163

pMo/UPadeSDN Expected   1501 CACTGTTATCTTCTGTGGCTGTATTATTTCATTAGTGATTGGCATGCGCCTCGCAAAGCG 1560
                              CACTGTTATCTTCTGTGGCTGTATTATTTCATTAGTGATTGGCATGCGCCTCGCAAAGCG
pMo/UPadeSDN FWadeS      164 CACTGTTATCTTCTGTGGCTGTATTATTTCATTAGTGATTGGCATGCGCCTCGCAAAGCG  223

pMo/UPadeSDN Expected   1561 TTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGCAAAAAAAAATTAGTCACGGCGACCT 1620
                              TTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGCAAAAAAAAATTAGTCACGGCGACCT
pMo/UPadeSDN FWadeS      224 TTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGCAAAAAAAAATTAGTCACGGCGACCT  283

pMo/UPadeSDN Expected   1621 CTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCGGAGCTTTTATATAA 1680
                              CTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCGGAGCTTTTATATAA
pMo/UPadeSDN FWadeS      284 CTCTGCTAGAGCTTACGATAATAGAATTCACTCCGCCGAAATGTCGGAGCTTTTATATAA  343

pMo/UPadeSDN Expected   1681 TTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGTCAAAAATGCGCAGGTTTGGAATGC 1740
                              TTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGTCAAAAATGCGCAGGTTTGGAATGC
pMo/UPadeSDN FWadeS      344 TTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGTCAAAAATGCGCAGGTTTGGAATGC  403

pMo/UPadeSDN Expected   1741 AGCTATCGCACATGAGTTAAGAACGCCTATAACGATATTACAAGGTCGTTTACAGGGAAT 1800
                              AGCTATCGCACATGAGTTAAGAACGCCTATAACGAT+TTACAAGGTCGTTTACAGGGAAT
pMo/UPadeSDN FWadeS      404 AGCTATCGCACATGAGTTAAGAACGCCTATAACGATWTTACAAGGTCGTTTACAGGGAAT  463

pMo/UPadeSDN Expected   1801 TATTGATGGCGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTTTTAAATCAAGTTGA 1860
                              TATTGATGGCGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTTTTAAATCAAGTTGA
pMo/UPadeSDN FWadeS      464 TATTGATGGCGTTTTTAAACCTGATGAAGTCCTATTTAAAAGCCTTTTAAATCAAGTTGA  523
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pMo/UPadeSDN Expected   1861 AGGTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTAGAGAACCAGCAACT 1920
                             AGGTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTAGAGAACCAGCAACT
pMo/UPadeSDN FWadeS      524 AGGTTTATCTCACTTAGTCGAAGACTTACGGACTTTAAGCTTAGTAGAGAACCAGCAACT  583

pMo/UPadeSDN Expected   1921 CCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGTAGTTGAAAAAGTTCTTAAAGCATT 1980
                             CCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGTAGTTGAAAAAGTTCTTAAAGCATT
pMo/UPadeSDN FWadeS      584 CCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGTAGTTGAAAAAGTTCTTAAAGCATT  643

pMo/UPadeSDN Expected   1981 TGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTAACGTCCACTCCTGT 2040
                             TGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTAACGTCCACTCCTGT
pMo/UPadeSDN FWadeS      644 TGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGAACTTGACCTAACGTCCACTCCTGT  703

pMo/UPadeSDN Expected   2041 ATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATTGATAATGCGATTCG 2100
                             ATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATTGATAATGCGATTCG
pMo/UPadeSDN FWadeS      704 ATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAATTGCTTTAATTGATAATGCGATTCG  763

pMo/UPadeSDN Expected   2101 CTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGAAGTGGTTGCAGACAACTGGATATT 2160
                             CTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGAAGTGGTTGCAGACAACTGGATATT
pMo/UPadeSDN FWadeS      764 CTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGAAGTGGTTGCAGACAACTGGATATT  823

pMo/UPadeSDN Expected   2161 AAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTTCGGGACGATTTATTTAA-GC 2219
                             AAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTTCGGGACGATTTATTT+A GC
pMo/UPadeSDN FWadeS      824 AAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGAGTTTCGGGACGATTTATTTWAAGC  883

pMo/UPadeSDN Expected   2220 CTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGCACAGGTTTAGGTCTTG 2279
                             CTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGG++CA GTTTAGGTCTTG
pMo/UPadeSDN FWadeS      884 CTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATTTGGCGGSMCAAGTTTAGGTCTTG  943

pMo/UPadeSDN Expected   2280 CTGTTGTACATGCAATTATTGTGGCACTGAAAGGCACTATTCAA-TATAGCAATCAAGGC 2338
                             CTGT GTACATGCAATTATTGTG++ CTGAAAGGC+CTATTC+A TA+AGCAATCAAGGC
pMo/UPadeSDN FWadeS      944 CTGT-GTACATGCAATTATTGTGSM-CTGAAAGGCMCTATTCMAATAWAGCAATCAAGGC 1001

pMo/UPadeSDN Expected   2339 TCGAAAAGTGTTTTCACCATAAAAATTTCTATGGGTC-ATGAAGAGATGGGGTAATTCGC 2397
                             TCGAAAAGTGTTT +  CATAAAAATTTCTATGGG+C ATGAA AGATGGG TAATTC+C
pMo/UPadeSDN FWadeS     1002 TCGAAAAGTGTTTCM--CATAAAAATTTCTATGGGKCCATGAAAAGATGGG-TAATTCSC 1058

pMo/UPadeSDN Expected   2398 TAAATTAAAAAATCTTAGAGTTAAAGTGCCCCCTCACTCTCTTTTATTCTTCTACGAATT 2457
                             T+A T  AAAAAT+ T+GAGTTAAAGTGCCCCCT+ACTC++ TTT+ TCTTCTACGAATT
pMo/UPadeSDN FWadeS     1059 TMATT--AAAAATYCTWGAGTTAAAGTGCCCCCTYACTCYYCTTTWATCTTCTACGAATT 1116

pMo/UPadeSDN Expected   2458 TCTTCTCGCCATTTTGTGGCATTTTCCTGTTGTTTGTTTAATAGGACACCTAACATATAA 2517
                              CTTC+++C A TTTG +G+ATT    TGTT  TT    AA+AG AC CCTAACAT+ +
pMo/UPadeSDN FWadeS     1117 CCTTCYSSC-AGTTTGGKGMATTCCTGTGTTAGTT----AAWAGAAC-CCTAACATWAR- 1169

pMo/UPadeSDN Expected   2518 GCTGTAACCGCAGCGCCAATTAAGGCTATACCGGTTTCATAAATAATATCTATAACAAAT 2577
                                +TAACCGC+  GCCA  TAAG  TA   CGGTT +A+AA +AAT T TA AACA  +
pMo/UPadeSDN FWadeS     1170 --CKTAACCGCMA-GCCA--TAAGCATA---CGGTTCYAWAATWAATCTATA-AACAGTY 1220

pMo/UPadeSDN Expected   2578 TCGAGCACTCCCTCCGACAAAAAATCTAATGAGCCTGAAAATTTAAGAAATTTAGCTATG 2637
                             T++
pMo/UPadeSDN FWadeS     1221 TMSGAGGA---------------------------------------------------- 1228
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c) Reverse sequencing of *adeRS* complementation construct (1).

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pMo/UPadeSDN Expected    901 CAGGCAGTCCTAAGACGTACTCAGTTTGCAAACAAA  936

pMo/UPadeSDN RVadeS          -----------------------------------

pMo/UPadeSDN Expected    937 GCAACTAATAAAAATAAACTCTATAAAAATATTGAAATTGATACCGACACTCATAGCGTT  996
                             G+  +T AT+ AAATA   +CT T AAAAT      ATTG TA CG CACTC+  +C
pMo/UPadeSDN RVadeS     1292 GMGCMTTATWGAAATA---YCTTTCAAAATT----GATTG-TATCG-CACTCWAGSC--- 1245

pMo/UPadeSDN Expected    997 TATATACACTCTGAGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGAATATAAAATT 1056
                               TATAC  TC   GAATA+GA +   +TGCT  ATC GACG TGACTGAA  TAAAATT
pMo/UPadeSDN RVadeS     1244 --TATACCTTC---GAATARGAGR--TYTGCT--ATC-GACG-TGACTGAAATTAAAATT 1196
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pMo/UPadeSDN Expected   1057 ATTTCATTCATGATTGATCAGCCTCATAAAGTTTTTACGCGCGGAGAGCTTATGAATCAC 1116
                             +TT    TCATGAT GATCAGCCTCATAAAGTTTT ACGCG+ G +    TATGA TCA
pMo/UPadeSDN RVadeS     1195 WTTCA--TCATGAT-GATCAGCCTCATAAAGTTTT-ACGCGSCGGRGAGCTATGA-TCA- 1142

pMo/UPadeSDN Expected   1117 TGCATGAATGATAGCGATGCACTAGAGCGAACCGTAGATAGCCATGTGAGTAAGCTGAGA 1176
                             TGCATGAATGATAGCGATGCA TAGAG   ACCGTAGATAGC   ++GAGTAAGCTGAGA
pMo/UPadeSDN RVadeS     1141 TGCATGAATGATAGCGATGCA-TAGAGGCGACCGTAGATAGC--AKKGAGTAAGCTGAGA 1085

pMo/UPadeSDN Expected   1177 AAAAAACTAGAAGAACAAGGCATATTTCAAATGTTAATTAATGTGCGTGGCGTGGGATAT 1236
                             AAAAAACTAGA   A    GCATATTTCAAAT++TAATTAATGTGCGTGGCGTGGGAT+T
pMo/UPadeSDN RVadeS     1084 AAAAAACTAGAGACA----GCATATTTCAAATKKTAATTAATGTGCGTGGCGTGGGATWT 1029

pMo/UPadeSDN Expected   1237 AGACTAGATAATCCCCTAGCTGTAAAAGATGATGCCTAAATAATATTAAAAAATAGCTAG 1296
                             AGACTAGATA   CCCTAGCTGTAAA GATGATGCCTAAATAATAT AAAAAATAGCTAG
pMo/UPadeSDN RVadeS     1028 AGACTAGATA--TCCCTAGCTGTAAA-GATGATGCCTAAATAATAT-AAAAAATAGCTAG  973

pMo/UPadeSDN Expected   1297 GGAATATTTTATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTAT 1356
                             GGAATATTTTATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTAT
pMo/UPadeSDN RVadeS      972 GGAATATTTTATGAAAAGTAAGTTAGGAATTAGTAAGCAACTTTTTATTGCCTTAACTAT  913

pMo/UPadeSDN Expected   1357 TGTGAATTTAAGCGTTACGCTATTTTCTATAGTATTGGGTTATATCATTTATAACTATGC 1416
                             TGTGAATTTAAGCGTTACGCTATTTTCTATAGTATTGGGTTATATCATTTATAACTATGC
pMo/UPadeSDN RVadeS      912 TGTGAATTTAAGCGTTACGCTATTTTCTATAGTATTGGGTTATATCATTTATAACTATGC  853

pMo/UPadeSDN Expected   1417 GATTGAAAAAGGCTGGATTAGCTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCA 1476
                             GATTGAAAAAGGCTGGATTAGCTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCA
pMo/UPadeSDN RVadeS      852 GATTGAAAAAGGCTGGATTAGCTTAAGCTCATTTCAACAAGAAGATTGGACCAGTTTTCA  793

pMo/UPadeSDN Expected   1477 TTTTGTAGACTGGATCTGGTTAGCCACTGTTATCTTCTGTGGCTGTATTATTTCATTAGT 1536
                             TTTTGTAGACTGGATCTGGTTAGCCACTGTTATCTTCTGTGGCTGTATTATTTCATTAGT
pMo/UPadeSDN RVadeS      792 TTTTGTAGACTGGATCTGGTTAGCCACTGTTATCTTCTGTGGCTGTATTATTTCATTAGT  733

pMo/UPadeSDN Expected   1537 GATTGGCATGCGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGC 1596
                             GATTGGCATGCGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGC
pMo/UPadeSDN RVadeS      732 GATTGGCATGCGCCTCGCAAAGCGTTTTATTGTGCCAATTAACTTCTTAGTCGAAGCAGC  673

pMo/UPadeSDN Expected   1597 AAAAAAAATTAGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGC 1656
                             AAAAAAAATTAGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGC
pMo/UPadeSDN RVadeS      672 AAAAAAAATTAGTCACGGCGACCTCTCTGCTAGAGCTTACGATAATAGAATTCACTCCGC  613

pMo/UPadeSDN Expected   1657 CGAAATGTCGGAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGT 1716
                             CGAAATGTCGGAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGT
pMo/UPadeSDN RVadeS      612 CGAAATGTCGGAGCTTTTATATAATTTTAATGATATGGCTCAAAAGCTAGAGGTTTCCGT  553

pMo/UPadeSDN Expected   1717 CAAAAATGCGCAGGTTTGGAATGCAGCTATCGCACATGAGTTAAGAACGCCTATAACGAT 1776
                             CAAAAATGCGCAGGTTTGGAATGCAGCTATCGCACATGAGTTAAGAACGCCTATAACGAT
pMo/UPadeSDN RVadeS      552 CAAAAATGCGCAGGTTTGGAATGCAGCTATCGCACATGAGTTAAGAACGCCTATAACGAT  493

pMo/UPadeSDN Expected   1777 ATTACAAGGTCGTTTACAGGGAATTATTGATGGCGTTTTTAAACCTGATGAAGTCCTATT 1836
                             ATTACAAGGTCGTTTACAGGGAATTATTGATGGCGTTTTTAAACCTGATGAAGTCCTATT
pMo/UPadeSDN RVadeS      492 ATTACAAGGTCGTTTACAGGGAATTATTGATGGCGTTTTTAAACCTGATGAAGTCCTATT  433

pMo/UPadeSDN Expected   1837 TAAAAGCCTTTTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACTTACGGACTTT 1896
                             TAAAAGCCTTTTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACTTACGGACTTT
pMo/UPadeSDN RVadeS      432 TAAAAGCCTTTTAAATCAAGTTGAAGGTTTATCTCACTTAGTCGAAGACTTACGGACTTT  373

pMo/UPadeSDN Expected   1897 AAGCTTAGTAGAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGT 1956
                             AAGCTTAGTAGAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGT
pMo/UPadeSDN RVadeS      372 AAGCTTAGTAGAGAACCAGCAACTCCGGTTAAATTATGAATTGTTTGACTTGAAGGCGGT  313

pMo/UPadeSDN Expected   1957 AGTTGAAAAAGTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGA 2016
                             AGTTGAAAAAGTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGA
pMo/UPadeSDN RVadeS      312 AGTTGAAAAAGTTCTTAAAGCATTTGAAGATCGTTTGGATCAAGCTAAGCTAGTACCAGA  253

pMo/UPadeSDN Expected   2017 ACTTGACCTAACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAAT 2076
                             ACTTGACCTAACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAAT
pMo/UPadeSDN RVadeS      252 ACTTGACCTAACGTCCACTCCTGTATATTGCGACCGCCGTCGTATTGAGCAAGTTTTAAT  193
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pMo/UPadeSDN Expected   2077 TGCTTTAATTGATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGA 2136
                             TGCTTTAATTGATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGA
pMo/UPadeSDN RVadeS      192 TGCTTTAATTGATAATGCGATTCGCTATTCAAATGCAGGCAAACTTAAAATCTCTTCAGA  133

pMo/UPadeSDN Expected   2137 AGTGGTTGCAGACAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCATTGCAACCGA 2196
                             AGTGGTTGCAGACAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCAT+GCAACCGA
pMo/UPadeSDN RVadeS      132 AGTGGTTGCAGACAACTGGATATTAAAAATTGAGGATGAAGGCCCCGGCATKGCAACCGA   73

pMo/UPadeSDN Expected   2197 GTTTCGGGACGATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGAATT 2256
                             GTTTCGGGACGATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGA TT
pMo/UPadeSDN RVadeS       72 GTTTCGGGACGATTTATTTAAGCCTTTCTTTAGATTAGAAGAATCAAGGAATAAAGA-TT   14

pMo/UPadeSDN Expected   2257 TGGCGGCACAGGT 2269
                              G +  C   +G
pMo/UPadeSDN RVadeS       13 GGCSCACCTTSGC    1
```

d) Reverse sequencing of *adeRS* complementation construct (2).

```
pMo/UPadeSDN Expected   2221 TTTCTTTAGATTAGAAGAATCAA 2243

pMo/UPadeSDN RVDOWN          -----------------------

pMo/UPadeSDN Expected   2244 GGAATAAAGAATTTGGCGGCACAGGTTTAGGTCTTGCTGTTGTACATGCAATTATTGTGG 2303
                             G   TAAAG   TT   G C        A    TTG+     TA   GC  +TA TG
pMo/UPadeSDN RVDOWN     1223 GATGTAAAGCCCTTCTAGTCGAGATCAGATAGATTGSGCAGTTAGTCGCTGKTACTGCAT 1164

pMo/UPadeSDN Expected   2304 CACTGAAAGGCACTATTCAATATAGCAATCAAGGCTCGAAAAGTGTTTTCACCATAAAAA 2363
                                TG  + G  C A+TCAAT  +GCA TCA      G AAAGTGTTT    CA  AAAA
pMo/UPadeSDN RVDOWN     1163 WTTTGGCMTGAGCAAWTCAAT--WGCA-TCAG---CTGGAAAGTGTTT----CACTAAAA 1114

pMo/UPadeSDN Expected   2364 TTTCTATGGGTCATGAAGAGATGGGGTAATTCGCTAAATTAAAAAATCTTAGAGTTAAAG 2423
                             TT  T   GGTCA+  AG++ATGGG         TA   TAA AAA   TAGAGTTAA G
pMo/UPadeSDN RVDOWN     1113 TTCWT---GGTCAK--AGRRATGGG---------TATCGTAATAAAATCTAGAGTTAA-G 1069

pMo/UPadeSDN Expected   2424 TGCCCCCTCACTCTCTTTTATTCTTCTACGAATTTCTTCTCGCCATTTTGTGGCATTTTC 2483
                             TGCCCC TCACTCT+TTT   T+TTCTACGA T TCT   CGC   TTTGTG CATTT
pMo/UPadeSDN RVDOWN     1068 TGCCCC-TCACTCTYTTT--ATYTTCTACGAGTCTCT---CGCA--TTTGTG-CATTTCT 1018

pMo/UPadeSDN Expected   2484 CTGTTGTTTGTTTAATAGGACACCTAACATATAAGCTGTAACCGCAGCGCCAATTAAGGC 2543
                              TGTT +TT T       GACAC   ACATATAAGCTGTA  CGCAGCGCCAATTAAGGC
pMo/UPadeSDN RVDOWN     1017 GTGTTTKTTAT------AGACACT--ACATATAAGCTGTA--CGCAGCGCCAATTAAGGC  968

pMo/UPadeSDN Expected   2544 TATACCGGTTTCATAAATAATATCTATAACAAATTCGAGCACTCCCTCCGACAAAAAATC 2603
                             TATACCGGTTTCATAAATAATATCTATA CAAATTCGAGCACTCC TCCGACAAAAAATC
pMo/UPadeSDN RVDOWN      967 TATACCGGTTTCATAAATAATATCTATA-CAAATTCGAGCACTCC-TCCGACAAAAAATC  910

pMo/UPadeSDN Expected   2604 TAATGAGCCTGAAAATTTAAGAAATTTAGCTATGGCAACCACTATAACTCCCAATAAAGC 2663
                             TAATGAGC TGAAA  TTAAGAAATTTAGCTATGGCAACCACTATAACTCCCAATAAAGC
pMo/UPadeSDN RVDOWN      909 TAATGAGC-TGAAA--TTAAGAAATTTAGCTATGGCAACCACTATAACTCCCAATAAAGC  853

pMo/UPadeSDN Expected   2664 GCCAGCCCATAAGAATATTTTATGAATATTGATAGGTGCCGTGACTTGATCGATTTGATC 2723
                             GCCAGCCCATAAGAATATTTTATGAATATTGATAGGTGCCGTGACTTGATCGATTTGATC
pMo/UPadeSDN RVDOWN      852 GCCAGCCCATAAGAATATTTTATGAATATTGATAGGTGCCGTGACTTGATCGATTTGATC  793

pMo/UPadeSDN Expected   2724 GGGGTACTTTCGATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTTAAATAA 2783
                             GGGGTACTTTCGATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTTAAATAA
pMo/UPadeSDN RVDOWN      792 GGGGTACTTTCGATAGTACTCAATTTCCTCATCTGTTACTTCAGAGGTAGATTTAAATAA  733

pMo/UPadeSDN Expected   2784 TAATTTAATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCG 2843
                             TAATTTAATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCG
pMo/UPadeSDN RVDOWN      732 TAATTTAATAAATAGCTTGTCTTGATCAAGCTTTGTATTTTTCTTCATAGCTGTGATGCG  673

pMo/UPadeSDN Expected   2844 TAATAAGTATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAG 2903
                             TAATAAGTATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAG
pMo/UPadeSDN RVDOWN      672 TAATAAGTATTCTAATGTTTAATGTTACTCACTTTTTCTTTAGGGAGTAACTGTTTTAAG  613
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pMo/UPadeSDN Expected   2904 AGCTTATCGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGA 2963
                             AGCTTATCGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGA
pMo/UPadeSDN RVDOWN      612 AGCTTATCGCTACCTTAACTCATTCATTTCTTCTCTAGCTTGTTGTGGTGTTTTACCCGA  553

pMo/UPadeSDN Expected   2964 CCACTCTTTATAAGCCCGTGTAAATGGACTGTGCTCGGCATAACCTAACAGTAAAGCTAT 3023
                             CC CTCTTTATAAGCCCGTGTAAATGGACTGTGCTCGGCATAACCTAACAGTAAAGCTAT
pMo/UPadeSDN RVDOWN      552 CCGCTCTTTATAAGCCCGTGTAAATGGACTGTGCTCGGCATAACCTAACAGTAAAGCTAT  493

pMo/UPadeSDN Expected   3024 TTCTACAATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGT 3083
                             TTCTACAATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGT
pMo/UPadeSDN RVDOWN      492 TTCTACAATTTGTAGCCTTAGATCTTTTAAATACGATTTTGCCAATTCGTAACGGACAGT  433

pMo/UPadeSDN Expected   3084 ATTTAATTCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACG 3143
                             ATTTAATTCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACG
pMo/UPadeSDN RVDOWN      432 ATTTAATTCTTTCCGAAAATTCGTACCTGCCTCGGTTAAACGGCGTTGCAAGGTTCTACG  373

pMo/UPadeSDN Expected   3144 TGAATAATTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTA 3203
                             TGAATAATTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTA
pMo/UPadeSDN RVDOWN      372 TGAATAATTTAATCGCTCTGCAAGCTGTTCAATAGTTGGTTCGCCTTGATGAAGTAAGTA  313

pMo/UPadeSDN Expected   3204 AGCAATCTGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAA 3263
                             AGCAATCTGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAA
pMo/UPadeSDN RVDOWN      312 AGCAATCTGTTTACGAACTTGATCTGTAATTTCATCAATATGTGGCAGGCTTGCCAGTAA  253

pMo/UPadeSDN Expected   3264 CTTGTCAGCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAG 3323
                             CTTGTCA+CATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAG
pMo/UPadeSDN RVDOWN      252 CTTGTCASCATGTTTTTCTAGTATGGCAACTAAGGCGGCGTCGGAGTTTTTAAGCGGTAG  193

pMo/UPadeSDN Expected   3324 ACTTAAGATTTCCTTATCAAAACGCATCAGTGCCACAGGCTGTTCAAAAAGCACAGGACA 3383
                             ACTTAAGATTTCCTTATCAAAACGCATCAGTGCC+CAGGCTGTTCAAAAAGC+CAGGACA
pMo/UPadeSDN RVDOWN      192 ACTTAAGATTTCCTTATCAAAACGCATCAGTGCCMCAGGCTGTTCAAAAAGCMCAGGACA  133

pMo/UPadeSDN Expected   3384 ACCAAAATACTCCTCATAAGGCTGAACATTTTCAGGCCGTTCATGAATAAAATGTATTTC 3443
                             ACCAAAATACTCCTCATAAGGCTGA+CATTTTCAGGCCGTTCATGAATAAAATGTATTTC
pMo/UPadeSDN RVDOWN      132 ACCAAAATACTCCTCATAAGGCTGAMCATTTTCAGGCCGTTCATGAATAAAATGTATTTC   73

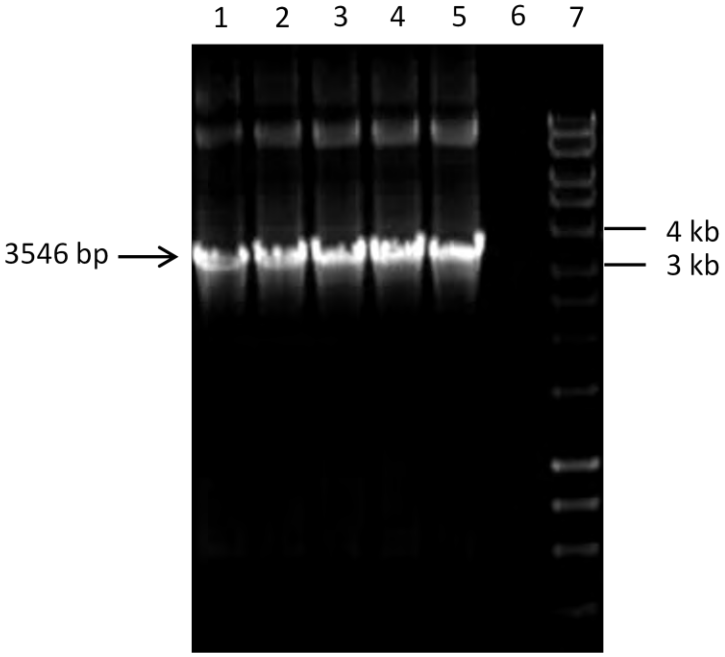
pMo/UPadeSDN Expected   3444 TTTGAGTCTTTCTTTTCCTCTAATGAGACTACGGCAAAATTGAACTATAACGGTACGGCC 3503
                             TTTGAGTCTTTCTTTTCCTCTAATGAGA+TACGGCAAAAT GAACTATAACGGTACG CC
pMo/UPadeSDN RVDOWN       72 TTTGAGTCTTTCTTTTCCTCTAATGAGAYTACGGCAAAAT-GAACTATAACGGTACGCCC   14

pMo/UPadeSDN Expected   3504 AGTTTCGTCCGATA 3517
                             AGT +C  CCG T
pMo/UPadeSDN RVDOWN       13 AGTCKC-CCCGGTG    1

pMo/UPadeSDN Expected   3518 AAGGGCCGACTTGCTCACCTGGATCCCCC 3546

pMo/UPadeSDN RVDOWN          ------------------------------
```

Figure 3.21 Verification of *adeRS* Complementation Construct in S17-1.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ 1 (Bioline)	---	---
2-5	Candidate S17-1 transformants	3546 bp	~ 3546 bp
6	pMo130-Tel^{R}/UP-*adeRS*-DOWN (positive control)	3546 bp	~ 3546 bp
7	---	No amplimer	No amplimer
8	Hyperladder™ 1 (Bioline)	---	---

PCR 16, Table 2.7

suggesting that these did not contain the construct (single recombinants are expected to be yellow when exposed to pyrocatechol). A conjugation was next carried out using 2 ml of both donor and recipient. This gave rise to one colony after overnight incubation and a further eight colonies after the plates had been left at room temperature for two days. All nine colonies remained white after pyrocatechol exposure. The original method used the donor and recipient in a 1:2 ratio, according to the absorbance of each stationary phase culture at OD_{600}. For the third conjugation, the donor and recipient (2 ml of each) were used in a 1:1 ratio since AYE grows faster than S17-1 and it was possible that increasing the concentration of donor would increase the chance of obtaining single recombinants. The bacteria were incubated on the filter for 6 hr (original method) and also 24 hr to allow conjugation to occur for a longer period of time.

A different strategy was to follow the original method, but using donor:recipient ratios of 2:1, 4:1, 6:1, 8:1 and 10:1, using approximately 0.6 ml donor for each ratio. The cells were incubated on the filters overnight and half of the resuspended cells were plated onto LB agar and the other half plated onto LB agar containing high magnesium (MgLB, Appendix 1) since high magnesium was previously found to increase conjugation efficiency in *Photorhabdus* (Watson, 2007). One colony was obtained from the 2:1 ratio on MgLB and one colony was obtained from the 10:1 ratio on LB. No more colonies were obtained after incubation for a further 24 hr. Both of these colonies were streaked onto LB (both remained white after pyrocatechol exposure) and onto LB containing tellurite (30 μg/ml) to confirm that they were definitely tellurite resistant.

They both grew on tellurite, suggesting that they were spontaneous tellurite resistant mutants, but did not contain the construct.

Altered strategy for Conjugation: The Patch Method (Section 2.4.4.2)

This method was followed using donor and recipient cells after approximately 2 hr of growth and also after S17-1/pMo130-Tel^R/UP-*adeRS*-DOWN had been incubated for an extra 2 hr, since AYE grew faster. After incubation for 48 hr, no candidate transconjugants were obtained when S17-1 was grown for the extra two hours and three colonies were obtained when the donor and recipient were grown for the same length of time. However, these three colonies remained white after subculture and pyrocatechol exposure.

A combination of Different Ratios and Different Growth Phase:

A conjugation using a range of ratios (donor:recipient; 1:1, 2:1, 5:1, 10:1) of actively growing donor and recipient was carried out, to investigate whether an active growth phase would increase conjugation frequency. Dilutions of AYEΔ*adeRS* (1/20) and S17-1/pMo130-Tel^R/UP-*adeRS*-DOWN (1/10) overnight culture were incubated for 1 hr (37^{o}C, 180 rpm). The absorbance of AYEΔ*adeRS* at OD_{600} was 0.49 and that of S17-1/pMo130-Tel^R/UP-*adeRS*-DOWN was 0.25. The donor and recipient were both dropped onto opposite sides of the filter disc and mixed on the disc to obtain a range of ratios. The filter was incubated for 20 hr before the cells were resuspended. One colony (from the 2:1 ratio conjugation) was obtained after 24 hr on selective media. This colony was confirmed to be tellurite-resistant but remained white after pyrocatechol exposure and was thought to not contain the construct.

The remaining conjugations used large volumes of donor and recipient in a range of ratios and combined all cells from all filters in the same NaCl before plating aliquots onto selective media.

Can AYEΔ*adeRS* act as a recipient?

In addition to the conjugations with complementation construct, two conjugations were carried out using S17-1/pMo130-Tel^R/*adeRS*UPDOWN or S17-1/pMo130-Tel^R/*pmrAB*UPDOWN (containing the *adeRS* and *pmrAB* deletion constructs previously conjugated into AYE) as the donor to investigate whether AYEΔ*adeRS* could receive plasmids by conjugation. AYE was used as a positive control as both constructs had previously been introduced into this, the parental strain. Seventy-eight colonies were obtained when S17-1/pMo130-Tel^R/*adeRS*UPDOWN was used as the donor. Most of these colonies were obtained after 21 hr incubation (37°C), but the plates were left at room temperature overnight before counting and sub-culturing 18 colonies onto LB agar. All subcultures were yellow after pyrocatechol exposure, suggesting that it was possible to a) introduce a plasmid into AYEΔ*adeRS* by conjugation and b) introduce a construct into the *adeS* UP or DOWN region of the chromosome, which were the target sites for recombination of the complementation construct into AYEΔ*adeRS.* One colony, which was yellow after pyrocatechol exposure, was also obtained when S17-1/pMo130-Tel^R/*pmrAB*UPDOWN was used as the donor. This further confirms that the conjugation conditions were not the limiting factor in complementing AYEΔ*adeRS*. It is interesting that only two colonies, which remained white upon pyrocatechol exposure, were obtained when a conjugation was carried out between S17-1/pMo130-Tel^R/*adeRS*UPDOWN and AYE. Therefore, the conjugation was unsuccessful on this occasion.

To confirm that the white colonies obtained after these conjugations were AYEΔ*adeRS*, which did not contain the complementation construct and had not undergone double recombination, two colony PCRs were used (Section 2.4; Figure 2.3; Figure 3.22), which were expected to give one product for the UP and DOWN fragments remaining on the chromosome and a larger product for the UP-*adeRS*-DOWN insert on the construct, if present in the chromosome. Since one primer in each reaction was specific for the chromosome, this could be used to investigate whether the plasmid was incorporated into the genome. Ten colonies were screened (Figure 3.22) they were all confirmed to be AYEΔ*adeRS*, with no construct incorporated into the chromosome and were not complements (i.e. they had not undergone double recombination).

In conclusion, despite using exactly the same method used to delete *adeRS* (in addition to other methods), it was not possible to re-insert *adeRS*.

Can the complementation constructs be introduced into AYE?

To investigate further whether the complementation construct could be introduced into AYE and whether it was just the deletion mutants, which would not accept the vector, conjugations were carried out to introduce both the *adeRS* and *pmrAB* complementation constructs into AYE. Conjugations (original method, Section 2.4.3) were carried out on two separate occasions. One conjugation was set up on the first occasion and five conjugations were set up in parallel on the second occasion. No colonies were obtained after 48 hr in both cases.

3.3.2. *pmrAB*

3.3.2.1. Deletion of *pmrAB*

3.3.2.1.1. Identification of *pmrB* in AYE

The search terms "*pmrB*" and "lipid A phosphoethanolamine" were used to search for *pmrB* in the AYE genome. Both search terms did not return *pmrB*, but "lipid A phosphoethanolamine returned *eptA*. Therefore, *pmrB* was unannotated in the AYE genome. The gene sequences of *pmrB*, *pmrA* and *pmrC* in AB0057 had been submitted to Genbank (accession numbers AB57_3172, AB57_3173 and AB57_3174 respectively, (Adams *et al.*, 2009)). *pmrB*, *pmrA* and *pmrC* in AB0057 aligned with "*qseC*", "*qseB*" and "*eptA*" in AYE (Figure 3.23). The sequences of *qseC*, *qseB* and *eptA* were blasted against the AYE genome and each returned one result, suggesting that there is only one copy of each gene in AYE. The alignment of *qseC* and AB57_3172 confirmed that *qseC* is the *pmrB* gene in AYE. (Figure 3.24).

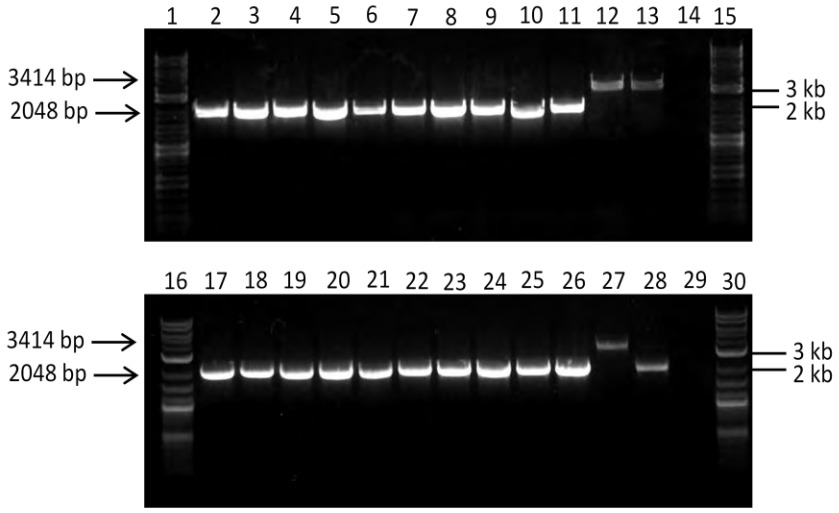
3.3.2.1.2. Construction of pMo130-Tel^{R}/*pmrAB*UPDOWN

The UP and DOWN fragments for *pmrAB* were amplified and the purified amplimers were verified by electrophoresis (Figure 3.25).

pMo130-Tel^{R} and the *pmrAB*UP fragment were digested with *Not*I and *Bam*HI (Table 2.8, Digestion 3), ligated and then transformed into DH5α. One candidate transformant was obtained, which was confirmed to harbour pMo130-Tel^{R}/*pmrAB*UP (Figure 3.26).

Twenty transformants were obtained when pMo130-Tel^{R}/*pmrAB*UP was ligated with DOWN fragment and transformed into DH5α. Eight of these were verified by PCR (Figure 3.27) and

Figure 3.22 Screening of *adeRS* Candidate Complementation Transconjugants by Colony PCR.



Lane	PCR[a]	DNA	Expected size	Actual size
1	---	2-Log ladder (New England Biolabs)	---	---
2-11	22	Candidate single recombinants	2048 or 3414 bp	~ 2048 bp
12	22	AYE (negative control for presence of construct)	3414 bp	~ 3414 bp
13	22	AYE/pMo130-Tel^R/*adeRS*UPDOWN (positive control for PCR[b])	2048 or 3414	~ 3414 bp
14	22	--- (Contamination control for PCR 1)	No amplimer	No amplimer
15, 16	---	2-Log ladder (New England Biolabs)	---	---
17-26	23	Candidate single recombinants	2048 or 3414 bp	~ 2048bp
27	23	AYE (negative control)	3414 bp	~ 3414 bp
28	23	AYE/pMo130-Tel^R/*adeRS*UPDOWN (positive control)	2048 or 3414	~ 2048 bp
29	23	--- (Contamination control for PCR 2)	No amplimer	No amplimer
30	---	2-Log ladder (New England Biolabs)	---	---

[a]PCR number, Table 2.7. PCR 22, primer flanking the UP fragment on the chromosome and a primer specific for the DOWN fragment; PCR 23, primer flanking the DOWN fragment on the chromosome and a primer specific for the UP fragment. [b] this strain was included to confirm that the PCR could indicate presence of a construct.

Figure 3.23 Alignment of the *pmrCAB* Operon in *A. baumannii* AB0057 and AYE.

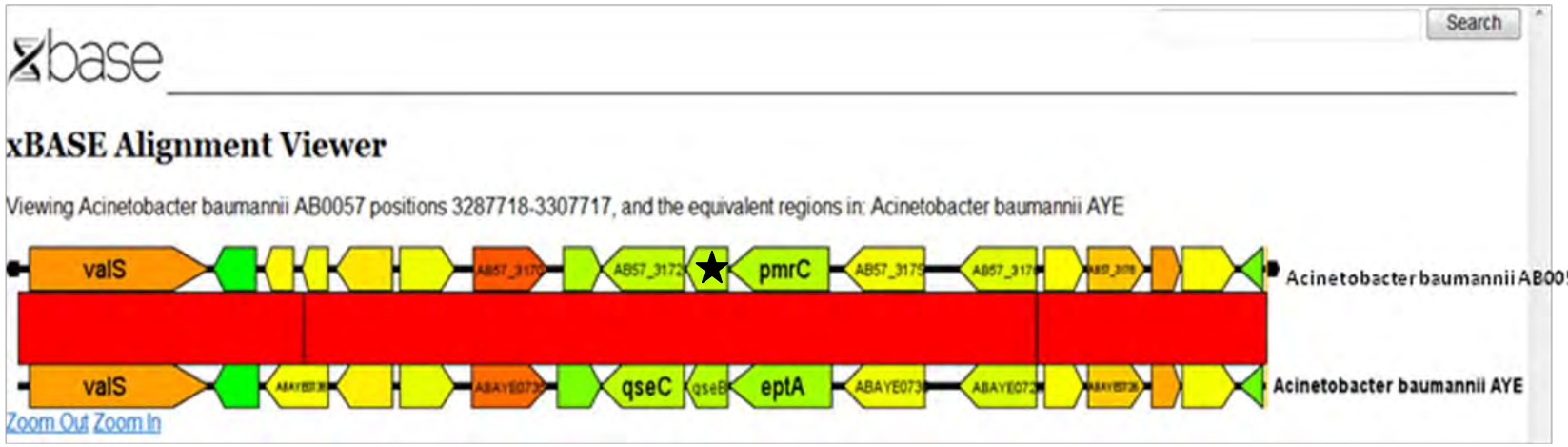


Figure 3.24 Alignment of *pmrB* in AB0057 and AYE.

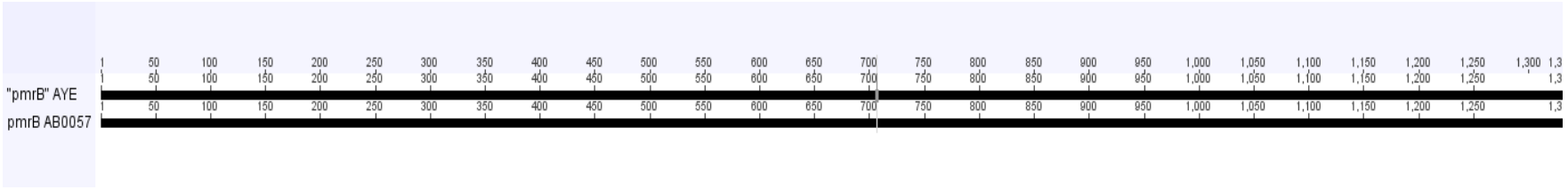
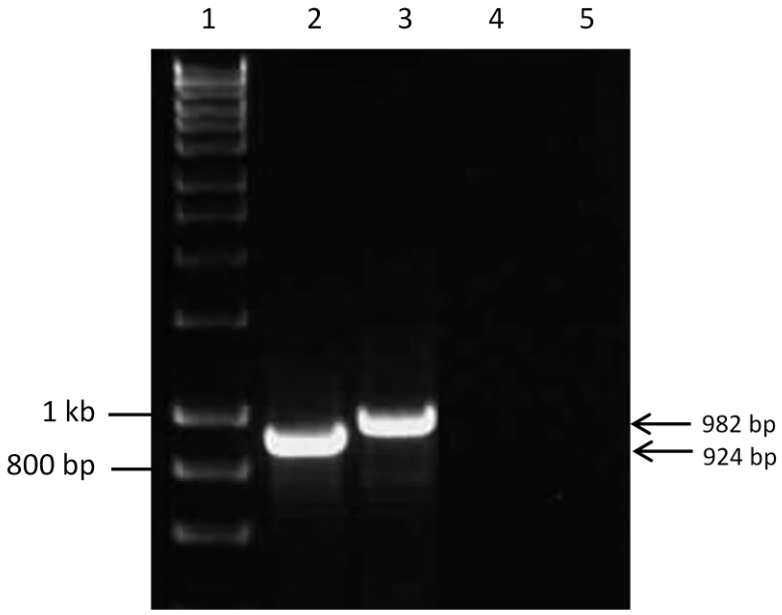


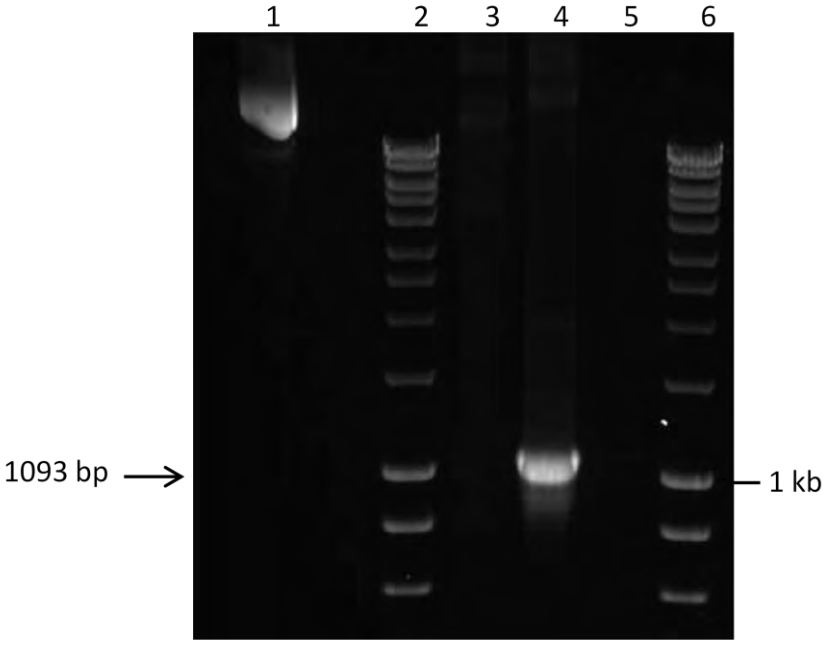
Figure 3.25 Amplification of *pmrAB* UP and DOWN Fragments



[a] PCR number, Table 2.7

Lane	PCR[a]	DNA	Expected size	Actual size
1, 6	---	Hyperladder™ 1 (Bioline)	---	---
2	6	Purified *pmrAB* UP fragment	924 bp	~ 924 bp
3	8	Purified *pmrAB* DOWN fragment	982 bp	~ 982 bp
4	6	Contamination control for *pmrAB* UP fragment	No amplimer	No amplimer
5	8	Contamination control for *pmrAB* DOWN fragment	No amplimer	No amplimer

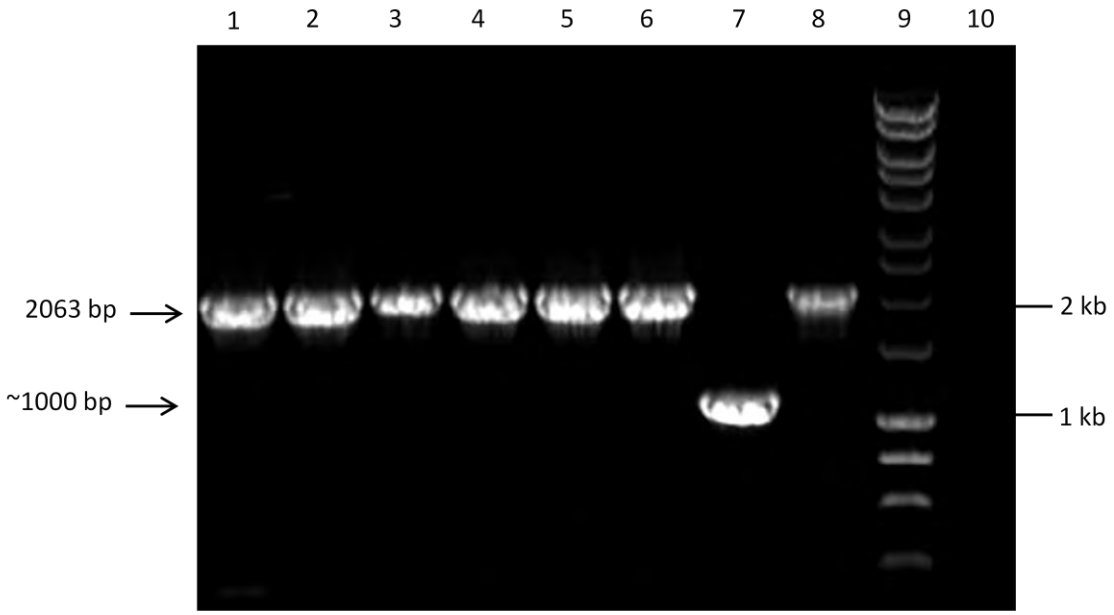
Figure 3.26 Verification of pMo130-TelR/*pmrAB*UP



Lane	DNA	Expected size	Actual size
1	pMo130-Tel^R/*pmrAB*UP extracted from transformant (No PCR)	---	---
2, 6	Hyperladder™ 1 (Bioline)	---	---
3	pMo130-Tel^R undigested (negative control)	No amplimer	No amplimer
4	pMo130-Tel^R/*pmrAB*UP from transformant	1093 bp	~ 1093 bp
5	---	No amplimer	No amplimer

PCR11, Table 2.7; Lane 1, undigested vector extracted from a candidate transformant and electrophoresed

Figure 3.27 Verification of pMo130-TelR/*pmrAB*UPDOWN.



Lane	DNA	Expected size	Actual size
1-8	Lysate from transformants 1-8	2063 bp	~ 2063 bp (transformants 1-6 and 8) ~ 1kb (transformant 7)
9	Hyperladder™ 1 (Bioline)	---	---
10	---	No amplimer	No amplimer

PCR 14, Table 2.7

candidate 1 was chosen for further verification. The vector from candidate 1 was extracted and confirmed to be pMo130-Tel^R/*pmrAB*UPDOWN by DNA sequencing (Figure 3.28).

3.3.2.1.3. Introduction of pMo130-Tel^R/*pmrAB*UPDOWN into AYE and Verification of Deletion

Two candidate S17-1/pMo130-Tel^R/*pmrAB*UPDOWN transformants were obtained. Plasmid was extracted from both candidates and used as the template in a PCR (Figure 3.29). One of these vectors (from candidate 2; Figure 3.29) yielded an amplimer of the correct size. Therefore, candidate 2 was used as the donor in a conjugation to introduce pMo130-Tel^R/*pmrAB*UPDOWN into AYE. Of six candidate AYE transconjugants obtained after 24hr incubation, three were yellow upon pyrocatechol exposure suggesting that these were single recombinants. These three colonies were passaged in LB (-NaCl) containing sucrose to cure the plasmid incorporated in the chromosome. Colonies from passages four and five that remained white after pyrocatechol exposure were streaked onto LB plates containing tellurite (30 µg/ml). Eleven colonies were completely sensitive to tellurite or only gave rise to one colony on LB plates containing tellurite (30 µg/ml), suggesting that these were deletion mutants. Two of the tellurite-sensitive colonies were confirmed to be deletion mutants by PCR (Figure 3.30) and DNA sequencing (Figure 3.31; Figure 3.32).

It was initially planned that just *pmrB* would be deleted. However, *pmrB,* 12 bp of *pmrA* and 244 bp of a gene encoding a hypothetical protein (ABAYE0734) were deleted because the primers,

Figure 3.28 Verification of pMo130-TelR/*pmrAB*UPDOWN Construct by Sequencing

a) Forward sequencing.

```
pMo/pmrBUPDN Expected      1 CCATCTACTTCTTCGACCCGTCCGGTAACCGCAACGAAGTGTTCTGCGGGGGAGATTACA   60
                                                   G AA+  AGTGTT+TGCGGGGGAGATTACA
pMo/pmrBUPDN FW Seq        1 ----------------------------GGGAAYASAGTGTTMTGCGGGGGAGATTACA   31

pMo/pmrBUPDN Expected     61 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT  120
                              ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT
pMo/pmrBUPDN FW Seq       32 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT   91

pMo/pmrBUPDN Expected    121 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGACCTGAGCGGCCGC  180
                              ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGAC     CTGAGCGGCCGC
pMo/pmrBUPDN FW Seq       92 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGAC-----CTGAGCGGCCGC  146

pMo/pmrBUPDN Expected    181 CTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTGAAA  240
                              CTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTGAAA
pMo/pmrBUPDN FW Seq      147 CTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTGAAA  206

pMo/pmrBUPDN Expected    241 GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAAAGT  300
                              GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAAAGT
pMo/pmrBUPDN FW Seq      207 GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAAAGT  266

pMo/pmrBUPDN Expected    301 TAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAAAACTCAGGTCA  360
                              TAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAAAACTCAGGTCA
pMo/pmrBUPDN FW Seq      267 TAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAAAACTCAGGTCA  326

pMo/pmrBUPDN Expected    361 TCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAGCGAGCCTAGAA  420
                              TCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAGCGAGCCTAGAA
pMo/pmrBUPDN FW Seq      327 TCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAGCGAGCCTAGAA  386

pMo/pmrBUPDN Expected    421 CATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCAACGATCACGTT  480
                              CATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCAACGATCACGTT
pMo/pmrBUPDN FW Seq      387 CATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCAACGATCACGTT  446

pMo/pmrBUPDN Expected    481 GCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGATGGTTTGGCTCA  540
                              GCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGATGGTTTGGCTCA
pMo/pmrBUPDN FW Seq      447 GCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGATGGTTTGGCTCA  506

pMo/pmrBUPDN Expected    541 ATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCTATGATGGATGG  600
                              ATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCTATGATGGATGG
pMo/pmrBUPDN FW Seq      507 ATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCTATGATGGATGG  566

pMo/pmrBUPDN Expected    601 TATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTAATTATTTCTGC  660
                              TATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTAATTATTTCTGC
pMo/pmrBUPDN FW Seq      567 TATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTAATTATTTCTGC  626

pMo/pmrBUPDN Expected    661 TCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGATGATTATTTAAT  720
                              TCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGATGATTATTTAAT
pMo/pmrBUPDN FW Seq      627 TCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGATGATTATTTAAT  686

pMo/pmrBUPDN Expected    721 TAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTACGCCGTAGTGG  780
                              TAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTACGCCGTAGTGG
pMo/pmrBUPDN FW Seq      687 TAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTACGCCGTAGTGG  746

pMo/pmrBUPDN Expected    781 AGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGATCTGGTTTTAAA  840
                              AGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGATCTGGTTTTAAA
pMo/pmrBUPDN FW Seq      747 AGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGATCTGGTTTTAAA  806

pMo/pmrBUPDN Expected    841 TGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTGATCTATCAAATCGTGAATG  900
                              TGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTGATCT+TCAA TCGTGAATG
pMo/pmrBUPDN FW Seq      807 TGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTGATCTMTCAA-TCGTGAATG  865

pMo/pmrBUPDN Expected    901 GGCAATCTTAATTCCACTTATGACTCACCCAAATAAAATCTTTTCTAAAGCCAACTTAGA  960
                              GGCAATCTTAATTCCACTTATGACTCACCCAA TAAAATCTTTTCTAAAGCCAACTTA+A
pMo/pmrBUPDN FW Seq      866 GGCAATCTTAATTCCACTTATGACTCACCCAA-TAAAATCTTTTCTAAAGCCAACTTAKA  924
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pMo/pmrBUPDN Expected    961 AGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATACTATTGAAGTATATGTTCA 1020
                             GATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATACTATTG+A+TATATGTTCA
pMo/pmrBUPDN FW Seq      925 TGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATACTATTGMASTATATGTTCA  984

pMo/pmrBUPDN Expected   1021 CCATTTAAGAGCGAAGCTGGGTAAAGATTTTATCCGAACCATCCGAGGACTGGGCTACCG 1080
                             +CATTTAAGA+CGAAGCTGG TAAA+ATTT ATCCGAA CATCCGAG ACTGG CTAC G
pMo/pmrBUPDN FW Seq      985 SCATTTAAGAKCGAAGCTGG-TAAASATTT-ATCCGAAGCATCCGAGAACTGG-CTAC-G 1040

pMo/pmrBUPDN Expected   1081 TTTGGGATCCCCTGCACTTGCATGACCGCCTGGAAAACAATGGCCATGTTTAGCGCTAAA 1140
                              TTGGGATCCC TGCACTTGCATGAC   C+ G AAACA TGGC ATGTT   CG+TAA
pMo/pmrBUPDN FW Seq     1041 GTTGGGATCCC-TGCACTTGCATGACG--CWTGCAAACA-TGGC-ATGTT--ACGMTAA- 1092

pMo/pmrBUPDN Expected   1141 GTCCCAAATATAGCTTCCTAAAGTATTAGGGTGAACCATATTCCAAGGGCAGGCGTGAGC 1200
                             GTCC A+++  AGCT     A+GTAT  G G   A CAT        GGCAG CGTGAGC
pMo/pmrBUPDN FW Seq     1093 GTCCTARWW--AGCTCT---ARGTATAGGTGACTATCAT--------GGCAG-CGTGAGC 1138

pMo/pmrBUPDN Expected   1201 AGATTGTGACTTTATTAGGCCAACTATACTGCTACCGAGCATACTGACAAAAAACATATA 1260
                              +AT G+GACTT ATTAG C  ACTATACTG    CGA CAT CTGACAA        TA
pMo/pmrBUPDN FW Seq     1139 GRAT-GKGACTT-ATTAGCC--ACTATACTGGCTACGA-CAT-CTGACAAC-----CTTA 1187

pMo/pmrBUPDN Expected   1261 GCCATATTGCCAGCGATAAGGTTTTAATTTTTTTAATTTAAATGACGCAAGCCATAAACC 1320
                             GC  TATTG CA+CGATA+G TT+TA  TT TT A TT+AA +G
pMo/pmrBUPDN FW Seq     1188 GC--TATTGACAKCGATAMGCTTKTASATTSTTCACTTYAA-YG---------------- 1228
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b) Reverse sequencing

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pMo/pmrBUPDN Expected    721 TAAACCTTATGAGTTTGAT  739

pMo/pmrBUPDN RV Seq          ------------------

pMo/pmrBUPDN Expected    740 GAGTTGCTTGCCCGTATTCATGCATTACTACGCCGTAGTGGAGTAGAAGCTC---AACTT  796
                             G  +   T G  C +A  CA  C T  C   G  +    +  +   AA CT    A+  +
pMo/pmrBUPDN RV Seq     1314 GGAWACGTAGATCAWAG-CACYCGTAGCAGAGATRCGARRAGSCTYAAACTTTGGAWTAY 1256

pMo/pmrBUPDN Expected    797 GCGAGTCAAGATCAACTATTAGAAAGTGGCGATCTG-GTTTTAAATGTTG---AACAGCA  852
                               G +TCA + TC   T T A A  + GG+ ++ +G  TTTT A T+T++   A+  GCA
pMo/pmrBUPDN RV Seq     1255 CAGTKTCACKKTCTTTTGT-AAAGTKCGGMWWYKWGWATTTTGAGTRTKKSAAAMAGGCA 1197

pMo/pmrBUPDN Expected    853 TATTGCGA-CGTTTAAAGGT-CAACGCATTGATCTATC--AAATCGTGAAT--GGGCAAT  906
                             T++T+C+A C T+  AAG+T CAACGCA T  + T T   A++TC + +AT  GGGCA
pMo/pmrBUPDN RV Seq     1196 TWWTKCRAACATYACAAGKTTCAACGCAATAGWKTGTTCAAMRTCTKAWATTTGGGCATS 1137

pMo/pmrBUPDN Expected    907 CTTAATTCCACTTATGACTCACCC--AAATAAAATCTTTTCTAAAGCCAA-CTTAGAAGA  963
                              TTA TTCC+ +   +A T++C C  A+ATAAA+TC+TTT T+AAGCC+A  TTA+A GA
pMo/pmrBUPDN RV Seq     1136 TTTATTTCCMAWMTMKAATSMCKCCWAMATAAAMTCWTTTTTWAAGCCMAGTTTAKACGA 1077

pMo/pmrBUPDN Expected    964 TAAGTTATA-TGATTTTGATAGTGATGTGACCAGTAATACTATTGAAGTATA--TGTTCA 1020
                               A+ TAT+ +GAT TT A AG+GA G GACC+GTAA+ C+ TTG AGTA +  + T +A
pMo/pmrBUPDN RV Seq     1076 AKARCTATRGWGATSTTAA-AGKGAAGCGACCMGTAAK-CKTTTGGAGTAAWATWTTCMA 1019

pMo/pmrBUPDN Expected   1021 CCATTTAAGAGCGAAG-CTGGGTAAAGATTTTATCCGAACCATCCGAGGACTGGGCTACC 1079
                             C AT+TA GAG+GAAG CTGG TA AGA +TTATCCGAACCATC G+GGA TGGGCTACC
pMo/pmrBUPDN RV Seq     1018 CAATWTAGGAGSGAAGKCTGGTTATAGAAWTTATCCGAACCATC-GRGGA-TGGGCTACC  961

pMo/pmrBUPDN Expected   1080 G-TTTGGGATCCCCTGCACTTGCATGACCGCCTGGAAAACAATGGCCATGTTTAGCGCTA 1138
                             G TT++GGATCCCCTGCA  TGC+TGACCGC++GG+AAACAA GG C  GTTTAG G TA
pMo/pmrBUPDN RV Seq      960 GGTTYSGGATCCCCTGCAACTGCWTGACCGCMWGGRAAACAAAGGTCCAGTTTAGTGTTA  901

pMo/pmrBUPDN Expected   1139 AAGT-CCCAAATATAGCTTCCTAAAGTATTAGGGTGAACCATATTCCAAGGGCAGGCGTG 1197
                             AA+T CCCAAATATAG T    AA GT +TAG GTGA CCATATTCCAAGGGCAGGCGTG
pMo/pmrBUPDN RV Seq      900 AARTTCCCAAATATAGATCGTAAAGGTGKTAGCGTGATCCATATTCCAAGGGCAGGCGTG  841

pMo/pmrBUPDN Expected   1198 AGCAGATT-GTGACTTTATTAGGCCAACTATACTGCTACCGAGCATACTGACAAAAAA-C 1255
                             AGCAGATT GT+ACTTT+TTAG+CCA+CT+T C+GCTAC+GAGCA+AC+GACAAAAAA C
pMo/pmrBUPDN RV Seq      840 AGCAGATTTGTKACTTTRTTAGKCCAWCTWTTCYGCTACMGAGCAKACWGACAAAAAAWC  781

pMo/pmrBUPDN Expected   1256 ATATAGCCATATTGCCAGCGATAAGGTTTTAATTTTTTTAATTTAAATGACGCAAGCCAT 1315
                             ATATAG+CAT+T+GCCAGCGATAAGGTTTT+ TTTTTTTAATTT+A+TG+CGC+AGCCAT
pMo/pmrBUPDN RV Seq      780 ATATAGYCATWTKGCCAGCGATAAGGTTTTW-TTTTTTTAATTTRARTGRCGCRAGCCAT  722

pMo/pmrBUPDN Expected   1316 AAACCAAAAAAGATAACATAAACGGCGGTGATGATTTGTTTT-ACAATTTCATGATTAAG 1374
                             AAACCAAAAAAGATAACATAA+CGGCGGTGATG+TTT TT+T ACAATTTCATGA+TAAG
pMo/pmrBUPDN RV Seq      721 AAACCAAAAAAGATAACATAARCGGCGGTGATGWTTTTTTWTTACAATTTCATGAYTAAG  662

pMo/pmrBUPDN Expected   1375 CCTTTCAAGATACCAATTGTCCCGATTGAAAAAATGACCTGTTGAATCAATCCAAGGTTG 1434
                             CCT++CAAGATACCA+TTGTCCCGATTGAAAAA++GACC+ +TGAATCAATCCA+GGTTG
pMo/pmrBUPDN RV Seq      661 CCTWWCAAGATACCAWTTGTCCCGATTGAAAAAMKGACCKAWTGAATCAATCCARGGTTG  602

pMo/pmrBUPDN Expected   1435 GATGAGGTACATATCAATTTTCCCACCGACTGGAAATACAAATAGAAGAATAAAAAAACT 1494
                             GATGAGGTACATATCAATTTTCCCACCG+CTGGAAATACAAAT+GAAGAATAAAAAAACT
pMo/pmrBUPDN RV Seq      601 GATGAGGTACATATCAATTTTCCCACCGRCTGGAAATACAAATRGAAGAATAAAAAAACT  542

pMo/pmrBUPDN Expected   1495 GAAAAAAAGAAAGAGAATATTAATTTGGAAAAAAAGTTTTTGATTAGTCATTCTGATGCA 1554
                             GAAAAAAAGAAAGAGAATATTAATT GGAAAAAAAGTTTTTGATTAGTCATT+TGATGCA
pMo/pmrBUPDN RV Seq      541 GAAAAAAAGAAAGAGAATATTAATT-GGAAAAAAAGTTTTTGATTAGTCATTMTGATGCA  483

pMo/pmrBUPDN Expected   1555 ATATCAATCGACAATAGGTGCGACTAATTACATAAAATTAATCTTAAATTGCTCTTAAAC 1614
                             ATATCAATCGACAATAGGTGCGACTAATTACATAAAATTAATCTTAAATTGCTCTTAAAC
pMo/pmrBUPDN RV Seq      482 ATATCAATCGACAATAGGTGCGACTAATTACATAAAATTAATCTTAAATTGCTCTTAAAC  423

pMo/pmrBUPDN Expected   1615 TATACTGGTGAAAGAAGTTCAATTTTCCTTAAAAAATAAAAAAGCGACGAATAGTATCGT 1674
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                            TATACTGGTGAAAGAAGTTCAATTTTCCTTAAAAAATAAAAAAGCGACGAATAGTATCGT
pMo/pmrBUPDN RV Seq      422 TATACTGGTGAAAGAAGTTCAATTTTCCTTAAAAAATAAAAAAGCGACGAATAGTATCGT  363

pMo/pmrBUPDN Expected   1675 CGCCTTAAGCACTTTAGAAAGTATTAAAACATACCTTCTTCAGAGTTTGCTGAGATCTTA 1734
                             CGCCTTAAGCACTTTAGAAAGTATTAAAACATAC+TT+TTCAGAGTTTGC+GAGAT+TTA
pMo/pmrBUPDN RV Seq      362 CGCCTTAAGCACTTTAGAAAGTATTAAAACATACSTTSTTCAGAGTTTGCWGAGATMTTA  303

pMo/pmrBUPDN Expected   1735 TGAATAGATAAGTCTGCACCACGGAACTCATCTTCTTGAGATAGGCGAATGCCTATGGTC 1794
                             +GAATAGATAAGT++GCACCACGGAA+TCAT+TT++TGAGATAGG+GAATGCCTATGGTC
pMo/pmrBUPDN RV Seq      302 WGAATAGATAAGTYKGCACCACGGAAYTCATMTTYWTGAGATAGGYGAATGCCTATGGTC  243

pMo/pmrBUPDN Expected   1795 GCTTTAAGGATGCCATAAACAATGAAACCGCCAGCCAGTGCAATAGCAATCGCGAGTGCT 1854
                             GCT+TAAGGATGCCATAAACAATGAAACCGCCAGCCAGTGCAATAGCAATCGCGAGT CT
pMo/pmrBUPDN RV Seq      242 GCTWTAAGGATGCCATAAACAATGAAACCGCCAGCCAGTGCAATAGCAATCGCGAGTACT  183

pMo/pmrBUPDN Expected   1855 GTACCGATGAGCTGAGAGATGAATGATACGCCGCCTAAACCACCTAGCCATTTTTGACCA 1914
                             GTACCGATGAG+TGAGAGATGAATGATACGCCGC+TAAACCACCTAGCCATTTTTGACCA
pMo/pmrBUPDN RV Seq      182 GTACCGATGAGSTGAGAGATGAATGATACGCCGCSTAAACCACCTAGCCATTTTTGACCA  123

pMo/pmrBUPDN Expected   1915 AAAATACCTACGGCAATTCCACCAAATGCACCGCATACACCATGTAGCGGCCAGACACCA 1974
                             AAAATACCTACGGCAATTCCACCAAATGCACCGCATACACCATGTAGCGGCCAGACACCA
pMo/pmrBUPDN RV Seq      122 AAAATACCTACGGCAATTCCACCAAATGCACCGCATACACCATGTAGCGGCCAGACACCA   63

pMo/pmrBUPDN Expected   1975 AGAACGTCATCAACTTTGAGTTTGTTTTGAGTATAGGTGAATAGATACACGAACATAGCA 2034
                             AGAACGTCATCAACTTTGAGTTTGTTT GAGTATAGG+ AATAGA    CGA C+TA C
pMo/pmrBUPDN RV Seq       62 AGAACGTCATCAACTTTGAGTTTGTTT-GAGTATAGGK-AATAGAMCATCGATCWTACCT    5

pMo/pmrBUPDN Expected   2035 CCCG 2038
                              ++G
pMo/pmrBUPDN RV Seq        4 ASSG    1

pMo/pmrBUPDN Expected   2064 CTGCACCACCAATCACGCATGCCCC 2088

pMo/pmrBUPDN RV Seq          -------------------------
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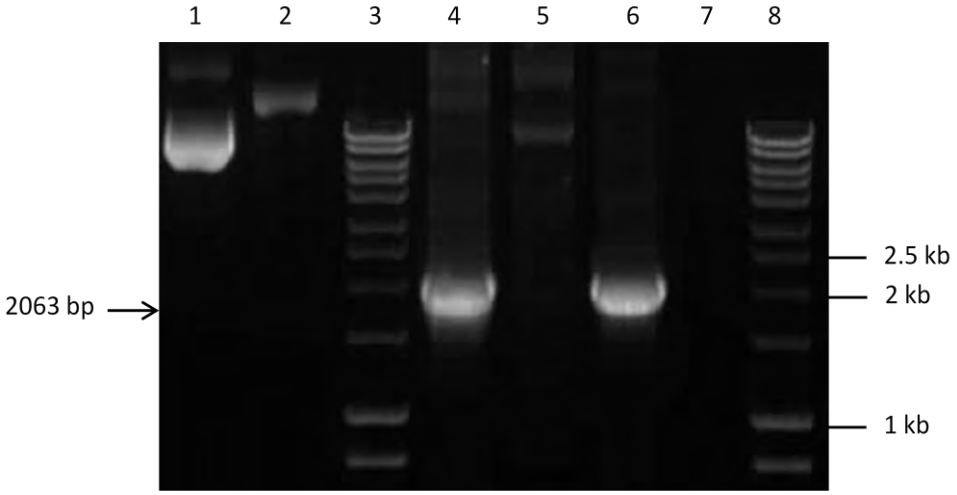
A region of pMo130-Tel^R containing *pmrAB* UP and DOWN fragments was amplified and sequenced. The result was aligned with the expected sequence.
a) pMo/*pmrB*UPDN FW Sequenced was generated using primer pMo130-Tel^R FW, which is specific for pMo130-Tel^R.
b) pMo/*pmrB*UPDNUPDN RV Sequenced was generated using primer DOWNRVpmrB, which is specific for the DOWN fragment. This sequence was reverse complemented before alignment. The first 1085 bp of the sequence corresponds to 174 bp of the vector and the entire UP fragment (a). The remainder of the sequence corresponds to the DOWN fragment (b).

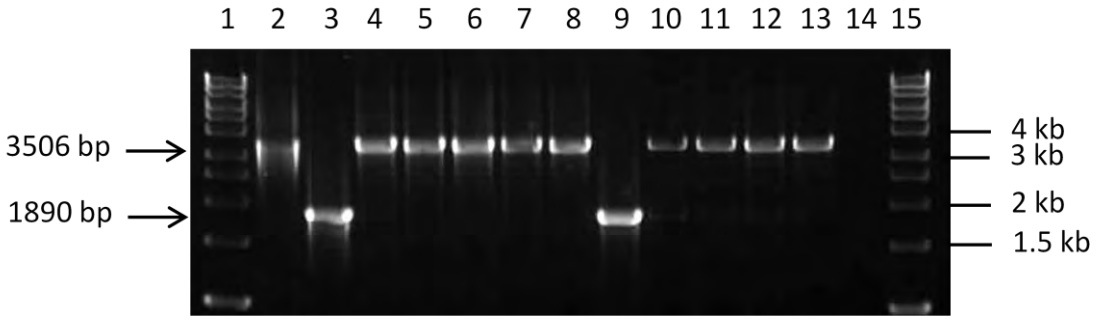
Figure 3.29 Verification of pMo130-Tel^R/*pmrAB*UPDOWN in S17-1



Lane	DNA	Expected size	Actual size
1	pMo130-Tel^R/*pmrAB*UPDOWN plasmid extraction from transformant 1	---	---
2	pMo130-Tel^R/*pmrAB*UPDOWN plasmid extraction from transformant 2	---	---
3, 8	Hyperladder™ 1 (Bioline)	---	---
4	pMo130-Tel^R/*pmrAB*UPDOWN (positive control)	2063 bp	~2063 bp
5	pMo130-Tel^R /*pmrAB*UPDOWN Transformant 1	2063 bp	No amplimer
6	pMo130-Tel^R /*pmrAB*UPDOWN Transformant 2	2063 bp	~2063 bp
7	---	No amplimer	No amplimer

PCR 14, Table 2.7

Figure 3.30 Verification of *pmrAB* gene Deletion in AYE by PCR.



Lane	DNA	Expected size	Actual size
1, 15	Hyperladder™ 1 (Bioline)	---	---
2	AYE (wild-type) genomic DNA	3506 bp	~3506 bp
3 - 13	AYEΔ*pmrAB* candidates 1-11	1890 bp	~ 1890 bp (AYEΔ*pmrAB)* ~ 3506 bp (AYE wild-type)
14	---	No amplimer	No amplimer

PCR 20, Table 2.7

Figure 3.31 Region Around *pmrB* in AYE and AYEΔ*pmrAB* (Expected Sequences) and Primers to Delete and Complement *pmrAB*.

Key for the following DNA sequences:

pmrC

pmrA

pmrB
-35: GTGACC
-10: TATATG
Shine dalgarno: ggga

ABAYE0734: gene encoding a hypothetical protein (xBASE)

UP primers

UPFWpmrB (Contains *Not*I recognition site)
GGGGCGGCCGCCTTATGCAATCGCACCGAGC

UPRVpmrB (Contains *Bam*HI recognition site)
GGGGGATCCCAAACGGTAGCCCAGTCCTC

DOWN primers

DOWNFWpmrB (Contains *Bam*HI recognition site)
GGGGGATCCCCTGCACTTGCATGACCGCC

DOWNRVpmrB(Contains *Sph*I recognition site)
GGGGCATGCGTGATTGGTGGTGCAGCGGG

UPFWpmrB and DOWNRVpmrB were also used to amplify the UP-*pmrAB*-DOWN insert for the complementation construct (Table 2.6).

a) Expected Sequence for Wild-type AYE.

tggctaaatatagcttctaagaacgattttagctaaaaatagaaaaggcgaggttaaac
ttatcctcgtctttttattttttaaatttaaaaaataataatgaaataaggcctctaaa
ttagatcatttttttatttaagtcatttttaagtttcaatttctacattaaagcatcataa
aaagattgtagtcactcacgATGCTGAATTTTTTTTCAACATTAAGAAATAAACAGATTT
CTCTGTTTATGTTTAATCTCATTATAGCCATTTGGCTAGGTGCAATTTTAAATATTGGTT
TTTATCATCAAGTCCATACTTTAACTCCATATTTTGGAGTAAAGGCTATTTTGTTTTTAG
CTGCTACTCTCGTAATTCTTGTTGCTACCTATTATGCGGTTTTACAAATCTTAAATTGGA
AATGGACTGCCAAAATCTTTGCAATTTTATTGATATTTATTGGTGGCTTTAGCTCTTATT
TTGTAAACACATTGGGTGTCATTATTTCACCTGACCAAATTCAAAACATGGTCCAGACAG
ATGTTTCAGAAGTTACCGATCTAATCTCTTTACGCTTTGTTTTATGGACAGTTTTTTTTG
TTATTTTACCCATTTTTTTAATTACTCAAGTTAAATTTAAACAAGAAAAAGTATCACGGT
TGTTATTGAAGAAAGTATTCTCACTGGTAGCTTCATTTGCAGTGGTCGGTGTTTTACTTT
TTACCTACTATGTCGATTTCGCTGCAATATTTCGTGAACATCGTGATTTAAAAGGGATGA
TTTCACCGCAAAATAGTATTTCATCGCTTATGTCTTACTATCATAAGAAGGCTCCGAAGA
AAAATCTGCCTCTTGTGATATATGGACAAGATGCTCATCAAGTTCAGCGCGTACAAAAGA
ACCTCCCTAAGTTAATGATACTTGTTGTAGGTGAAACGGCACGTGCCGAAAGTTTCTCTC
TAAATGGGTATGCAAAAAATACGAATCCGGAGCTTTCTAAACAAGATATTTTCAACTTTT
CGCAAGTGAGCTCATGCGGTACGGCGACAGCAGTTTCTGTGCCATGTATGTTCTCGGGTA
TGCCACGTGTAGATTATGATGAGCAATTAGCCAGTCACCGCGAAGGTTTACTAGATATTG
CAAAACGTGCGGGTTACCAAGTGACTTGGATTGATAATAACTCGGGTTGTAAAGGTGCAT
GTGATCGTGTTGAACAATACCAGATTCCAGAAAACTTAAAGAAAAAATGGTGTAAAGATG
GCGAATGTTATGATGACATTCTCATTGACAGCTTAAAGCAGTATTTGGCTACTATTGCCA
AAGATGATGATCGCCCACGTTTGATTGTTTTGCATCAGGTGGGTAGTCACGGGCCTGCAT
ATTACAAGCGTGCGCCTGAGGCATATCAACCCTTTAAACCGACTTGTGATACGAATGCGA
TACAGGGCTGTTCGCAAACCGAATTGCTAAATAGTTATGATAATACAATCGTATATACAG
ACCATGTATTAAGCCAAATGATTAATACTCTAAAAGAAATATCAAAATATCAGACAGGTT
TATGGTATTTATCTGATCATGGCGAATCAACCGGAGAACATGGTTTATATTTACATGGTT
CACCTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTG
AAAGTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAA
AGTTAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTG
gatgtaaaaactcaggtcatcaaccctcaactggacatgttgcactcttgtgcccatgta
aactaaagcgagcctagaacATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTG
CAGAATCAACGATCACGTTGCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACG
GTTTAGATGGTTTGGCTCAATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAG
GATTGCCTATGATGGATGGTATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACAC
CAGTATTAATTATTTCTGCTCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGG
GTGCAGATGATTATTTAATTAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATG
CATTACTACGCCGTAGTGGAGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAA
GTGGCGATCTGGTTTTAAATGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTG
ATCTATCAAATCGTGAATGGGCAATCTTAATTCCACTTATGACTCACCCAAATAAAATCT
TTTCTAAAGCCAACTTAGAAGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATA
CTATTGAAGTATATGTTCACCATTTAAGAGCGAAGCTGGGTAAAGATTTTATCCGAACCA
TCCGAGGACTGGGCTACCGTTTGGGGCAATCATAAtttaaaatttcgggacttcataaaa
GTGCATTATTCATTAAAAAAACGACTGATTTGGGGCACCTCAATTTTCAGTGTCATCTTA
GGTTGTATTTTAATTTTTAGTGCTTACAAGGTTGCACTTCAAGAAGTCGATGAAATTCTA
GATACTCAAATGAAGTATTTAGCGGAAAGAACAGCTGAGCACCCTTTAAAAACTGTAAGC
AGTAAGTTCGATTTTCATAAAACTTACCACGAAGAAGATCTGTTTATCGATATTTGGGCT
TATAAGGATCAGGCTCATTTGTCTCATCATTTACATTTGCTGGTTCCACCTGTTGAGCAA
GCGGGATTTTATTCTCATAAAACCGCTCAAGGTATAGTCAGAACTTATGTTTTACCTTTG
AAGGATTACCAGATTCAGGTCAGCCAGCAAGAGAGGGTTCGTGAAGCTTTCGCTTGGGAG
CTTGCGGGCAGTATGTTTATTCCGTATTTAATTATTTTACCTTTTGCAATATTTGCTTTA
GCAGCCATTATTCGTCGTGGTTTAAAACCAATAGATGATTTTAAAAATGAGTTAAAAGAA
CGCGATTCCGAAGAACTCACCCCAATTGAAGTACATGATTATCCTCAAGAGCTTTTACCT
ACTATTGACGAAATGAACCGTCTTTTTGAGCGAATTTCTAAAGCTCAAAATGAACAGAAG
CAATTTATTGCCGATGCTGCTCATGAACTACGAACACCTGTGACTGCATTGAACTTACAA
ACCAAGATTTTGCTAAGTCAGTTCCCTGAGCATGAATCATTGCAAAACTTAAGCAAAGGT
TTGGCACGTATTCAGCATTTGGTGACTCAGCTTTTAGCATTGGCAAAGCAAGATGTAACT
TTAAGTATGGTCGAGCCTACTGGATATTTTCAACTCAATGATGTGGCATTAAATTGTGTG
GAGCAGTTGGTTAACTTGGCTATGCAAAAAGAAATCGATTTAGGTTTTGTTAGAAATGAA

CCCATCGAAATGCATAGTATTGAACCTACTGTACATTCGATTATTTTTAATTTAATTGAT
AATGCAATTAAGTACACCCCGCATCAGGGTGTTATTAATATTTCAGTTTATACCGATCAA
GATCACTACGCATGTATTCAAATTGAAGATAGCGGTGCAGGAATAGACCCTGAAAATTAC
GATAAAGTCCTTAAGCGTTTTTATCGCGTGCATCACCATCTTGAGGTGGGAAGTGGTCTA
GGTTTATCTATTGTAGATCGTGCAACTCAAAGGCTTGGTGGGACTTTAACTCTCGATAAG
AGCTTAGAGCTTGGCGGTCTTTCTGTATTAGTGAAATTACCTAAAGTCTTACATTTACAT
GAAACAAGAGCGTGAttacgtaagctcttgtttcacttgtttttcgaactcaaaacgatg
aatacaaatcgcataaaataaaatatttattgcccaaacgacccagcctgtccataagtt
atggcttaggaaatgtgctcctcgcatcatttgcgcccaacccatcatgaaacctaataa
aataccggagaataaataaaagtaagcacgtttaggttgttcaagacgataaacaaaata
acctgtcattaaaataaaacctgcacttgcatgaccgcctggaaaacaatggccatgttt
agcgctaaagtcccaaatatagcttcctaaagtattagggtgaaccatattccaagggca
ggcgtgagcagattgtgactttattaggccaactatactgctaccgagcatactgacaaa
aaacatatagccatattgccagcgataaggttttaatttttttaatttaaatgacgcaag
ccataaaccaaaaaagataacataaacggcggtgatgatttgttttacaatttcatgatt
aagcctttcaagataccaattgtcccgattgaaaaaatgacctgttgaatcaatccaagg
ttggatgaggtacatatcaattttcccaccgactggaaatacaaatagaagaataaaaaa
actgaaaaaaagaaagagaatattaatttggaaaaaaagttttgattagtcattctgat
gcaatatcaatcgacaataggtgcgactaattacataaaattaatcttaaattgctctta
aactatactggtgaaagaagttcaattttccttaaaaaataaaaaagcgacgaatagtat
cgtcgccttaagcactttagaaagtattaaaacataccttcttcagagtttgctgagatc
ttatgaatagataagtctgcaccacggaactcatcttcttgagataggcgaatgcctatg
gtcgctttaaggatgccataaacaatgaaaccgccagccagtgcaatagcaatcgcgagt
gctgtaccgatgagctgagagatgaatgatacgccgcctaaaccacctagccatttttga
ccaaaaatacctacggcaattccaccaaatgcaccgcatacaccatgtagcggccagaca
ccaagaacgtcatcaactttgagtttgttttgagtataggtgaatagatacacgaacata
gcacccgctgcaccaccaatcacaagtgcactgactggatgaacaatgtcagaacctgcg
caaatagcgactaaacccgcaagtggaccgttgtgtaagaaaccagggtcatttttccca
atcgcatttgctgtaattgtaccgccaaccattgccataagtgagttaatcgcaacgaga
ccagagattgcatcaacacgctgagcactcatcacattaaagccaaaccagcctacaatc
aaaatccatgaaccaagcgccaaaaatggaatagaagaaggtgggtgagcacttacacga

b) Expected Sequence for AYEΔ*pmrAB*.

CTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTG
AAAGTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAA
AGTTAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTG
gatgtaaaaactcaggtcatcaaccctcaactggacatgttgcactcttgtgcccatgta
aactaaagcgagcctagaacATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTG
CAGAATCAACGATCACGTTGCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACG
GTTTAGATGGTTTGGCTCAATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAG
GATTGCCTATGATGGATGGTATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACAC
CAGTATTAATTATTTCTGCTCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGG
GTGCAGATGATTATTTAATTAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATG
CATTACTACGCCGTAGTGGAGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAA
GTGGCGATCTGGTTTTAAATGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTG
ATCTATCAAATCGTGAATGGGCAATCTTAATTCCACTTATGACTCACCCAAATAAAATCT
TTTCTAAAGCCAACTTAGAAGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATA
CTATTGAAGTATATGTTCACCATTTAAGAGCGAAGCTGGGTAAAGATTTTATCCGAACCA
TCCGAGGACTGGGCTACCGTTTG ***GGATCC***
cctgcacttgcatgaccgcctggaaaacaatggccatgttt
agcgctaaagtcccaaatatagcttcctaaagtattagggtgaaccatattccaagggca
ggcgtgagcagattgtgactttattaggccaactatactgctaccgagcatactgacaaa
aaacatatagccatattgccagcgataaggttttaatttttttaatttaaatgacgcaag
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aagcctttcaagataccaattgtcccgattgaaaaaatgacctgttgaatcaatccaagg
ttggatgaggtacatatcaattttcccaccgactggaaatacaaatagaagaataaaaaa
actgaaaaaaagaaagagaatattaatttggaaaaaaagttttgattagtcattctgat
gcaatatcaatcgacaataggtgcgactaattacataaaattaatcttaaattgctctta
aactatactggtgaaagaagttcaattttccttaaaaaataaaaaagcgacgaatagtat
cgtcgccttaagcactttagaaagtattaaaacataccttcttcagagtttgctgagatctt
atgaatagataagtctgcaccacggaactcatcttcttgagataggcgaatgcctatggtcgc
tttaaggatgccataaacaatgaaaccgccagccagtgcaatagcaatcgcgagtgctgtacc
gatgagctgagagatgaatgatacgccgcctaaaccacctagccatttttgaccaaaaatacc
tacggcaattccaccaaatgcaccgcatacaccatgtagcggccagacaccaagaacgtcatc
aactttgagtttgttttgagtataggtgaatagatacacgaacatagcacccgctgcaccacc
aatca

It was expected that a *Bam*HI site (***GGATCC***) would be introduced in AYEΔ*adeRS*.

Figure 3.32 Verification of *pmrAB* Deletion in AYE by DNA Sequencing.

a) Forward sequencing

```
pmrB candidate7 FW        1 ---------------------------------ACCACAAMMATGTACCAATGATTATGTG   28
                                                              A CA A ++ATGTACCAATGATTATGTG
pmrB mutant expected      1 GGGGCGGCCGCCTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTG   60

pmrB candidate7 FW       29 GTTCTCTGAA-GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAAC   87
                            GTTCTCTGAA GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAAC
pmrB mutant expected     61 GTTCTCTGAAAGTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAAC  120

pmrB candidate7 FW       88 TAAACAAAAGTTAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAA  147
                            TAAACAAAAGTTAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAA
pmrB mutant expected    121 TAAACAAAAGTTAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAA  180

pmrB candidate7 FW      148 AACTCAGGTCATCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAG  207
                            AACTCAGGTCATCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAG
pmrB mutant expected    181 AACTCAGGTCATCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAG  240

pmrB candidate7 FW      208 CGAGCCTAGAACATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCA  267
                            CGAGCCTAGAACATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCA
pmrB mutant expected    241 CGAGCCTAGAACATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCA  300

pmrB candidate7 FW      268 ACGATCACGTTGCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGAT  327
                            ACGATCACGTTGCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGAT
pmrB mutant expected    301 ACGATCACGTTGCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGAT  360

pmrB candidate7 FW      328 GGTTTGGCTCAATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCT  387
                            GGTTTGGCTCAATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCT
pmrB mutant expected    361 GGTTTGGCTCAATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCT  420

pmrB candidate7 FW      388 ATGATGGATGGTATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTA  447
                            ATGATGGATGGTATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTA
pmrB mutant expected    421 ATGATGGATGGTATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTA  480

pmrB candidate7 FW      448 ATTATTTCTGCTCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGAT  507
                            ATTATTTCTGCTCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGAT
pmrB mutant expected    481 ATTATTTCTGCTCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGAT  540

pmrB candidate7 FW      508 GATTATTTAATTAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTA  567
                            GATTATTTAATTAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTA
pmrB mutant expected    541 GATTATTTAATTAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTA  600

pmrB candidate7 FW      568 CGCCGTAGTGGAGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGAT  627
                            CGCCGTAGTGGAGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGAT
pmrB mutant expected    601 CGCCGTAGTGGAGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGAT  660

pmrB candidate7 FW      628 CTGGTTTTAAATGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTGATCTATCA  687
                            CTGGTTTTAAATGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTGATCTATCA
pmrB mutant expected    661 CTGGTTTTAAATGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTGATCTATCA  720

pmrB candidate7 FW      688 AATCGTGAATGGGCAATCTTAATTCCACTTATGACTCACCCAAATAAAATCTTTTCTAAA  747
                            AATCGTGAATGGGCAATCTTAATTCCACTTATGACTCACCCAAATAAAATCTTTTCTAAA
pmrB mutant expected    721 AATCGTGAATGGGCAATCTTAATTCCACTTATGACTCACCCAAATAAAATCTTTTCTAAA  780

pmrB candidate7 FW      748 GCCAACTTAGAAGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATACTATTGAA  807
                            GCCAACTTAGAAGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATACTATTGAA
pmrB mutant expected    781 GCCAACTTAGAAGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATACTATTGAA  840

pmrB candidate7 FW      808 GTATATGTTCACCATTTAAGAGCGAAGCTGGGTAAAGATTTTATCCGAACCATCCGAGGA  867
                            GTATATGTTCACCATTTAAGAGCGAAGCTGGGTAAAGATTTTATCCGAACCATCCGAGGA
pmrB mutant expected    841 GTATATGTTCACCATTTAAGAGCGAAGCTGGGTAAAGATTTTATCCGAACCATCCGAGGA  900

pmrB candidate7 FW      868 CTGGGCTACCGTTTGGGATCCCCTGCACTTGCATGACCGCCTGGAAAC--ATGGCCATGT  925
                            CTGGGCTACCGTTTGGGATCCCCTGCACTTGCATGACCGCCTGGAAA   ATGGCCATGT
pmrB mutant expected    901 CTGGGCTACCGTTTGGGATCCCCTGCACTTGCATGACCGCCTGGAAAACAATGGCCATGT  960

pmrB candidate7 FW      926 TTAGCGCTAAAGTCCCAA-TATAGCTTCCTAA-GTAT--AGGTGAAC-ATATTCCA-GGG  979
```

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                            TTAGCGCTAAAGTCCCAA TATAGCTTCCTAA GTAT   GGTGAAC ATATTCCA GGG
pmrB mutant expected    961 TTAGCGCTAAAGTCCCAAATATAGCTTCCTAAAGTATTAGGGTGAACCATATTCCAAGGG 1020

pmrB candidate7 FW      980 CAG-CGTGAGCAGAT-GTGACTTW--TAGCC--ACTATACTGCTACCGAGCATACTGACA 1033
                            CAG CGTGAGCAGAT GTGACTT+  TAG C  ACTATACTGCTACCGAGCATACTGACA
pmrB mutant expected   1021 CAGGCGTGAGCAGATTGTGACTTTATTAGGCCAACTATACTGCTACCGAGCATACTGACA 1080

pmrB candidate7 FW     1034 AAAMW---AWAGC-ATATTGC-AGCGATAAGGTTTAA--TTTTTTAATTTAA-TGACGCA 1085
                            AAA++   A+AGC ATATTGC AGCGATAAGGTTT A  TTTTTTAATTTAA TGACGCA
pmrB mutant expected   1081 AAAAACATATAGCCATATTGCCAGCGATAAGGTTTTAATTTTTTTAATTTAAATGACGCA 1140

pmrB candidate7 FW     1086 G--CAWAAC---AAAAAGAWAM--WAAACGGSGGKGATGATTG--TTTACA--TTCATGA 1134
                               CA+AA    AAAAAGA+A+  +AAACGG+GG+GATGATT   TTTACA  TTCATGA
pmrB mutant expected   1141 AGCCATAAACCAAAAAAGATAACATAAACGGCGGTGATGATTTGTTTTACAATTTCATGA 1200

pmrB candidate7 FW     1135 TTAG--CTTTCARAW---CMATTGTCC-GAT--GAAAAATGAC-TGTKAWY---AYYCAG 1182
                            TTA   CTTTCA+ +   C+ATTGTCC GAT   AAAAATGAC TGT+ +    A++CA
pmrB mutant expected   1201 TTAAGCCTTTCAAGATACCAATTGTCCCGATTGAAAAAATGACCTGTTGAATCAATCCAA 1260

pmrB candidate7 FW     1183 GKT--GAKRARGWMYW-AYWMATTTCCAC---GMSKGAWWCCATAGR-------------- 1223
                            G+T  GA++A+G++++ A+ +ATTT C C   G+++G ++  A A +
pmrB mutant expected   1261 GGTTGGATGAGGTACATATCAATTTTCCCACCGACTGGAAATACAAATAGAAGAATAAAA 1320
```

b) Reverse sequencing

```
pmrB candidate7 RV          ---------------------------------------------

pmrB mutant expected    481 ATTATTTCTGCTCGAGATCAATTACAAAACCGTGTCGATGGTTTA  525

pmrB candidate7 RV     1278 CACTCGAGCGCAGAKKCMTATTTATTTA----TCATTRGTTSTRTGTGCTGC-----CGA 1228
                             A T G G GCAGA++  TATTTA TTA    T AT +GTT  +TG G TGC     CG
pmrB mutant expected    526 AATTTGGGTGCAGATGATTATTTAATTAAACCTTATGAGTTTGATGAGTTGCTTGCCCGT  585

pmrB candidate7 RV     1227 TTTCATGCATTAC-GCGTACGAGTGGAGTC--------ACTTG--ARTCAG--TCAACT- 1182
                              TTCATGCATTAC  CG    AGTGGAGT         ACTTG  A+TCA   TCAACT
pmrB mutant expected    586 ATTCATGCATTACTACGCCGTAGTGGAGTAGAAGCTCAACTTGCGAGTCAAGATCAACTA  645

pmrB candidate7 RV     1181 ---KGRAATGGGCRTTCGTTAKTGACAGCWR----------TGCGASRTK---AAGTCAS 1138
                                + +A TGG  +T  G T +T A  G ++          TGCGA++T+   A GTCA
pmrB mutant expected    646 TTAGAAAGTGGCGATCTGGTTTTAAATGTTGAACAGCATATTGCGACGTTTAAAGGTCAA  705

pmrB candidate7 RV     1137 CATGATCATCAATC------KGAATSGGCATCTATCACT------WTGACTCACCCGAAT 1090
                             C    T ATC ATC      +GAAT+GGCA    T A T      +TGACTCACCC AAT
pmrB mutant expected    706 CGCATTGATCTATCAAATCGTGAATGGGCAATCTTAATTCCACTTATGACTCACCCAAAT  765

pmrB candidate7 RV     1089 CAAATCWTT-CTAAAGCYAACT-MGWAGMTAAGY-ATATGATT--GATAGTGATGTGACC 1035
                              AAATC+TT CTAAAGC+AACT +G+AG+TAAG+ ATATGATT  GATAGTGATGTGACC
pmrB mutant expected    766 AAAATCTTTTCTAAAGCCAACTTAGAAGATAAGTTATATGATTTTGATAGTGATGTGACC  825

pmrB candidate7 RV     1034 AGTAATACTATS--AGTATATGT-CAYCATT-AAGAGCGAWGCTGGGTAAMGATTT-ATC  980
                             AGTAATACTAT   AGTATATGT CA+CATT AAGAGCGA+GCTGGGTAA+GATTT ATC
pmrB mutant expected    826 AGTAATACTATTGAAGTATATGTTCACCATTTAAGAGCGAAGCTGGGTAAAGATTTTATC  885

pmrB candidate7 RV      979 CGATCCATCYSAGGACTGGGCTACCGTT-GGGATCCC-TGCACTTGCATGACCGCATGGA  922
                             CGA CCATC++AGGACTGGGCTACCGTT GGGATCCC TGCACTTGCATGACCGC TGGA
pmrB mutant expected    886 CGAACCATCCGAGGACTGGGCTACCGTTTGGGATCCCCTGCACTTGCATGACCGCCTGGA  945

pmrB candidate7 RV      921 AAACAATGGCCATGTKW-GCGCTAAAGTCCCAAATATASCTTCCTAAAGTMTTAGGGTGA  863
                             AAACAATGGCCATGT++ GCGCTAAAGTCCCAAATATA+CTTCCTAAAGT+TTAGGGTGA
pmrB mutant expected    946 AAACAATGGCCATGTTTAGCGCTAAAGTCCCAAATATAGCTTCCTAAAGTATTAGGGTGA 1005

pmrB candidate7 RV      862 ACCATATTCCAAGGGCAGGCGTGAGCAGATTGTGWCTTTAKTAGGCCAACTATACTGCTA  803
                             ACCATATTCCAAGGGCAGGCGTGAGCAGATTGTG+CTTTA+TAGGCCAACTATACTGCTA
pmrB mutant expected   1006 ACCATATTCCAAGGGCAGGCGTGAGCAGATTGTGACTTTATTAGGCCAACTATACTGCTA 1065

pmrB candidate7 RV      802 CCGAGCATACTGACAAAAAACATATAKCCATATTGCCAKCGATAAGGTTTTAATTTTTTT  743
                             CCGAGCATACTGACAAAAAACATATA+CCATATTGCCA+CGATAAGGTTTTAATTTTTTT
pmrB mutant expected   1066 CCGAGCATACTGACAAAAAACATATAGCCATATTGCCAGCGATAAGGTTTTAATTTTTTT 1125

pmrB candidate7 RV      742 AATTTAAATGACGCAAGCCATAAACCAAAAAAGATAACATAAACGGCGGTGATGATTTGT  683
                             AATTTAAATGACGCAAGCCATAAACCAAAAAAGATAACATAAACGGCGGTGATGATTTGT
pmrB mutant expected   1126 AATTTAAATGACGCAAGCCATAAACCAAAAAAGATAACATAAACGGCGGTGATGATTTGT 1185

pmrB candidate7 RV      682 TTTACAATTTCATGATTAAGCCTYTCAAGATACCAATTSTCCCGATTGAAAAAATGMCCT  623
                             TTTACAATTTCATGATTAAGCCT+TCAAGATACCAATT+TCCCGATTGAAAAAATG+CCT
pmrB mutant expected   1186 TTTACAATTTCATGATTAAGCCTTTCAAGATACCAATTGTCCCGATTGAAAAAATGACCT 1245

pmrB candidate7 RV      622 GTTGAATCAATCCAAGGTTGGATGAGGTACATATCAATTTTCCCACCGACTGGAAATACA  563
                             GTTGAATCAATCCAAGGTTGGATGAGGTACATATCAATTTTCCCACCGACTGGAAATACA
pmrB mutant expected   1246 GTTGAATCAATCCAAGGTTGGATGAGGTACATATCAATTTTCCCACCGACTGGAAATACA 1305

pmrB candidate7 RV      562 AATAGAAGAATAAAAAAACTGAAAAAAAGAAAGAGAATATTAATTTGGAAAAAAAGTTTT  503
                             AATAGAAGAATAAAAAAACTGAAAAAAAGAAAGAGAATATTAATTTGGAAAAAAAGTTTT
pmrB mutant expected   1306 AATAGAAGAATAAAAAAACTGAAAAAAAGAAAGAGAATATTAATTTGGAAAAAAAGTTTT 1365

pmrB candidate7 RV      502 YGATTAGTCATTCTGATSCAATATCAATCGACAATAGGTGCGACTAATTACATAAAATTA  443
                             +GATTAGTCATTCTGAT+CAATATCAATCGACAATAGGTGCGACTAATTACATAAAATTA
pmrB mutant expected   1366 TGATTAGTCATTCTGATGCAATATCAATCGACAATAGGTGCGACTAATTACATAAAATTA 1425

pmrB candidate7 RV      442 ATCTTAAATTGCTCTTAAACTATACTGGTGAAAGAAGTTCAATTTTCCTTAAAAAATAAA  383
                             ATCTTAAATTGCTCTTAAACTATACTGGTGAAAGAAGTTCAATTTTCCTTAAAAAATAAA
pmrB mutant expected   1426 ATCTTAAATTGCTCTTAAACTATACTGGTGAAAGAAGTTCAATTTTCCTTAAAAAATAAA 1485
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pmrB candidate7 RV      382 AAAGCGACGAATAGTATCGTCGCCTTAAGCACTTTAGAAAGTATTMAAACATACCTTCTT  323
                            AAAGCGACGAATAGTATCGTCGCCTTAAGCACTTTAGAAAGTATT+AAACATACCTTCTT
pmrB mutant expected   1486 AAAGCGACGAATAGTATCGTCGCCTTAAGCACTTTAGAAAGTATTAAAACATACCTTCTT 1545

pmrB candidate7 RV      322 CAGAGTTTGCTGAGATCTTATGAATAGATAAGTCTGCACCACGGAACTCATCTTCTTGAG  263
                            CAGAGTTTGCTGAGATCTTATGAATAGATAAGTCTGCACCACGGAACTCATCTTCTTGAG
pmrB mutant expected   1546 CAGAGTTTGCTGAGATCTTATGAATAGATAAGTCTGCACCACGGAACTCATCTTCTTGAG 1605

pmrB candidate7 RV      262 ATAGGCGAATGCCTATGGTCGCYTTAAGGATGCCATAAACAATGAAACCGCCAGCCAGTG  203
                            ATAGGCGAATGCCTATGGTCGC+TTAAGGATGCCATAAACAATGAAACCGCCAGCCAGTG
pmrB mutant expected   1606 ATAGGCGAATGCCTATGGTCGCTTTAAGGATGCCATAAACAATGAAACCGCCAGCCAGTG 1665

pmrB candidate7 RV      202 CAATAGCAATCGCGAGTGCTGYACCGAYGAGCTGAGAGATGAATSATACGCCGCCTAAAC  143
                            CAATAGCAATCGCGAGTGCTG+ACCGA+GAGCTGAGAGATGAAT+ATACGCCGCCTAAAC
pmrB mutant expected   1666 CAATAGCAATCGCGAGTGCTGTACCGATGAGCTGAGAGATGAATGATACGCCGCCTAAAC 1725

pmrB candidate7 RV      142 CACCTAGCCATTYTTGACCAAAAATACCTACGGCAATTCCACCAAATGCACCGCATACAC   83
                            CACCTAGCCATT+TTGACCAAAAATACCTACGGCAATTCCACCAAATGCACCGCATACAC
pmrB mutant expected   1726 CACCTAGCCATTTTTGACCAAAAATACCTACGGCAATTCCACCAAATGCACCGCATACAC 1785

pmrB candidate7 RV       82 CATGTAGCGGCCAGACACCAAGAACGTCATCAACTTTGAGTTTGTTTTGWGTATAGGK-A   24
                            CATGTAGCGGCCAGACACCAAGAACGTCATCAACTTTGAGTTTGTTTTG+GTATAGG+ A
pmrB mutant expected   1786 CATGTAGCGGCCAGACACCAAGAACGTCATCAACTTTGAGTTTGTTTTGAGTATAGGTGA 1845

pmrB candidate7 RV       23 ATAGATACACGAA-ACATMACCTG    1
                            ATAGATACACGAA A A +ACC G
pmrB mutant expected   1846 ATAGATACACGAACATAGCACCCG 1869

pmrB candidate7 RV          -------------------------

pmrB mutant expected   1870 CTGCACCACCAATCACGCATGCCCC 1894
```

The region of the AYEΔ*pmrAB* genome flanking *pmrB* (UP and DOWN fragments) was amplified by a single PCR (1894 bp amplimer) and sequenced. The result was aligned with the expected sequence.

a) pmrB candidate7 FW was generated using primer UPFWpmrB (Table 2.6); b) pmrB candidate7 RV was generated using primer DOWNRVpmrB (Table 2.6). This sequence was reverse complemented before alignment.

The first 915 bp of the sequence corresponds to the UP fragment and 922-1894 bp corresponds to the DOWN fragment. A *Bam*HI site has been introduced at 916-921 bp in the mutant (highlighted).

which were designed to delete the whole of *pmrB*, also flanked part of *pmrA* and ABAYE0734 (Figure 3.31). Candidate 7 was selected for phenotypic characterisation.

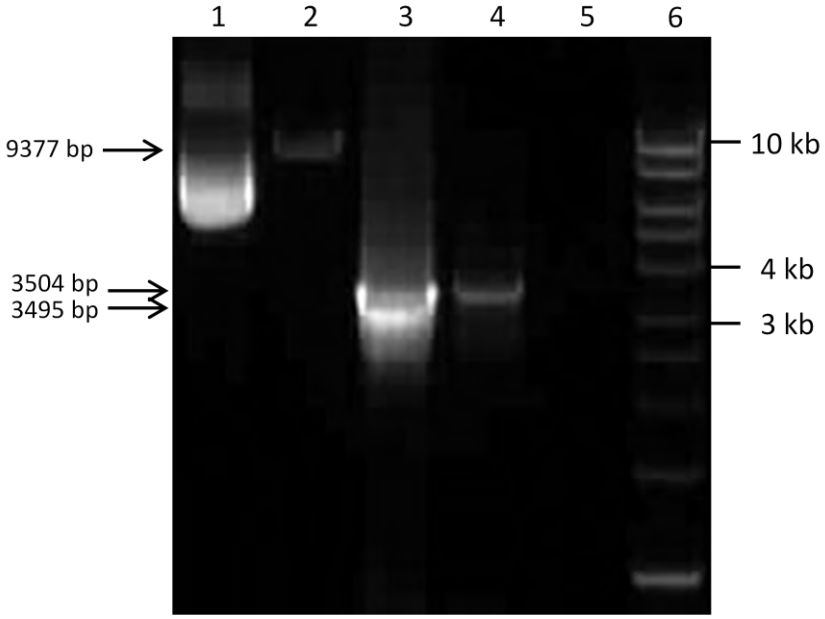
3.3.2.2. Complementation of *pmrAB* Gene Deletion

The fragment containing the deleted region (including *pmrB* and 12 bp of *pmrA*) and the UP and DOWN fragments used to delete *pmrAB* was amplified and verified by PCR (Figure 3.33; Section 2.4). This amplimer and pMo130-Tel^{R} were digested and ligated (Figure 3.33). The ligation mixture was transformed into DH5α and 15 candidate transformants were obtained, and four of these were confirmed by PCR to harbour pMo130-Tel^{R}/UP-*pmrAB*-DOWN (Figure 3.34). The construct from candidate 3 was further verified by digestion and DNA sequencing. Digestion with *Not*I (Figure 3.35) confirmed that the construct contained an insert. DNA sequencing confirmed that the insert contained the UP and DOWN fragments and the region deleted in AYEΔ*pmrAB* (Figure 3.36).

The verified construct was transformed into S17-1 and four candidate transformants were obtained. Plasmid was extracted from each transformant and its identity was verified by PCR (Figure 3.37).

Despite 12 attempts at conjugation and several modifications to the method, no AYEΔ*pmrAB*/pMo130-Tel^{R}/UP-*pmrAB*-DOWN single recombinants were obtained and AYEΔ*pmrAB* was not complemented. The strategies and method modifications used to attempt to introduce the complementation construct into AYEΔ*pmrAB* were as described previously (Section 3.3.1.2). As for the conjugations carried out to complement AYEΔ*adeRS*, colonies were usually not obtained until after 48 hr incubation. All colonies remained white after pyrocatchol

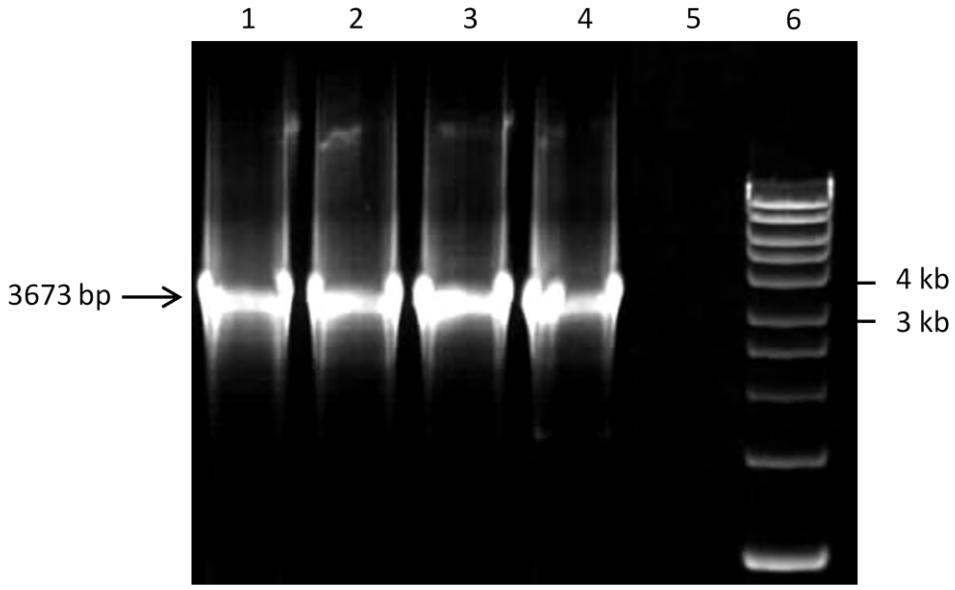
Figure 3.33 Digestion of pMo130-TelR and UP-*pmrAB*-DOWN.



Lane	DNA	Expected size	Actual size
1	pMo130-Tel^{R} undigested	---	---
2	pMo130-Tel^{R} + *Not*I + *sph*I	9377bp	~ 9377bp
3	UP-*pmrAB*-DOWN undigested	3504 bp	~3504bp
4	UP-*pmrAB*-DOWN + *Not*I + *sph*I	3495 bp	~ 3495 bp
5	--- (Contamination control for UP-*pmrAB*-DOWN amplification)	No amplimer	No amplimer
6	Hyperladder™ 1 (Bioline)	---	---

Lanes 4 and 6, PCR 20, Table 2.7; Lanes 3 and 5, Digestion 5, Table 2.8.

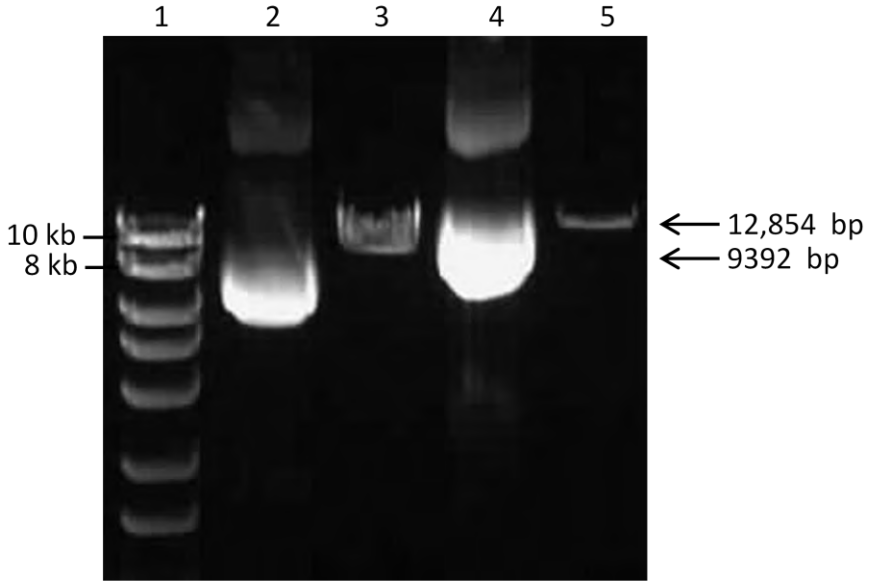
Figure 3.34 Verification of *pmrAB* Complementation Construct by PCR.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ 1 (Bioline)	---	---
2	Plasmid from candidate transformant 3	3673 bp	~3673 bp
3	Plasmid from candidate transformant 4	3673 bp	~3673 bp
4	Plasmid from candidate transformant 6	3673 bp	~3673 bp
5	Plasmid from candidate transformant 7	3673 bp	~3673 bp
6	---	No amplimer	No amplimer
7	Hyperladder™ 1 (Bioline)	---	---

PCR 14, Table 2.7

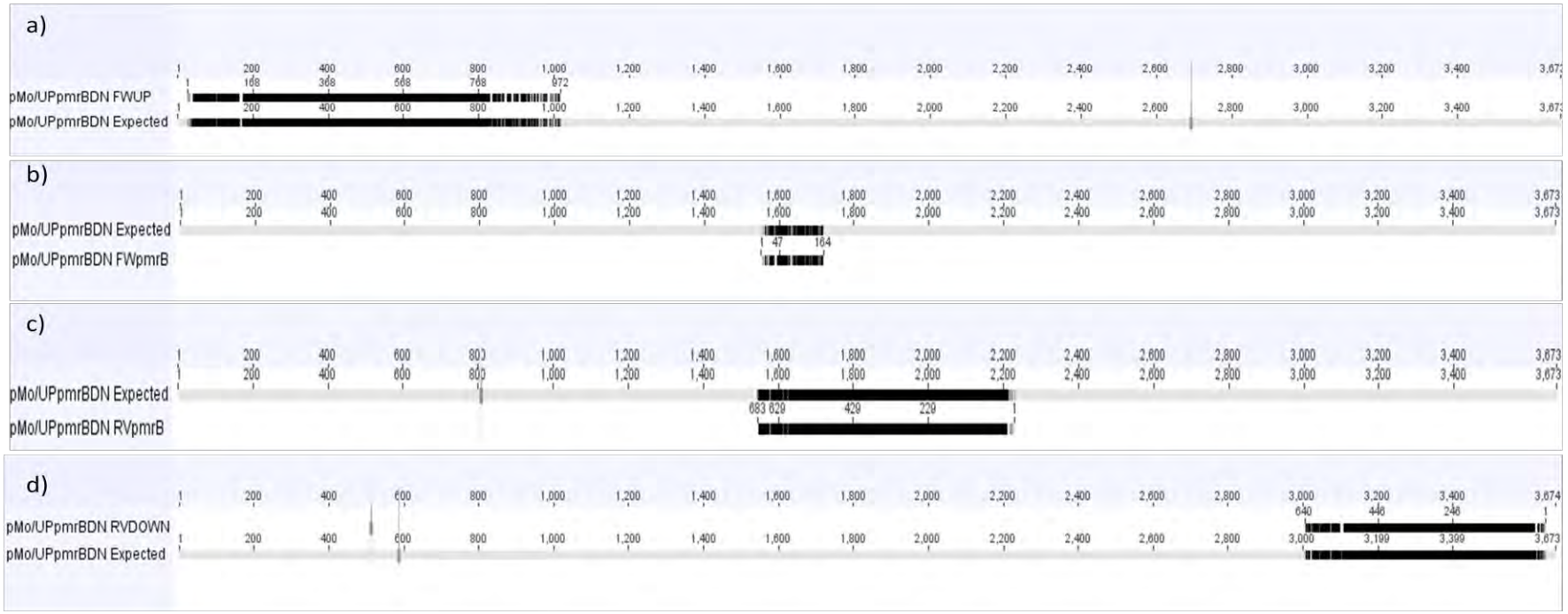
Figure 3.35 Verification of *pmrAB* Complementation Construct by Digestion.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ 1 (Bioline)	---	---
2	pMo130-Tel^{R} undigested	---	---
3	pMo130-Tel^{R} + *Not*I	9392 bp	~ 9392 bp
4	pMo130-Tel^{R}/UP-*pmrAB*-DOWN undigested	---	---
5	pMo130-Tel^{R}/UP-*pmrAB*-DOWN + *Not*I	12, 854 bp	~ 12, 854 bp
6	Hyperladder™ 1 (Bioline)	---	---

Digestion 2, Table 2.8

Figure 3.36 Verification of *pmrAB* Complementation Construct by DNA Sequencing.



A region of pMo130-Tel^{R} containing *the* UP-*pmrAB*-DOWN complementation fragment was amplified and sequenced (the whole amlimer is shown above in four independent sequencing reactions a-d). The result was aligned with the expected sequence.
a) pMo/UPpmrBDN FWUP was generated using primer pMo130-Tel^{R} FW, which is specific for pMo130-Tel^{R}.
b) pMo/UPpmrBDN FWpmrB was generated using primer pmrBgeneFW, which binds inside *pmrB*.
c) pMo/UPpmrBDN RVpmrB was generated using primer pmrBgeneRV, which binds inside *pmrB.* This sequence was reverse complemented before alignment.
d) pMo/UPpmrBDN RVDOWN was generated using primer DOWNRVpmrB, which is specific for the DOWN fragment. This sequence was reverse complemented before alignment.
The first 1084 bp of the sequence corresponds to 174 bp of the vector and the entire UP fragment; 1122-2456 bp corresponds to *pmrB* ; 2701-3673 bp corresponds to the DOWN fragment. The sequences of the above figure (a-d) are shown below.

a) Forward sequencing of *pmrAB* complementation construct (1).

```
pMo/UPpmrBDN Expected      1 CCATCTACTTCTTCGACCCGTCCGGTAACCGCAACGAAGTGTTCTGCGGGGGAGATTACA   60
                                                A  G ++C+A GTGTTCTGCGGGGGAGATTACA
pMo/UPpmrBDN FWUP          1 --------------------------TATGGAWWCSA-GTGTTCTGCGGGGGAGATTACA   33

pMo/UPpmrBDN Expected     61 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTT  120
                              ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGG+CAAGGCGATCTTTT
pMo/UPpmrBDN FWUP         34 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGCTGGSCAAGGCGATCTTTT   93

pMo/UPpmrBDN Expected    121 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGACCTGAGCGGCCGC  180
                              ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTG     AGCGGCCGC
pMo/UPpmrBDN FWUP         94 ACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTG-----AGCGGCCGC  148

pMo/UPpmrBDN Expected    181 CTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTGAAA  240
                              CTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTGAAA
pMo/UPpmrBDN FWUP        149 CTTATGCAATCGCACCGAGCCAACAAACACATGTACCAATGATTATGTGGTTCTCTGAAA  208

pMo/UPpmrBDN Expected    241 GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAAAGT  300
                              GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAAAGT
pMo/UPpmrBDN FWUP        209 GTTGGAAACAACGTAATCTTGCTCAAGTGAATTGTTTAAGCCAACAAACTAAACAAAAGT  268

pMo/UPpmrBDN Expected    301 TAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAAAACTCAGGTCA  360
                              TAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAAAACTCAGGTCA
pMo/UPpmrBDN FWUP        269 TAAGTCAGGATAATTTATTCCCAAGTTTGTTAAGTTTGCTGGATGTAAAAACTCAGGTCA  328

pMo/UPpmrBDN Expected    361 TCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAGCGAGCCTAGAA  420
                              TCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAGCGAGCCTAGAA
pMo/UPpmrBDN FWUP        329 TCAACCCTCAACTGGACATGTTGCACTCTTGTGCCCATGTAAACTAAAGCGAGCCTAGAA  388

pMo/UPpmrBDN Expected    421 CATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCAACGATCACGTT  480
                              CATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCAACGATCACGTT
pMo/UPpmrBDN FWUP        389 CATGACAAAAATCTTGATGATTGAAGATGATTTTATGATTGCAGAATCAACGATCACGTT  448

pMo/UPpmrBDN Expected    481 GCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGATGGTTTGGCTCA  540
                              GCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGATGGTTTGGCTCA
pMo/UPpmrBDN FWUP        449 GCTGCAATATCATCAGTTTGAGGTGGAATGGGTCAATAACGGTTTAGATGGTTTGGCTCA  508

pMo/UPpmrBDN Expected    541 ATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCTATGATGGATGG  600
                              ATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCTATGATGGATGG
pMo/UPpmrBDN FWUP        509 ATTGGCGAAGACTAAATTTGATCTTATTCTTTTGGATTTAGGATTGCCTATGATGGATGG  568

pMo/UPpmrBDN Expected    601 TATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTAATTATTTCTGC  660
                              TATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTAATTATTTCTGC
pMo/UPpmrBDN FWUP        569 TATGCAAGTTTTGAAGCAGATCCGTCAAAGAGCAGCAACACCAGTATTAATTATTTCTGC  628

pMo/UPpmrBDN Expected    661 TCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGATGATTATTTAAT  720
                              TCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGATGATTATTTAAT
pMo/UPpmrBDN FWUP        629 TCGAGATCAATTACAAAACCGTGTCGATGGTTTAAATTTGGGTGCAGATGATTATTTAAT  688

pMo/UPpmrBDN Expected    721 TAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTACGCCGTAGTGG  780
                              TAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTACGCCGTAGTGG
pMo/UPpmrBDN FWUP        689 TAAACCTTATGAGTTTGATGAGTTGCTTGCCCGTATTCATGCATTACTACGCCGTAGTGG  748

pMo/UPpmrBDN Expected    781 AGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGATCTGGTTTTAAA  840
                              AGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGATCTGGTTT AAA
pMo/UPpmrBDN FWUP        749 AGTAGAAGCTCAACTTGCGAGTCAAGATCAACTATTAGAAAGTGGCGATCTGGTTT-AAA  807

pMo/UPpmrBDN Expected    841 TGTTGAACAGCATATTGCGACGTTTAAAGGTCAACGCATTGATCTATCAAATCGTGAATG  900
                              TGTTGAACA+CATATTGC+AC+TTTAA+GG CAACGCATTGATCTATCAA TCGTGAA+G
pMo/UPpmrBDN FWUP        808 TGTTGAACARCATATTGCSACKTTTAARGG-CAACGCATTGATCTATCAA-TCGTGAAWG  865

pMo/UPpmrBDN Expected    901 GGCAATCTTAATTCCACTTATGACTCACCCAAATAAAATCTTTTCTAAAGCCAACTTAGA  960
                              GGCAA C TAATT+CACTTATGACTC+CCC+AATAAA TCTTTTC AAAGCCA CTTA A
pMo/UPpmrBDN FWUP        866 GGCAA-CCTAATTYCACTTATGACTCMCCCWAATAAA-TCTTTTC-AAAGCCA-CTTAAA  921

pMo/UPpmrBDN Expected    961 AGATAAGTTATATGATTTTGATAGTGATGTGACCAGTAATACTATTGAAGTATATGTTCA 1020
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```
                                A   AA ++A A GAT+TTGA +G    GTGA+ A  AA    ATTG A +ATAT TT
pMo/UPpmrBDN FWUP        922 A---AAAWKAAAAGATYTTGAARG----GTGASGACAAAATACATTGGAAKATATTTT--  972

pMo/UPpmrBDN Expected   1021 CCATTTAAGAGCGAAGCTGGGTAAAGATTTTATCCGAACCATCCGAGGACTGGGCTACCG 1080

pMo/UPpmrBDN FWUP            ------------------------------------------------------------
```

b) Forward sequencing of *pmrAB* complementation construct (2).

```
pMo/UPpmrBDN Expected   1501 TCAGCCAGCAAGAGAGGGTTCGTGAAGCTTTCGCTTGGGAGCTTGCGGGCAGTATGTTTA 1560
                                                                                + + +T+
pMo/UPpmrBDN FWpmrB        1 ----------------------------------------------------CSRRRAYTW    9

pMo/UPpmrBDN Expected   1561 TTCCGTATTTAATTATTTTACCTTTTGCAATATTTGCTTTAGCAGCCATTATTCGTCGTG 1620
                                CCG+ATT+A TTATTTTACCTTTTG+A TATTTGCTTTAGCAGCCATTATTCGTCGTG
pMo/UPpmrBDN FWpmrB       10 AACCGWATTWA-TTATTTTACCTTTTGMA-TATTTGCTTTAGCAGCCATTATTCGTCGTG   67

pMo/UPpmrBDN Expected   1621 GTTTAAAACCAATAGATGATTTTAAAAATGAGTTAAAAGAACGCGATTCCGAAGAACTCA 1680
                                GTTTA AAC AAT++A+GATTTTAAA+ATGAGTTAAAAGAACGCGATTCC AAGAACTCA
pMo/UPpmrBDN FWpmrB       68 GTTTAGAAC-AATWKAWGATTTTAAAWATGAGTTAAAAGAACGCGATTCCCAAGAACTCA  126

pMo/UPpmrBDN Expected   1681 CCCCAATTGAAGTACATGATTATCCTCAAGAGCTTTTACCTACTATTGACGAAATGAACC 1740
                                C+CCAA+TGAAGTACATGATTATCC+C+A+AGCTTTTA
pMo/UPpmrBDN FWpmrB      127 CMCCAAWTGAAGTACATGATTATCCYCMAKAGCTTTTA----------------------  164
```

c) Reverse sequencing of *pmrAB* complementation construct (1).

```
pMo/UPpmrBDN Expected   1501 TCAGCCAGCAAGAGAGGGTTCGTGAAGCTTTCGCTTGGGAGCTTG 1545

pMo/UPpmrBDN RVpmrB          ---------------------------------------------

pMo/UPpmrBDN Expected   1546 CGGGCAGTATGTTTATTCCGTATTTAATTATTTTACCTTTTGCAATATTTGCTTTAGCAG 1605
                                CGGGCAGTATGTTTATTCCGTATTTAATTAT++TACCTTTTGCAATATTTGCTTTAGCAG
pMo/UPpmrBDN RVpmrB       683 CGGGCAGTATGTTTATTCCGTATTTAATTATWKTACCTTTTGCAATATTTGCTTTAGCAG  624

pMo/UPpmrBDN Expected   1606 CCATTATTCGTCGTGGTTTAAAACCAATAGATGATTTTAAAAATGAGTTAAAAGAACGCG 1665
                                CCATTATT+GTCGTGGTTTA AACCAATAGATGATTTTAAAAATGAGTTAAAAGAACGCG
pMo/UPpmrBDN RVpmrB       623 CCATTATTYGTCGTGGTTTAGAACCAATAGATGATTTTAAAAATGAGTTAAAAGAACGCG  564

pMo/UPpmrBDN Expected   1666 ATTCCGAAGAACTCACCCCAATTGAAGTACATGATTATCCTCAAGAGCTTTTACCTACTA 1725
                                ATTCCGAAGAACTCACCCCAATTGAAGTACATGATTATCCTCAAGAGCTTTTACCTACTA
pMo/UPpmrBDN RVpmrB       563 ATTCCGAAGAACTCACCCCAATTGAAGTACATGATTATCCTCAAGAGCTTTTACCTACTA  504

pMo/UPpmrBDN Expected   1726 TTGACGAAATGAACCGTCTTTTTGAGCGAATTTCTAAAGCTCAAAATGAACAGAAGCAAT 1785
                                TTGACGAAATGAACCGTCTTTTTGAGCGAATTTCTAAAGCTCAAAATGAACAGAAGCAAT
pMo/UPpmrBDN RVpmrB       503 TTGACGAAATGAACCGTCTTTTTGAGCGAATTTCTAAAGCTCAAAATGAACAGAAGCAAT  444

pMo/UPpmrBDN Expected   1786 TTATTGCCGATGCTGCTCATGAACTACGAACACCTGTGACTGCATTGAACTTACAAACCA 1845
                                TTATTGCCGATGCTGCTCATGAACTACGAACACCTGTGACTGCATTGAACTTACAAACCA
pMo/UPpmrBDN RVpmrB       443 TTATTGCCGATGCTGCTCATGAACTACGAACACCTGTGACTGCATTGAACTTACAAACCA  384

pMo/UPpmrBDN Expected   1846 AGATTTTGCTAAGTCAGTTCCCTGAGCATGAATCATTGCAAAACTTAAGCAAAGGTTTGG 1905
                                AGATTTTGCTAAGTCAGTTCCCTGAGCATGAATCATTGCAAAACTTAAGCAAAGGTTTGG
pMo/UPpmrBDN RVpmrB       383 AGATTTTGCTAAGTCAGTTCCCTGAGCATGAATCATTGCAAAACTTAAGCAAAGGTTTGG  324

pMo/UPpmrBDN Expected   1906 CACGTATTCAGCATTTGGTGACTCAGCTTTTAGCATTGGCAAAGCAAGATGTAACTTTAA 1965
                                CACGTATTCAGCATTTGGTGACTCAGCTTTTAGCATTGGCAAAGCAAGATGTAACTTTAA
pMo/UPpmrBDN RVpmrB       323 CACGTATTCAGCATTTGGTGACTCAGCTTTTAGCATTGGCAAAGCAAGATGTAACTTTAA  264

pMo/UPpmrBDN Expected   1966 GTATGGTCGAGCCTACTGGATATTTTCAACTCAATGATGTGGCATTAAATTGTGTGGAGC 2025
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                                GTATGGTCGAGCCTACTGGATATTTTCAACTCAATGATGTGGCATTAAATTGTGTGGAGC
pMo/UPpmrBDN RVpmrB      263 GTATGGTCGAGCCTACTGGATATTTTCAACTCAATGATGTGGCATTAAATTGTGTGGAGC  204

pMo/UPpmrBDN Expected   2026 AGTTGGTTAACTTGGCTATGCAAAAAGAAATCGATTTAGGTTTTGTTAGAAATGAACCCA 2085
                                AGTTGGTTAACTTGGCTATGCAAAAAGAAATCGATTTAGGTTTTGTTAGAAATGAACCCA
pMo/UPpmrBDN RVpmrB      203 AGTTGGTTAACTTGGCTATGCAAAAAGAAATCGATTTAGGTTTTGTTAGAAATGAACCCA  144

pMo/UPpmrBDN Expected   2086 TCGAAATGCATAGTATTGAACCTACTGTACATTCGATTATTTTTAATTTAATTGATAATG 2145
                                TCGAAATGCATAGTATTGAACCTACTGTACATTCGATTATTTTTAATTTAATTGATAATG
pMo/UPpmrBDN RVpmrB      143 TCGAAATGCATAGTATTGAACCTACTGTACATTCGATTATTTTTAATTTAATTGATAATG   84

pMo/UPpmrBDN Expected   2146 CAATTAAGTACACCCCGCATCAGGGTGTTATTAATATTTCAGTTTATACCGATCAAGATC 2205
                                CAATTAAGTACACCCCGCATCAGGGTGTTATTAATATTTCAGTTTATACCGATCAAGATC
pMo/UPpmrBDN RVpmrB       83 CAATTAAGTACACCCCGCATCAGGGTGTTATTAATATTTCAGTTTATACCGATCAAGATC   24

pMo/UPpmrBDN Expected   2206 ACTACGCATGTATTCAAATTGAAG 2229
                                ACTACGCA G +T +AA +++++G
pMo/UPpmrBDN RVpmrB       23 ACTACGCA-GAWTCMAAKKWRRWG    1
```

d) Reverse sequencing of *pmrAB* complementation construct (2).

```
pMo/UPpmrBDN Expected   3001 TTGT 3004

pMo/UPpmrBDN RVDOWN          ----

pMo/UPpmrBDN Expected   3005 CCCGATTGAAAAAA-TGACCTGTTGAATCAATCCAAGGTTGGATGAGGTACATATCAATT 3063
                              +CGATT+AAAAAA TG+CCTGTTGAATCAATCCAAGGT+GGATGAGGTACATATCAATT
pMo/UPpmrBDN RVDOWN       640 AYCGATTRAAAAAAATGRCCTGTTGAATCAATCCAAGGTWGGATGAGGTACATATCAATT  581

pMo/UPpmrBDN Expected   3064 TTCCCACCGACTGGAAATACAAATAGAAGAATAAAAAAACTGAAAAAAAGAAAGAGAATA 3123
                              TTCCCACCG CTGGAAATACAAATAGAAGAATAAAAAA CTGAAAAAAAGAAAGAGAATA
pMo/UPpmrBDN RVDOWN       580 TTCCCACCGTCTGGAAATACAAATAGAAGAATAAAAAA-CTGAAAAAAAGAAAGAGAATA  522

pMo/UPpmrBDN Expected   3124 TTAATTTGGAAAAAAAGTTTTTGATTAGTCATTCTGATGCAATATCAATCGACAATAGGT 3183
                              TTAATTTGGAAAAAAAGTTTTTGATTAGTCATTCTGATGCAATATCAATCGACAATAGGT
pMo/UPpmrBDN RVDOWN       521 TTAATTTGGAAAAAAAGTTTTTGATTAGTCATTCTGATGCAATATCAATCGACAATAGGT  462

pMo/UPpmrBDN Expected   3184 GCGACTAATTACATAAAATTAATCTTAAATTGCTCTTAAACTATACTGGTGAAAGAAGTT 3243
                              GCGACTAATTACATAAAATTAATCTTAAATTGCTCTTAAACTATACTGGTGAAAGAAGTT
pMo/UPpmrBDN RVDOWN       461 GCGACTAATTACATAAAATTAATCTTAAATTGCTCTTAAACTATACTGGTGAAAGAAGTT  402

pMo/UPpmrBDN Expected   3244 CAATTTTCCTTAAAAAATAAAAAAGCGACGAATAGTATCGTCGCCTTAAGCACTTTAGAA 3303
                              CAATTTTCCTTAAAAAATAAAAAAGCGACGAATAGTATCGTCGCCTTAAGCACTTTAGAA
pMo/UPpmrBDN RVDOWN       401 CAATTTTCCTTAAAAAATAAAAAAGCGACGAATAGTATCGTCGCCTTAAGCACTTTAGAA  342

pMo/UPpmrBDN Expected   3304 AGTATTAAAACATACCTTCTTCAGAGTTTGCTGAGATCTTATGAATAGATAAGTCTGCAC 3363
                              AGTATTAAAACATACCTTCTTCAGAGTTTGCTGAGATCTTATGAATAGATAAGTCTGCAC
pMo/UPpmrBDN RVDOWN       341 AGTATTAAAACATACCTTCTTCAGAGTTTGCTGAGATCTTATGAATAGATAAGTCTGCAC  282

pMo/UPpmrBDN Expected   3364 CACGGAACTCATCTTCTTGAGATAGGCGAATGCCTATGGTCGCTTTAAGGATGCCATAAA 3423
                              CACGGAACTCATCTTCTTGAGATAGGCGAATGCCTATGGTCGCTTTAAGGATGCCATAAA
pMo/UPpmrBDN RVDOWN       281 CACGGAACTCATCTTCTTGAGATAGGCGAATGCCTATGGTCGCTTTAAGGATGCCATAAA  222

pMo/UPpmrBDN Expected   3424 CAATGAAACCGCCAGCCAGTGCAATAGCAATCGCGAGTGCTGTACCGATGAGCTGAGAGA 3483
                              CAATGAAACCGCCAGCCAGTGCAATAGCAATCGCGAGTGCTGTACCGATGAGCTGAGAGA
pMo/UPpmrBDN RVDOWN       221 CAATGAAACCGCCAGCCAGTGCAATAGCAATCGCGAGTGCTGTACCGATGAGCTGAGAGA  162

pMo/UPpmrBDN Expected   3484 TGAATGATACGCCGCCTAAACCACCTAGCCATTTTTGACCAAAAATACCTACGGCAATTC 3543
                              TGAATGATACGCCGCCTAAACCACCTAGCCATTTTTGACCAAAAATACCTACGGCAATTC
pMo/UPpmrBDN RVDOWN       161 TGAATGATACGCCGCCTAAACCACCTAGCCATTTTTGACCAAAAATACCTACGGCAATTC  102

pMo/UPpmrBDN Expected   3544 CACCAAATGCACCGCATACACCATGTAGCGGCCAGACACCAAGAACGTCATCAACTTTGA 3603
                              CACCAAATGCACCGCATACACCATGTAGCGGCCAGACACCAAGAACGTCATCAACTTTGA
pMo/UPpmrBDN RVDOWN       101 CACCAAATGCACCGCATACACCATGTAGCGGCCAGACACCAAGAACGTCATCAACTTTGA   42
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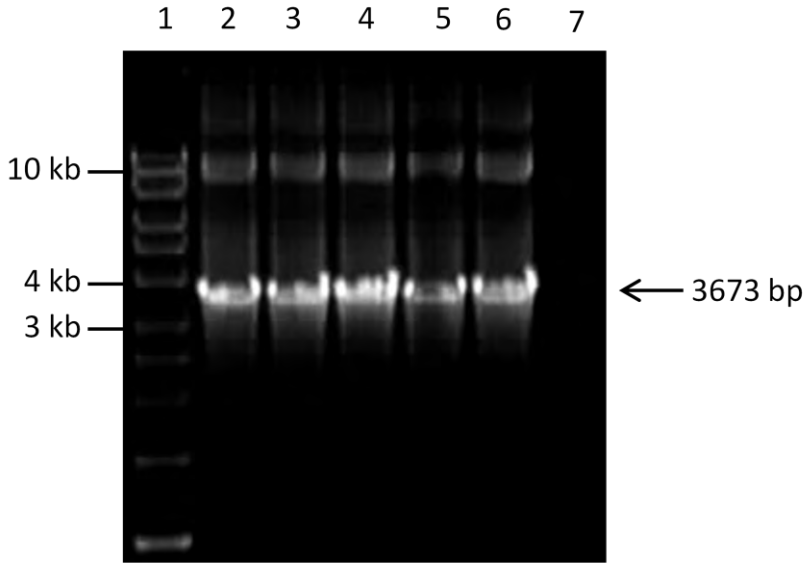
pMo/UPpmrBDN Expected   3604 GTTTGTTTTGAGTATAGGTGAATAGATACACGAACATAGCACC 3646
                             GTTTGTTTTGAGTATAGG+ AATAGA ACACGA CA+A  AC
pMo/UPpmrBDN RVDOWN       41 GTTTGTTTTGAGTATAGGK-AATAGA-ACACGATCAWAARACT    1

pMo/UPpmrBDN Expected   3648 CGCTGCACCACCAATCACGCATGCCCC 3674

pMo/UPpmrBDN RVDOWN          ----------------------------
```

Figure 3.37 Verification of *pmrAB* Complementation Construct in S17-1.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ 1 (Bioline)	---	---
2	Plasmid from candidate transformant 1	3673 bp	~3673 bp
3	Plasmid from candidate transformant 2	3673 bp	~3673 bp
4	Plasmid from candidate transformant 3	3673 bp	~3673 bp
5	Plasmid from candidate transformant 4	3673 bp	~3673 bp
6	pMo130-Tel^{R}/UP-*pmrAB*-DOWN (positive control)	3673 bp	~3673 bp
7	---	No amplimer	No amplimer

PCR 14, Table 2.7

exposure.

3.4. *bfmRS*

3.4.1. Deletion of *bfmRS*

3.4.1.1. Identification of *bfmS* in AYE

bfmS was unannotated in AYE, but a search of *bfmS* in the whole of xBASE retrieved the *bfmS* gene sequence in *A. baumannii* ATCC 17978 (A1S_0749), which was used to identify *bfmS* in AYE (Figure 3.38). This gene sequence was BLASTED against the AYE genome and one result was obtained, suggesting that there is only one copy of this gene in AYE. An alignment of *bfmS* in ATCC 17978 and that identified in AYE confirmed the identity of the gene in AYE (Figure 3.38; Figure 3.39).

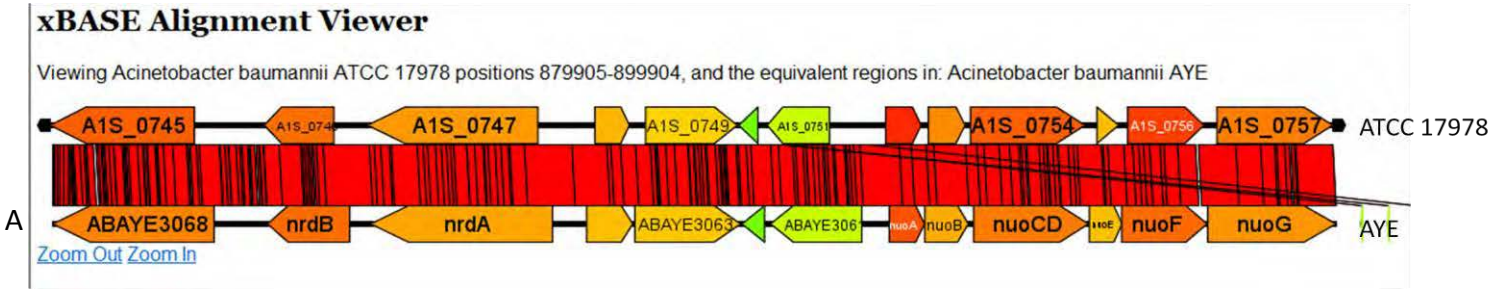
3.4.1.2. Construction of pMo130-Tel^R/*bfmRS*UPDOWN

The UP and DOWN fragments for *bfmRS* were amplified and the purified amplimers were verified by electrophoresis (Figure 3.40).

3.4.1.2.1. Cloning of the UP Fragment into pMo130-Tel^R and Verification of Construct

pMo130-Tel^R and the *bfmRS*UP fragment were digested with *Not*I and *Bam*HI (Table 2.8, digestion 3). They were ligated and transformed into DH5α. Three candidate transformants were obtained, two of which were confirmed to harbour the correct construct (pMo130-Tel^R/*bfmRS*UP) (Figure 3.41).

Figure 3.38 Alignment of *bfmS* in *A. baumannii* ATCC 17978 and AYE



A

1S_0749 and ABAYE3063, *bfmS*

Figure 3.39 Alignment of *bfmS* in *A. baumannii* ATCC 17978 and AYE.

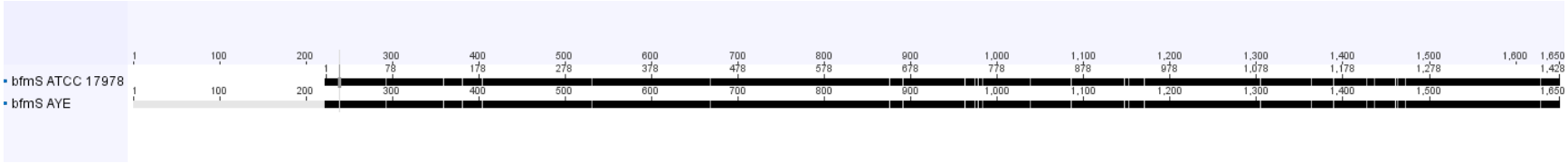
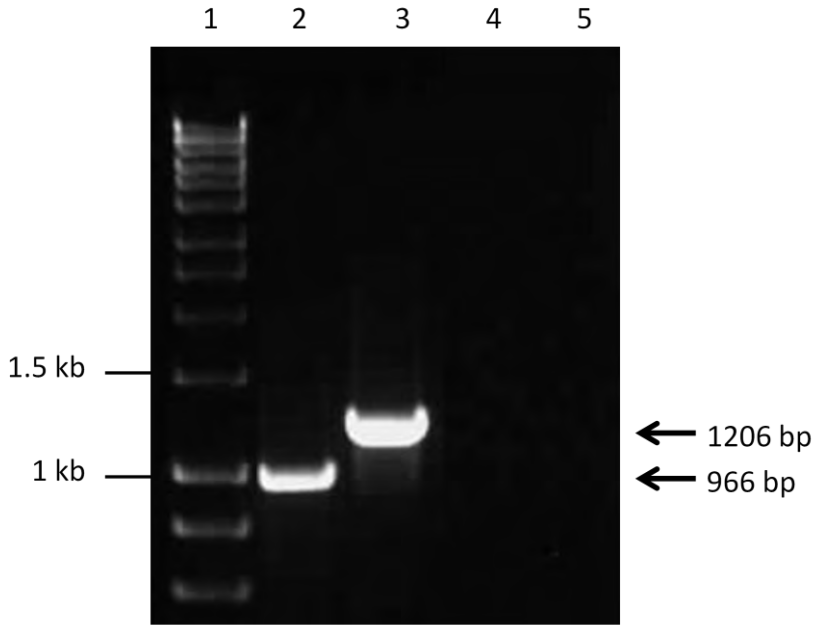


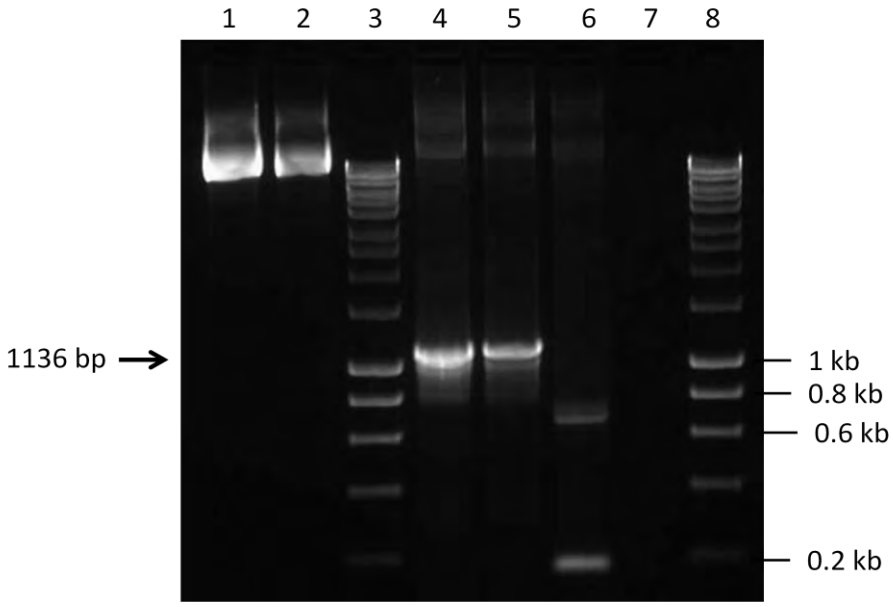
Figure 3.40 Amplification of *bfmRS* UP and DOWN fragments.



Lane	PCR[a]	DNA	Expected size	Actual size
1, 6	---	Hyperladder™ 1 (Bioline)	---	---
2	7	Purified *bfmRS* UP fragment	966 bp	~ 966 bp
3	9	Purified *bfmRS* DOWN fragment	1206 bp	~1206
4	7	Contamination control for *bfmRS* UP fragment	No amplimer	No amplimer
5	9	Contamination control for *bfmRS* DOWN fragment	No amplimer	No amplimer

[a]PCR number, Table 2.7

Figure 3.41 Verification of pMo130-Tel^R/*bfmRS*UP



Lane	DNA	Expected size	Actual size
1	Undigested pMo130-Tel^R/UP*bfmRS* extracted from candidate 2 (no PCR)	---	---
2	Undigested pMo130-Tel^R/UP*bfmRS* extracted from candidate 3 (no PCR)	----	----
3, 8	Hyperladder™ 1 (Bioline)	---	---
4	pMo130-Tel^R/UP*bfmRS* from candidate 2	1136 bp	~1136 bp
5	pMo130-Tel^R/UP*bfmRS* from candidate 3	1136 bp	~1136 bp
6	pMo130-Tel^R unmanipulated (negative control)	No amplimer	~0.7 bp and ~ 0.2 bp
7	___	No amplimer	No amplimer

PCR 12, Table 2.

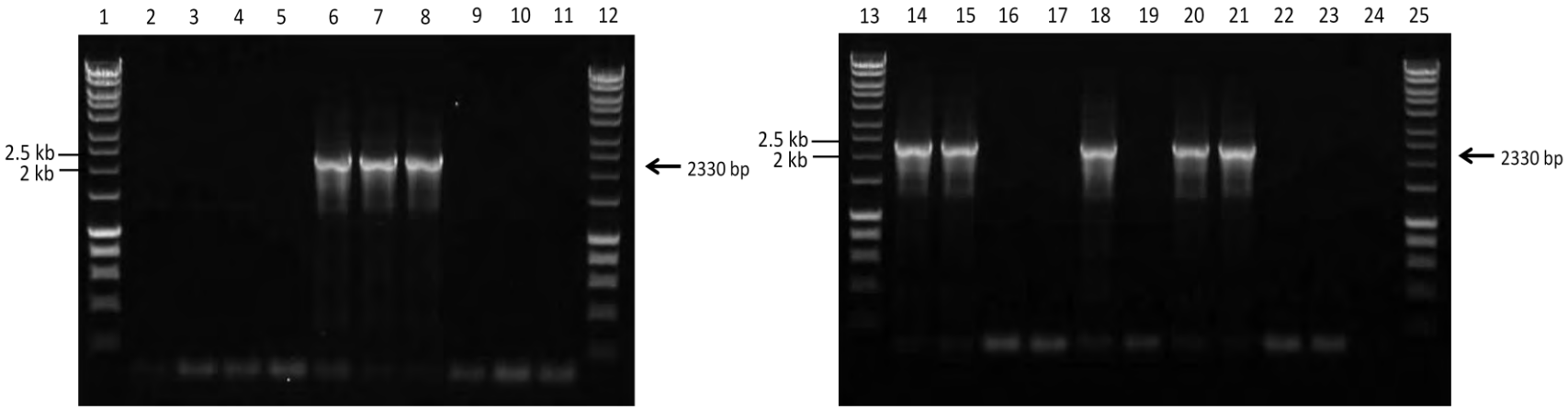
3.4.1.2.2. Cloning of the DOWN Fragment into pMo130-Tel^R/*bfmRS*UP and Verification of Construct

Approximately 100 candidate transformants were obtained when pMo130-Tel^R/*bfmRS*UP was ligated with the DOWN fragment and transformed into DH5α. Twenty of these were screened by PCR (using lysate as the template; Figure 3.42) and eight yielded the correct sized amplimer. Plasmid was extracted from candidate 5 (Figure 3.42) and sequencing (Figure 3.43) of the plasmid multiple cloning site confirmed that the correct construct had been obtained.

3.4.1.3. Introduction of pMo130-Tel^R/*bfmRS*UPDOWN into AYE

The construct was transformed into *E. coli* S17-1 and four candidate S17-1/pMo130-Tel^R/*bfmRS* transformants were confirmed by PCR to harbour the plasmid. One transformant (transformant 1) was carried forward to introduce the construct into AYE (Figure 3.44, lanes 1 and 5). Two conjugations between S17-1/pMo130-Tel^R/*bfmRS* and AYE did not give rise to any single recombinants. Transformation of S17-1 with pMo130-Tel^R/*bfmRS*UPDOWN was repeated (transformation 2) and two more conjugations carried out, using a verified transformant from both transformations (Figure 3.44). Colonies were obtained when both donors were used, but the candidates remained white after pyrocatechol exposure. To increase the likelihood of recombination occurring, conjugation was carried out using larger volumes of donor and recipient strains. All colonies remained white after exposure to pyrocatechol and were therefore not single recombinants. To investigate whether any of these colonies were in fact

Figure 3.42 Screening of pMo130-Tel^{R}/*bfmRS*UPDOWN Candidate DH5α Transformants.



Lane	DNA	Expected size	Actual size
1	Hyperladder™ 1 (Bioline)	---	---
2-11	Lysate from candidates 1-10	2330 bp	~ 2330 bp
12, 13	Hyperladder™ 1 (Bioline)	---	---
14-23	Lysate from candidates 11-20	2330 bp	~ 2330 bp
24	---	No amplimer	No amplimer
25	Hyperladder™ 1 (Bioline)	---	---

PCR 15, Table 2.7

Figure 3.43 Verification of pMo130-TelR/*bfmRS*UPDOWN construct by sequencing.

a) Forward sequencing

```
pMo/bfmSUPDN Expected      1 CCATCTACTTCTTCGACCCGTCCGGTAACCGCAACGAAGTGTTCTGCGGGGGAGATTACA   60
                                                   G+A    AGTGTTCTGCGGGGGAGATTACA
pMo/bfmSUPDN FW Seq        1 ----------------------------GTGSAYACGAGTGTTCTGCGGGGGAGATTACA   32

pMo/bfmSUPDN Expected     61 ACTACCCGGACCACAAACCGGTGACCTGGACCACCGACCAGC-TGGGCAAGGCGATCTTT  119
                              ACTACCCGGACCACAAACCGG++ACCTGGACC+++G+C+AGC TG++C+ +GCGATCTTT
pMo/bfmSUPDN FW Seq       33 ACTACCCGGACCACAAACCGGKRACCTGGACCMSMGWCSAGCMTGRSCMCRGCGATCTTT   92

pMo/bfmSUPDN Expected    120 TACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGACCTGAGCGGCCG  179
                              TACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGA     GCGGCCG
pMo/bfmSUPDN FW Seq       93 TACCACGACCGCATTCTCAACGAACGATTCATGACCGTGCTGACCTGA-----GCGGCCG  147

pMo/bfmSUPDN Expected    180 CGAGATAGCATACCAAAGCTGTGAAAAAACACTTATAGATAACTTTGTGGATAACTCAAA  239
                              CGAGATAGCATACCAAAGCTGTGAAAAAACACTTATAGATAACTTTGTGGATAACTCAAA
pMo/bfmSUPDN FW Seq      148 CGAGATAGCATACCAAAGCTGTGAAAAAACACTTATAGATAACTTTGTGGATAACTCAAA  207

pMo/bfmSUPDN Expected    240 AAACAAACACTTGAGTGAATTTAAATTTAGTGAAGAAATAATTTGTTTAATATTCATACA  299
                              AAACAAACACTTGAGTGAATTTAAATTTAGTGAAGAAATAATTTGTTTAATATTCATACA
pMo/bfmSUPDN FW Seq      208 AAACAAACACTTGAGTGAATTTAAATTTAGTGAAGAAATAATTTGTTTAATATTCATACA  267

pMo/bfmSUPDN Expected    300 TAGGAAATATGAATAGATTATGACATTTAAGAACAATAAACAGATAATAAAAATACCTGA  359
                              TAGGAAATATGAATAGATTATGACATTTAAGAACAATAAACAGATAATAAAAATACCTGA
pMo/bfmSUPDN FW Seq      268 TAGGAAATATGAATAGATTATGACATTTAAGAACAATAAACAGATAATAAAAATACCTGA  327

pMo/bfmSUPDN Expected    360 AAATTCAAAGATTAATAAAATGCAACAAAACATGGTTGTCATGTATCAGTTTGGTGAACG  419
                              AAATTCAAAGATTAATAAAATGCAACAAAACATGGTTGTCATGTATCAGTTTGGTGAACG
pMo/bfmSUPDN FW Seq      328 AAATTCAAAGATTAATAAAATGCAACAAAACATGGTTGTCATGTATCAGTTTGGTGAACG  387

pMo/bfmSUPDN Expected    420 CCTACTTGTTTACAATGTAAACGTGTGTATATTGCAAATGATAAACGAATGTATCTGCAA  479
                              CCTACTTGTTTACAATGTAAACGTGTGTATATTGCAAATGATAAACGAATGTATCTGCAA
pMo/bfmSUPDN FW Seq      388 CCTACTTGTTTACAATGTAAACGTGTGTATATTGCAAATGATAAACGAATGTATCTGCAA  447

pMo/bfmSUPDN Expected    480 GATTTTTAAGATACATGTAATGAGATTTATAGGGGCAATGATATGAGCCAAGAAGAAAAG  539
                              GATTTTTAAGATACATGTAATGAGATTTATAGGGGCAATGATATGAGCCAAGAAGAAAAG
pMo/bfmSUPDN FW Seq      448 GATTTTTAAGATACATGTAATGAGATTTATAGGGGCAATGATATGAGCCAAGAAGAAAAG  507

pMo/bfmSUPDN Expected    540 TTACCAAAGATTCTGATCGTTGAAGACGACGAGCGTTTAGCGCGATTAACTCAAGAATAT  599
                              TTACCAAAGATTCTGATCGTTGAAGACGACG GCGTTTAGCGCGATTAACTCAAGAATAT
pMo/bfmSUPDN FW Seq      508 TTACCAAAGATTCTGATCGTTGAAGACGACGGGCGTTTAGCGCGATTAACTCAAGAATAT  567

pMo/bfmSUPDN Expected    600 TTAATCCGTAATGGTTTGGAAGTTGGTGTAGAAACCGATGGTAACCGTGCAATTCGTCGT  659
                              TTAATCCGTAATGGTTTGGAAGTTGGTGTAGAAACCGATGGTAACCGTGCAATTCGTCGT
pMo/bfmSUPDN FW Seq      568 TTAATCCGTAATGGTTTGGAAGTTGGTGTAGAAACCGATGGTAACCGTGCAATTCGTCGT  627

pMo/bfmSUPDN Expected    660 ATTATTAGTGAGCAACCGGATCTTGTGGTCTTGGATGTCATGTTGCCGGGTGCAGATGGT  719
                              ATTATTAGTGAGCAACCGGATCTTGTGGTCTTGGATGTCATGTTGCCGGGTGCAGATGGT
pMo/bfmSUPDN FW Seq      628 ATTATTAGTGAGCAACCGGATCTTGTGGTCTTGGATGTCATGTTGCCGGGTGCAGATGGT  687

pMo/bfmSUPDN Expected    720 TTAACCGTTTGTCGTGAAGTTCGCCCACACTATCATCAACCAATCTTAATGTTGACTGCA  779
                              TTAACCGTTTGTCGTGAAGTTCGCCCACACTATCATCAACCAATCTTAATGTTGACTG++
pMo/bfmSUPDN FW Seq      688 TTAACCGTTTGTCGTGAAGTTCGCCCACACTATCATCAACCAATCTTAATGTTGACTGYW  747

pMo/bfmSUPDN Expected    780 CGTACTGAAGATATGGATCAGGTACTTGGTCTGGAAATGGGTGCAGACGATTATGTCGCG  839
                              CGTACTGAAGATATGGATCAGGTACTTGGTCTGGAAATGGGTGCAGACGATTATGTC+CG
pMo/bfmSUPDN FW Seq      748 CGTACTGAAGATATGGATCAGGTACTTGGTCTGGAAATGGGTGCAGACGATTATGTCKCG  807

pMo/bfmSUPDN Expected    840 AAACCAGTTCAACCACGTGTATTATTAGCGCGTATTCGTGCTTTGCTACGCCGTACGGAT  899
                              AAACCAGTTCA CCACGTGTATTATTAGCGCGTATTCGTGCTTTGCTACGCCGTACGGAT
pMo/bfmSUPDN FW Seq      808 AAACCAGTTCA-CCACGTGTATTATTAGCGCGTATTCGTGCTTTGCTACGCCGTACGGAT  866

pMo/bfmSUPDN Expected    900 AAAACTGTTGAAGATGAAGTTGCTCAACGTATTGAGTTTGACGACCTTGTTATCGACAAT  959
                              AAAACTGTTGAAGATGAAGTTGCTCAACGTATTGAGT+TGA+GACC+T+TTATCGA+AAT
pMo/bfmSUPDN FW Seq      867 AAAACTGTTGAAGATGAAGTTGCTCAACGTATTGAGTKTGASGACCWTRTTATCGAYAAT  926
```

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pMo/bfmSUPDN Expected    960 GGTGGCCGTTCGGTAACGTTGAACGGTGAGCTTGTTGACTTTACAAGTGCTGAATATGAC 1019
                             G TGGCCGTTCG TAACGTTGAACGGTGAGCTTGTTGACTTT+CA GTGCTGAATATGAC
pMo/bfmSUPDN FW Seq      927 GATGGCCGTTCG-TAACGTTGAACGGTGAGCTTGTTGACTTTRCA-GTGCTGAATATGAC  984

pMo/bfmSUPDN Expected   1020 TTGTTATGGTTGCTTGCATCAAACGCTGGCCGTATTTTATCGCGTGAAGATATCTTCGAA 1079
                             TTGT ATGGTTGCTTGC+TCAA CG++GGCCG +TTT ATCGCGTG+  ++  CTTCGAA
pMo/bfmSUPDN FW Seq      985 TTGT-ATGGTTGCTTGCMTCAA-CGMYGGCCGARTTT-ATCGCGTGWGAWW--CTTCGAA 1039

pMo/bfmSUPDN Expected   1080 CGTTTACGTGGTATCGAATACGATGGTCAAGACCGTTCAATTGACGTGGATCCAGGTTTG 1139
                             C+TT AC+T GTATCGA TA+GATGGTC+ +ACCG+TCAAT GACG+GGATC   GTTTG
pMo/bfmSUPDN FW Seq     1040 CRTT-ACRTWGTATCGA-TASGATGGTCR-SACCGYTCAAT-GACGWGGATC--AGTTTG 1093

pMo/bfmSUPDN Expected   1140 CCGCTTCTCAGGCTATTGAGTGCATCTGTATTAAAGTCAATCAAGAGCGAATTATTTTTA 1199
                             C G+T CTCAG CTAT    TGCATCTG AT  +AGTCA    A AGCGAAT AT+T
pMo/bfmSUPDN FW Seq     1094 CAGMT-CTCAG-CTAT--GMTGCATCTGAAT--RAGTCATC--ACAGCGAAT-ATYTACS 1144

pMo/bfmSUPDN Expected   1200 GCATCAATTTGATATTTTTGAATAAGAGGCTGATCCCCATTCAAAGTCTCAACTCTATAT 1259
                             ++A          ++TTTTGAA++    GCTGATC  CATTC A  TC   AC  T++
pMo/bfmSUPDN FW Seq     1145 RSACTSG------MWTTTTGAAYRTA--GCTGATC--CATTCCAGCTC---ACCTTMWGA 1191

pMo/bfmSUPDN Expected   1260 GAGTCTG-CAATATGTAAAATGCCTTCTAGATTTGTTGTGGTTGATGCGAAATCTTGCCT 1318
                             + GTC+  +AAT+   +AA                 TG  G TGA GCGAA  CT GCCT
pMo/bfmSUPDN FW Seq     1192 RTGTCWATSAATRCTCRAA-----------------TGATGCTGA-GCGAA--CTGGCCT 1231

pMo/bfmSUPDN Expected   1319 AAAAATTTCATAGACATCTCGCAAGTCAAAAATGGCATTATCTGCATCTGGGTGTTTATG 1378
                             A+AA T   +++     + CGC+AGT+  A A  G
pMo/bfmSUPDN FW Seq     1232 ARAA-TCCTRWRACTYAYACGCMAGTMTCAGACTG------------------------- 1265
```

b) Reverse sequencing

```
pMo/bfmSUPDN Expected    901 AAACTGTTGAAGATGAAGTTGCTCAACGTATTGAGTTTGACGACCTTGTT  950

pMo/bfmSUPDN RV Seq          --------------------------------------------------

pMo/bfmSUPDN Expected    951 ATCGACAATGGTGGCCGTTCGGTAACGTTGAACGGTGAGCTTGTTGACTTTACAAGTGCT 1010
                             A   A+ A+G T++C  T++++ AA  TT +   + G +CT   + AC+  +C +   +T
pMo/bfmSUPDN RV Seq     1323 ACGTASGAKG-TRSC--TKMKKGAAKMTTTMCAMKGGKKCTCAGYAACKSRRCKMTGWMT 1267

pMo/bfmSUPDN Expected   1011 GAATATGACTTGTTATGGTTGCTTGCATCAAACGCTGGCCGTATTTTATCGCGTGAAGAT 1070
                              +    +     TT++  ++ CTT+    +  +G T     TATT  A ++ +  A G++
pMo/bfmSUPDN RV Seq     1266 CMGGGSRGTC--TTMYYYKWTCTTSGSGRRGCSGGTATTTTTATTC-AGSSGKAAACGWY 1210

pMo/bfmSUPDN Expected   1071 ATCTTCGAACGTTTACGTGGTATCGAATACGATGGTCAAGACCGTTCAATTGACGTGGAT 1130
                              T T+ G+  +TT+ C +G+TAT GA++ C+AT++TCAAGA CG TCAATT+A+ TGG T
pMo/bfmSUPDN RV Seq     1209 TTATKTGRGAKTTKTCAYGKTATTGAMKTCSATKKTCAAGAGCGRTCAATTRAS-TGGGT 1151

pMo/bfmSUPDN Expected   1131 CCAGGTTTGCCGCTTCTCAGGCTATTGAGTGCATCTGTATTAAAGTCAATCAAGAGCGAA 1190
                              C+GGTTT+C+  +T ++ GG T +T AG++C TCT   TT+AA+TCAATCA GAG++A+
pMo/bfmSUPDN RV Seq     1150 TCMGGTTTSCS-TKTTKYYGGGTTKTTAGKKCTTCT---TTRAARTCAATCA-GAGSRAM 1096

pMo/bfmSUPDN Expected   1191 TTATTTTTAGCATCAATTTGATATTTTTGAATAAGAGGCTGATCCCCATTCAAAGTCTCA 1250
                              T ++TTT+++C +CA  T+GA A T +   A+ AG   CTGATC CCA+T AAA T T+A
pMo/bfmSUPDN RV Seq     1095 T-MYTTTYWRCTYCA--TWGAAAATGY--GAWTAGGCCCTGATCTCCAWTWAAATTTTSA 1041

pMo/bfmSUPDN Expected   1251 ACTCTATATGAGTCTGCAATATGTAAAATGCCTTCTAGATTTGTTGTGGTTGATGCGAAA 1310
                              A T T+ A GA+T T +AA +  TAAAAT C TT   G+ TTG TG GG+TGATGC+AAA
pMo/bfmSUPDN RV Seq     1040 ATTTTWAA-GAKTTTAMAAAWATTAAAATCCTTT---GRGTTGGTGGGGWTGATGCSAAA  985

pMo/bfmSUPDN Expected   1311 TCTTGCCTAAAAATTTCATAGACATCTCGCAAGTCAAAAATGGCATTATCTGCATCTGGG 1370
                              T+T GC+TAAAAA++TCAT+GAC TCT +CAAGTCAAAA +G C+TT T  GCAT+TGGG
pMo/bfmSUPDN RV Seq      984 TMT-GCYTAAAAAWKTCATRGACTTCTARCAAGTCAAAATKGCCWTTTTGGGCATSTGGG  926

pMo/bfmSUPDN Expected   1371 TGTTTATGAATATAGCTTTCTATATGTCGCACCAGTAATTGTGCAAACAAGTAATAGTCT 1430
                               +TT   GA + TAGCTTT+T T+ GTCGCACCAG+AAT G  CAAACAAGTAATAGTCT
pMo/bfmSUPDN RV Seq      925 ARTTA--GATWKTAGCTTTYT-TMCGTCGCACCAGKAATCG-ACAAACAAGTAATAGTCT  870

pMo/bfmSUPDN Expected   1431 GGTTTATGCGTATATTCTAGATT-CATGCATTTTTCTCCTT-ATTATCTGTTATTAGCAT 1488
                              GGTTTATG GTATATTCTAGATT CAT+CATTTTT T CTT ATTATC+GTT+TTAGCAT
pMo/bfmSUPDN RV Seq      869 GGTTTATGGGTATATTCTAGATTTCATKCATTTTTTTTCTTTATTATCKGTTRTTAGCAT  810

pMo/bfmSUPDN Expected   1489 ACATGGCAAAACAGAAGATATTGTATAAAATTCATTACACTAGATGATGACTTGGAAATA 1548
                              ACATGGC+AAACAGAAGATATTGTAT+AA+TTCATTACACT+GATGATGACTTGGAAATA
pMo/bfmSUPDN RV Seq      809 ACATGGCRAAACAGAAGATATTGTATMAAWTTCATTACACTWGATGATGACTTGGAAATA  750

pMo/bfmSUPDN Expected   1549 TTACAAAGAAATAAAAACTCTATTACGCCCTTGGCGCTTTGCTTGATAGAGTGCATCGTC 1608
                              TTACAAAGAAATAAAAACTCTATTACGCCCT+GGCGCTTTGC++GATAGAGTGCATCGTC
pMo/bfmSUPDN RV Seq      749 TTACAAAGAAATAAAAACTCTATTACGCCCTKGGCGCTTTGCKWGATAGAGTGCATCGTC  690

pMo/bfmSUPDN Expected   1609 TGCTTCTTTAAACAAACTTTCAATAGAACGGTGATAGCTTGGAGAATATAAGCTAATACC 1668
                              TG+TTCT+TAAACAAACTTTCAATAGA+CGGTGATAGCTTGGAGAATATAAG+TAATACC
pMo/bfmSUPDN RV Seq      689 TGYTTCTYTAAACAAACTTTCAATAGARCGGTGATAGCTTGGAGAATATAAGYTAATACC  630

pMo/bfmSUPDN Expected   1669 AATACTTACCTGCACAAAGATGGGTAATTTGCCATATACATAAATCGGTTGCTGACTAAT 1728
                              AATACTTACCTGCACAAAGATGGGTAATTTGCCATATACATAAATCGGTTGCTGACTAAT
pMo/bfmSUPDN RV Seq      629 AATACTTACCTGCACAAAGATGGGTAATTTGCCATATACATAAATCGGTTGCTGACTAAT  570

pMo/bfmSUPDN Expected   1729 TTGAATGCGTATTCTTTCCGCGAGTTCAAAGGCAGCCTCTCTGGTGGTATCCGGAATAAA 1788
                              TTGAATG+GTATTCTTTCCGCGAGTTCAAAGGCAGCCTCTCTGGTGGTATCCGGAATAAA
pMo/bfmSUPDN RV Seq      569 TTGAATGMGTATTCTTTCCGCGAGTTCAAAGGCAGCCTCTCTGGTGGTATCCGGAATAAA  510

pMo/bfmSUPDN Expected   1789 AATTGCGAATTCTTCTCCACCAAAACGTCCTAGTAAGTCATGAGGATGTAAAATATTTTG 1848
                              AATTGCGAATT+TTCTCCACCAAAACGTCCTAGTAAGTCATGAGGATGTAAAATATTTTG
pMo/bfmSUPDN RV Seq      509 AATTGCGAATTYTTCTCCACCAAAACGTCCTAGTAAGTCATGAGGATGTAAAATATTTTG  450

pMo/bfmSUPDN Expected   1849 TATGGTCGTTACACAGGTCTGTAAGGCTCTATCCCCAATTAAGTGGCCATAACTATCATT 1908
                              TATGGTCGTTACACAG+ CTGTAAGGCTCTATCCCCAATTAAGTGGCCATAACTATCATT
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pMo/bfmSUPDN RV Seq      449 TATGGTCGTTACACAGSCCTGTAAGGCTCTATCCCCAATTAAGTGGCCATAACTATCATT  390

pMo/bfmSUPDN Expected   1909 CACTTTTTTAAAATGATCGACGTCCAACATAAAAAATGCAGAGGTCTTATTTTCCTTTTG 1968
                             CACTTTTTTAAAATGATCGACGTCCAACATAAAAAATGCAGAGGTCTTATTTTCCTTTTG
pMo/bfmSUPDN RV Seq      389 CACTTTTTTAAAATGATCGACGTCCAACATAAAAAATGCAGAGGTCTTATTTTCCTTTTG  330

pMo/bfmSUPDN Expected   1969 TACTTTTTCTTGGAGTAAGCGGACTGACTGTAAAAATTGGCGTCGAGTTAAACTTGAGGT 2028
                             TACTTTTTCTTGGAGTAAGCGGACTGACTGTAAAAATTGGCGTCGAGTTAAACTTGAGGT
pMo/bfmSUPDN RV Seq      329 TACTTTTTCTTGGAGTAAGCGGACTGACTGTAAAAATTGGCGTCGAGTTAAACTTGAGGT  270

pMo/bfmSUPDN Expected   2029 GAGTTCATCATGGGCAATTGCATGTTGTAACTTCTGAATTAATTCAGAATGGAGTGCGCT 2088
                             GAGTTCATCATGGGCAATTGCATGTTGTAACTTCTGAATTAATTCAGAATGGAGTGCGCT
pMo/bfmSUPDN RV Seq      269 GAGTTCATCATGGGCAATTGCATGTTGTAACTTCTGAATTAATTCAGAATGGAGTGCGCT  210

pMo/bfmSUPDN Expected   2089 AATACTGGAAACAGTAAGTGGCGCAACCGTCATCATGATCAGCCCTATACGTATAGAAAT 2148
                             AATACTGGAAACAGTAAGTGGCGCAACCGTCATCATGATCAGCCCTATACGTATAGAAAT
pMo/bfmSUPDN RV Seq      209 AATACTGGAAACAGTAAGTGGCGCAACCGTCATCATGATCAGCCCTATACGTATAGAAAT  150

pMo/bfmSUPDN Expected   2149 GTTATTGCTTAAATAGTCTTGCGGATACAGTAATAAATAATGCCCTGAGACATGATAAAT 2208
                             GTTATTGCTTAAATAGTCTTGCGGATACAGTAATAAATAATGCCCTGAGACATGATAAAT
pMo/bfmSUPDN RV Seq      149 GTTATTGCTTAAATAGTCTTGCGGATACAGTAATAAATAATGCCCTGAGACATGATAAAT   90

pMo/bfmSUPDN Expected   2209 AATATATGCACTGGTAAAGGATGTAATGAGAGCAACAATAATCGGTTTATAGCGGATAGC 2268
                             AATATATGCACT  T A  G T+T + G   G   C  T A CG  ++  AG++ +T+G
pMo/bfmSUPDN RV Seq       89 AATATATGCACTCCTTATAGTTSTTMGGCCCGTC-CGGTTACCGCAWYGAAGYKTMTRGG   31

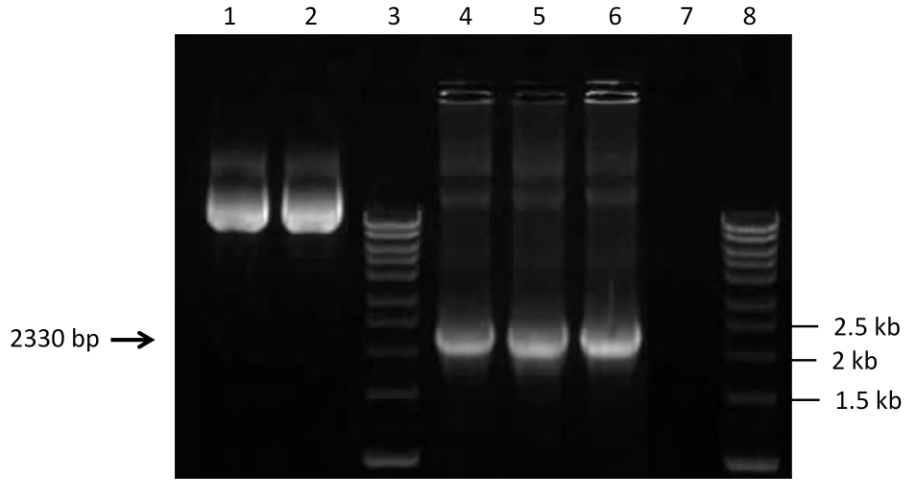
pMo/bfmSUPDN Expected   2269 GCACCAGATTAATGCGGCGATGGGGTAGAGTAG 2301
                             GC        AAT C  CGAT   G AGA
pMo/bfmSUPDN RV Seq       30 GCGGAGATACAATACC-CGATC--GCAGATCCA    1

pMo/bfmSUPDN Expected   2304 GGAACCTGGACCCGAGTCGGGATCCCCC 2331

pMo/bfmSUPDN RV Seq          ----------------------------
```

A region of pMo130-Tel^R containing *bfmRS* UP and DOWN fragments was amplified and sequenced. The result was aligned with the expected sequence.
a) pMo/*bfmS*UPDN FW Sequenced was generated using primer pMo130-Tel^R FW, which is specific for pMo130-Tel^R.
b) pMo/*bfmS*UPDNUPDN RV Sequenced was generated using primer DOWNRVbfmS, which is specific for the DOWN fragment. This sequence was reverse complemented before alignment. The first 1070bp of the sequence corresponds to 174 bp of the vector and the entire UP fragment (a). The remainder of the sequence corresponds to the DOWN fragment (b).

Figure 3.44 Verification of pMo130-Tel^R/*bfmRS*UPDOWN in S17-1



Lane	DNA	Expected size	Actual size
1	pMo130-Tel^R/*bfmRS*UPDOWN from transformation 1	---	---
2	pMo130-Tel^R/*bfmRS*UPDOWN from transformation 2	---	---
3	Hyperladder™ 1 (Bioline)	---	---
4	pMo130-Tel^R/*bfmRS*UPDOWN (positive control)	2330 bp	~ 2330 bp
5	pMo130-Tel^R/*bfmRS*UPDOWN from transformation 1 into S17-1	2330 bp	~ 2330 bp
6	pMo130-Tel^R/*bfmRS*UPDOWN from transformation 2 into S17-1	2330 bp	~ 2330 bp
7	---	No amplimer	No amplimer
8	Hyperladder™ 1 (Bioline)	---	---

PCR 15, Table 2.7

double recombinants (which would account for their white phenotype), 12 were screened by PCR for gene deletion (Table 2.7, PCR 21). Only one amplimer was obtained and this was of the size expected for wild-type AYE. Twenty more colonies were screened, but no double recombinants were identified. Since a single recombinant had not been obtained by conjugation, transformation was carried out to introduce pMo130-Tel^{R}/*bfmRS*UPDOWN directly into AYE (Section 2.4.4.1). All candidate transformants obtained from two transformations remained white after pyrocatechol exposure. At this stage, construction of a *bfmRS* deletion mutant was suspended to focus on the phenotypic characterisation and complementation of AYEΔ*adeRS* and AYEΔ*pmrAB.*

It was initially planned that only *bfmS* would be deleted. However, due to the location of the deletion primers, the whole of *bfmS,* 112 bp of *bfmR* and 30 bp of ABAYE3062 would have been deleted (Figure 3.45).

3.5. Discussion

Investigation of the role of TCSs in *A. baumannii* was carried out in AYE. The conservation of *adeRS* in the strains investigated in this study was high; there was at least 95% identity between the aligned regions of the genes from different strains. However, the length of the genes did vary between strains, giving rise to different sized proteins, particularly in ATCC 17978 (Section 3.2.2). Strain ATCC 17978 was not used as it was antibiotic-susceptible, rather than its lack of *adeC* or its smaller *adeRS* genes. In a study by Nemec *et al.* (2007a), 41% of 116 strains did not have an *adeC* gene suggesting that this is a common feature in *A. baumannii* strains. AB0057 and ACICU were other possible candidate strains in which to delete *adeRS*, as they are MDR

Figure 3.45 Region around *bfmS* in AYE (Expected Sequences) and Primers Designed to Delete *bfmRS*.

Key for the following DNA sequences:

Expected deleted region

bfmR

bfmS

ABAYE3062 hypothetical protein (xBASE)

UP primers

UPFWbfmS (Contains *Not*I recognition site)
GGGGCGGCCGCGAGATAGCATACCAAAGCTG

UPRVbfmS (Contains *Bam*HI recognition site)
GGGGGATCCACGTCAATTGAACGGTCTTG

DOWN primers

DOWNFWbfmS (Contains *Bam*HI recognition site)
GGGGGATCCAGGTTTGCCGCTTCTCAGGC

DOWNRVbfmS (Contains *Sph*I recognition site)
GGGGCATGCCGACTCGGGTCCAGGTTCCC

a) Wild-type AYE Expected Sequence.

ggtgaaagcaaatcaagctctacacctttttttcaagaatgcttcaagtgcattgttttca
gctgtatcggccggtaaacctaagaatgctaagccagtgcttaccaactcatcacgaagc
aaacgtgcagttacgtatgtatagttaggttcttgttcaatacgtgtacgtgttgccatc
atcatggtggtagcaatatcgctttcttttacgccgttatataagtttttaacggtttca
tcgataattgcttgaacatcaataccttctaagccttcagcagctcgatttacagttgct
tgcagtgcacttaaatcaagtggttgaagctgaccgtttgcatcggtaacttgcaaggta
gggtgatgattagaatttgtgtctttacgggcacttgcgcgttgatcgcgatagataacg
taagcacgagcgactttgtgctccccagtacgcatcagtgcaagttcaacttgatcttga
atctcttcaatatgaatggtaccgccagaaggtaaacggcgagtgaaagtgttcagtacc
atttccgtcagttgggtaatacggtcgtgaatacggctcgaatccgcactttgttgacct
tccacagcaagaaaagctttaccaatcgcaacagaaattttctctgcatcaaatgcagca
acatctccggtgcgtttaatcacctgaatttgaccgggagtcgaagtaattacgctcatg
ccagcatagctccattgtcacttttgttatgttcatagaccgtttgaaccgagcaaaaaa
catgccacgatatttgagggataaacacaagacttagtagttaaagtttatttcaacac
aagatacgggatttttacggtagatcaatcttgacttttagtaatttttatctgtat
gatttgtcgggagatagcataccaaagctgtgaaaaaacacttatagataactttgtgga
taactcaaaaaacaaacacttgagtgaatttaaatttagtgaagaaataatttgtttaat
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aaaccgatta

b) AYEΔ*bfmRS* Expected Sequence

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It was expected that a *Bam*HI site (***GGATCC***) would be introduced in AYEΔ*bfmRS*.

clinical isolates (Adams *et al.*, 2008). AYE was chosen as it had the most BLAST hits with other *A. baumannii* genomes and was considered to have the core set of genes for the species and therefore to be a representative strain.

The *adeRS* deletion in AYE was verified by PCR and DNA sequencing. In addition, an *adeS* transcript was detected in AYE, but not in AYEΔ*adeRS* (as shown in RNA sequencing; Richmond and Piddock, unpublished). However, despite repeated attempts, it was not possible to delete *bfmRS* in AYE. This gene is not essential in other strains of *A. baumannii* (clinical isolate and type strain, ATCC 19606 and clinical isolate, M2), since it has previously been inactivated with a transposon (Clemmer *et al.*, 2011; Tomaras *et al.*, 2008). However, there have been no reports of targeted deletion or inactivation of *bfmR* or *bfmS* in AYE. It is possible that there are some regions of the chromosome into which pMo130-Tel^R is harder to introduce.

It was also not possible to re-introduce the wild-type *adeRS* or *pmrAB* into AYEΔ*adeRS* and AYEΔ*pmrAB*, respectively after multiple attempts. It was initially suspected that AYEΔ*adeRS* and AYEΔ*pmrAB* had a reduced ability compared with AYE to accept DNA by conjugation. However, AYE, AYEΔ*adeRS* and AYEΔ*pmrAB* were all able to accept the *adeRS* and *pmrAB* deletion constructs by conjugation and incorporate them into their chromosome. Moreover, AYE was able to incorporate the deletion constructs, but not the complementation constructs. Therefore, the problem is with the complementation constructs, rather than the method, or the recipient strains. It is possible that the presence of *adeRS* or *pmrAB* in the complementation construct is responsible for an inability of the constructs to recombine into the chromosome, or maybe the increased construct size had an effect on conjugation. The most likely explanation for the white tellurite-resistant colonies is that they were

spontaneous tellurite-resistant mutants, since the colonies obtained when attempting to complement AYEΔ*adeRS* were confirmed by PCR to be deletion mutants, with no complementation vector in their chromosome.

3.6. Further Work

3.6.1. Problems with gene deletion - how to resolve

An alternative strategy to delete *bfmRS* could be to use different UP and DOWN fragments for recombination. For example, the UP and DOWN pairs of primers could be designed closer together, to delete an internal gene region, rather that the whole gene. Nonetheless, if pMo130-Tel^{R} were to be used for further work, it would be important at this stage to verify that certain genes on the vector have not been disrupted (genes responsible for conjugation, replication and gene expression). This should also be carried out for the *bfmRS* deletion construct and complementation constructs (Section 3.6.2) to check that the genes have not been disrupted during construction.

3.6.2. Problems with complementation - how to resolve

Although the reasons for using the current complementation strategy are discussed later (Chapter 6), an alternative strategy would be to use a plasmid known to replicate in *A. baumannii* to complement the gene deletions. A third and preferable option would be to introduce the tellurite-resistance cassette onto a new suicide vector and use the same complementation strategy used in this study. This is because replacing the gene directly into its original genomic location is more stable than using a plasmid (which could be lost) returns the strain to its original state and also removes any problems with increased or decreased gene expression due to plasmid copy number.

There was not enough time to investigate the white tellurite-resistant colonies obtained during *pmrAB* complementation. However, it would be more important at this stage to change the complementation strategy or to establish the problems with the current constructs.

3.7. Key Findings

- Identifying representative strain to make mutants
- Making AYEΔ*adeRS* and AYEΔ*pmrAB*
- Making vector to delete *bfmRS*
- Making vectors to complement AYEΔ*adeRS* and AYEΔ*pmrAB*

4. Role of *adeRS* in Growth, Antimicrobial and Biocide Susceptibility and Accumulation

4.1. Background

adeRS are involved in antimicrobial susceptibility due to their regulation of *adeABC.* Deletion of *adeS* in BM4454 resulted in increased susceptibility to gentamicin and kanamycin (Marchand *et al.*, 2004) and deletion of *adeS* in clinical isolates resulted in increased susceptibility to meropenem, cefotaxime, gentamicin, amikacin and ciprofloxacin (Wong *et al.*, 2009). Mutations in *adeR* and/or *adeS* give increased MICs (MDR) (Table 1.2; Table 1.3). Investigation into the role of *adeRS* in growth, efflux, biocide susceptibility and virulence has not been reported to date. Based on MIC data for an *A. baumannii* clinical isolate, AdeABC transports biocides such as benzalkonium chloride, chlorhexidine and tetraphenylphosphonium chloride (Rajamohan *et al.*, 2010b). The antibiotic and biocide susceptibility profiles for AYE have not been reported.

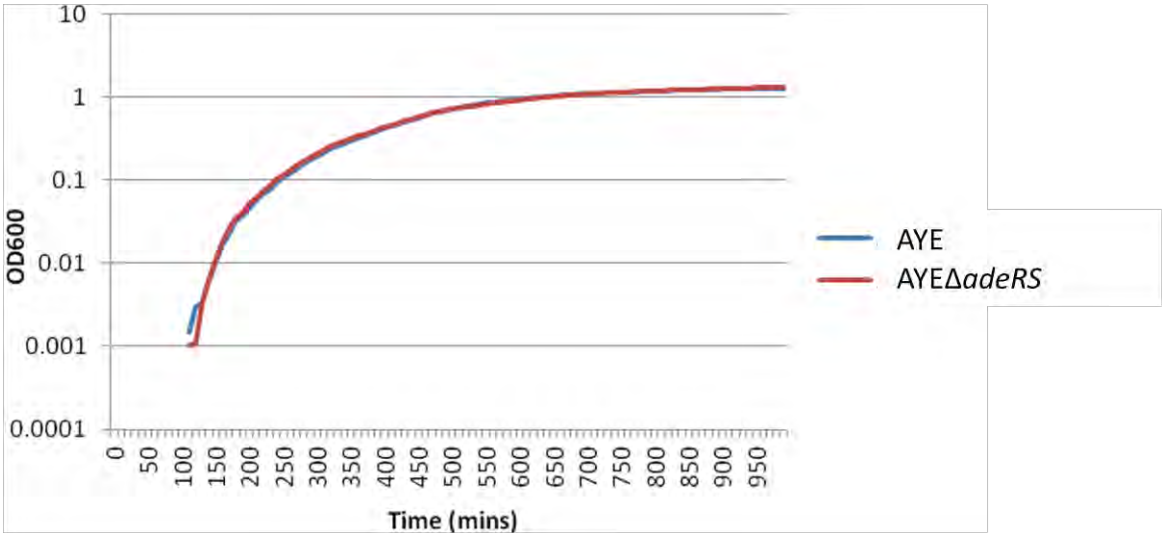
4.2. Aims and hypotheses

AYEΔ*adeRS* was hypothesised to be more susceptible to antimicrobials and to accumulate higher levels of these compounds, due to decreased expression of *adeABC*. Therefore, the aims were to investigate whether deletion of *adeRS* affected growth, antibiotic and biocide susceptibility, efflux and virulence in AYE.

4.3 Effect of *adeRS* Deletion on Growth of Planktonic Cells and Biofilm *in vitro*

There was no significant difference in generation time or final optical density at 600nm between planktonic AYE and AYEΔ*adeRS* grown in LB broth (Figure 4.1; Table 4.1). There was

Figure 4.1 Growth of AYE and AYEΔ*adeRS* in LB broth.



Data are the mean of three separate experiments, each carried out in duplicate.

Table 4.1 Mean Generation time and Optical Density at Stationary Phase (± standard deviation).

Strain	Mean generation time (min)	T-test (*P*)	Mean OD_{600} at stationary phase	T-test (*P*)
AYE	167.25 ± 22.62	---	1.31 ± 0.05	---
AYEΔ*adeRS*	183.89 ± 23.87	0.43	1.30 ± 0.06	0.89

Data are the mean of three separate experiments, each carried out in duplicate. The generation times were determined from the steepest part of the exponential curve. A two-tailed, unpaired Student's *t* test was used to determine any significant difference ($P < 0.05$) between strains.

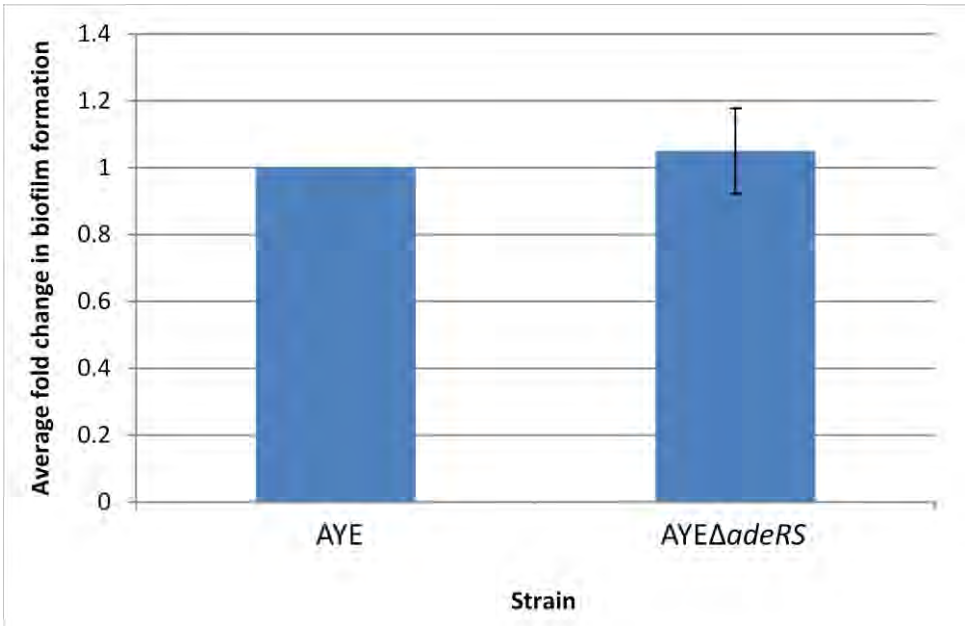
also no significant difference in the ability of AYE and AYEΔ*adeRS* to grow as a biofilm in TSB (Figure 4.2).

4.4. Effect of *adeRS* Deletion on Antibiotic and Biocide Susceptibility

MICs of various antibiotics were determined for AYE and AYEΔ*adeRS* (Table 4.2). These antibiotics were chosen for three reasons: a) to compare the antibiotic susceptibility of AYEΔ*adeRS* in the present study with published data for other strains, b) to ensure that many antibiotic classes were investigated, to obtain a wide susceptibility profile and c) to ensure that clinically relevant antibiotics (Table 4.2) were investigated. The mutant strain was significantly more susceptible to gentamicin, tobramycin and tigecycline. There was two-fold difference in susceptibility to kanamycin and amikacin (MIC value for AYE was always at least 2-fold higher than for AYEΔ*adeRS* on five separate occasions for both antibiotics). There was no difference in susceptibility to any of the other antibiotics tested.

The MIC and MBC of eight biocides were determined for AYE and AYEΔ*adeRS*. Five of these biocides were surface biocides used to clean inanimate objects. Three antiseptics were also investigated. These two groups of biocides were of interest because the hospital environment and infected patients are two reservoirs of *A. baumannii* infection (Dijkshoorn *et al.*, 2007). There was no difference in the MIC or MBC of any of the surface biocides or for cetylpyridinium chloride for planktonic AYE compared with planktonic AYEΔ*adeRS* (Table 4.3; Table 4.4). However, there was a significant increase in chlorhexidine and benzalkonium chloride susceptibility when *adeRS* were deleted (Table 4.4). The two-fold increase in the MIC and MBC of benzalkonium chloride was recorded on three separate occasions. There may be a significant difference in the MBC of chlorhexidine for AYE and AYEΔ*adeRS*, but there was too much variation in the results to draw a conclusion, despite the starting OD_{600}

Figure 4.2 Effect of *adeRS* Deletion on the Ability of AYE to Grow as a Biofilm.



Data are the mean of eight separate experiments each carried out four times. The OD_{570} (indicating biofilm formation) for AYEΔ*adeRS* is presented as fold change compared with the OD_{570} for AYE ± standard deviation. An unpaired, two-tailed Student's *t* test on fold change values generated a *P* value of 0.29.

Table 4.2 Minimum inhibitory concentrations of antibiotics for AYE and AYEΔ*adeRS*.

	CIP	AMK	GEN	KAN[a]	TOB	TIG	TET[a]	COL	PMX[a]	AMP[a]
AYE	**64**	**16[I]**	**256**	512	**16**	1	128	1	0.5	>1024
AYEΔ*adeRS*	**32**	8	**8**	256	4	0.25	128	1	0.5	>1024

	CAZ[a]	CTX[a]	IMP	MER	CHL[a]	ERY[a]	AMP/SUL[a]	EtBr[a]
AYE	>1024	1024	0.38	1.5	256	128	8	512
AYEΔ*adeRS*	>1024	512	0.38	1.5	256	128	8	512

CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; TIG, tigecycline; TET, tetracycline ; COL, colistin; PMX, polymyxin B; AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; IMP, imipenem; MER, meropenem; CHL, chloramphenicol; ERY, erythromycin; AMP/SUL, ampicillin/sulbactam combination; EtBr, ethidium bromide.

The minimum inhibitory concentration (MIC) values are the mode value of at least three agar-dilution experiments, each carried out in triplicate. The MIC of IMP and MER, were determined three times by E-test.
Significant decreases in MIC are indicated in blue text and MICs which correspond to resistance ([I] intermediate resistance) to that agent are in bold ([a]breakpoint concentration not available). Clinically relevant antibiotics (used to treat *Acinetobacter* infection) are highlighted in yellow.

Table 4.3 Minimum Inhibitory and Bactericidal Concentrations of Surface Biocides for AYE and AYEΔ*adeRS*.

		Peracetic acid %		TBQ %		Hydrogen peroxide %		SporKlenz %		Ethanol %	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
AYE	**planktonic**	0.08	0.16	0.375	0.75	0.06	0.06	3.125	3.125[b]	6.25	25
	biofilm	0.08	0.16	---	6.25	0.06	>2	3.125	12.5	---	25
AYEΔ*adeRS*	**planktonic**	0.08	0.08 or 0.16[a]	0.375	0.75	0.06	0.06	3.125	6.25[c]	6.25	25
	biofilm	0.08	0.16	---	6.25/12.5	0.06	≥2	3.125	12.5	---	25

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for planktonic cells were each determined in duplicate for three independent cultures. MICs for cells in biofilm were determined for three independent cultures, each with four technical repeats. MBC concentrations for cells in biofilm were determined for two independent cultures, each with four technical repeats.
[a] MBC values of 0.08 and 0.16 µg/ml were each obtained three times; [b] An MBC of 6.25 µg/ml was obtained twice; [c] An MBC of 1.5µg/ml was obtained once and 3.125 µg/ml was obtained twice. Significant increases in MBC for cells in biofilm compared with planktonic cells are indicated in red text.

Table 4.4 Minimum Inhibitory and Bactericidal Concentrations of Antiseptics for AYE and AYEΔ*adeRS*.

	Chlorhexidine (µg/ml)		Benzalkonium chloride (µg/ml)		Cetylpyridinium chloride (µg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
AYE	18.75	18.75 - >150	4	16	2	4
AYEΔ*adeRS*	4.5	9 - 75	2	2 – 8[a]	2	4

Minimum inhibitory concentrations and minimum bactericidal concentrations were each determined in duplicate on at least two separate occasions. [a] MBC values of 2 µg/ml and 8 µg/ml were each obtained twice. Significant decreases in MIC or MBC are indicated in blue text.

being the same for each experiment.

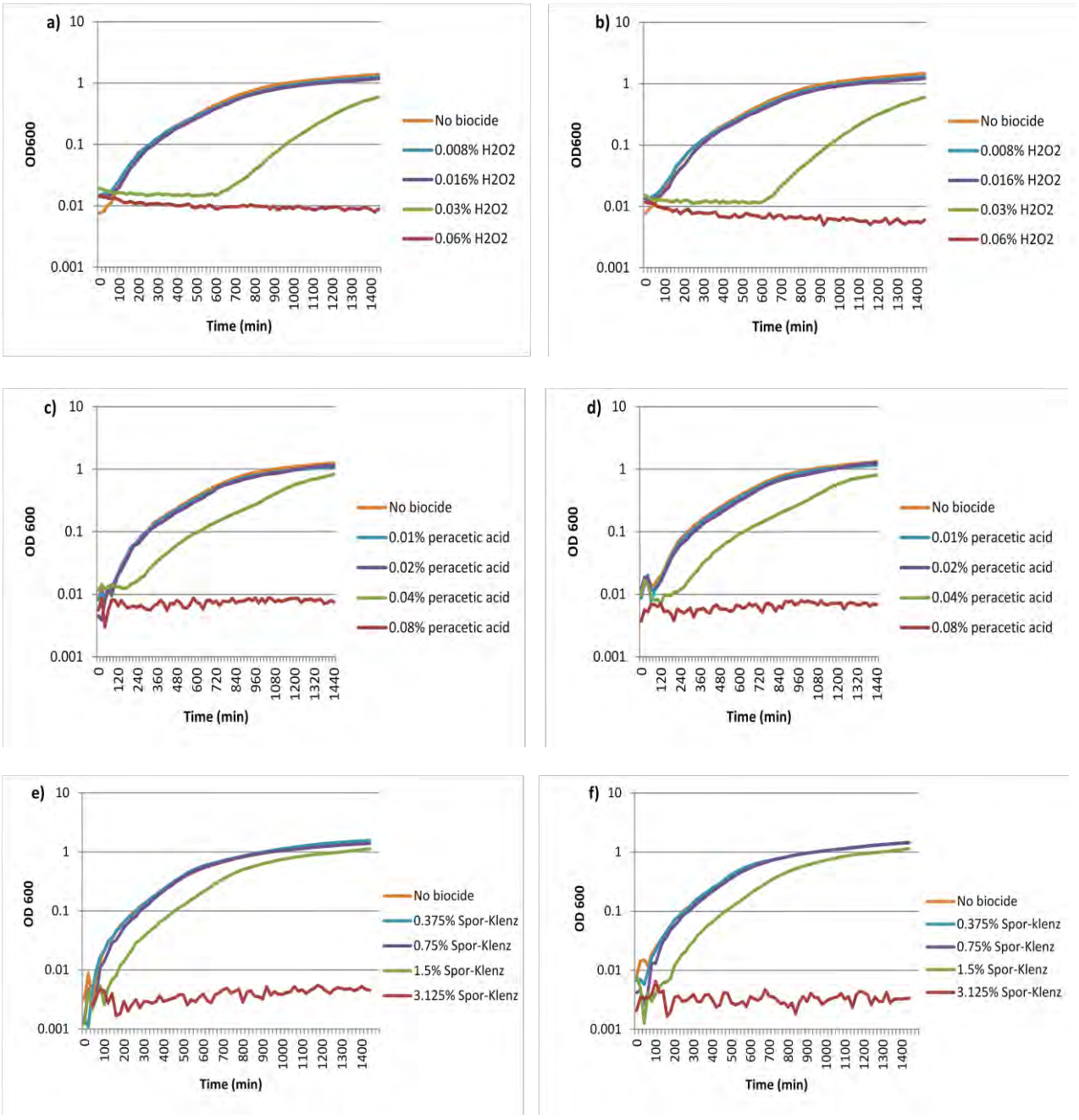
Cells naturally survive on inanimate objects as a biofilm (Smith and Hunter, 2008). Therefore, the ability of planktonic cells to form a biofilm (Section 2.5.4.2) in the presence of surface biocides was investigated to determine whether the presence of biocides induces biofilm formation. The ability of an established biofilm to tolerate surface biocides (Section 2.5.4.3) was also compared with the biocide susceptibility of planktonic cells. Three surface biocides were chosen for more detailed investigation (growth curves and growth of a biofilm). H_2O_2 was selected because interest in this biocide for the elimination of hospital pathogens has increased recently (Otter *et al.*, 2010; Piskin *et al.*, 2011). Peracetic acid was chosen because it is a peroxygen compound, like hydrogen peroxide, but is considered to be a more potent biocide and is active in the presence of organic material. SporKlenz was included since it is a mixture of hydrogen peroxide and peracetic acid, and to investigate whether the combination of these disinfectants is more effective that each used alone.

There was no difference in the ability of either AYE or AYEΔ*adeRS* cells to grow as a planktonic culture or as a biofilm in the presence of H_2O_2, peracetic acid or Spor-Klenz (MIC values, Table 4.3). However, cells in biofilm were more tolerant of TBQ, Spor-Klenz and H_2O_2 than planktonic cells (MBC values, Table 4.3).

Although there was no difference in the MICs of each surface biocide for AYE and AYEΔ*adeRS*, growth kinetics were determined in the presence of H_2O_2, peracetic acid and Spor-Klenz to establish whether there were any growth differences between the two strains. Deletion of *adeRS* had no effect on the ability of the strains to grow in the presence of each surface biocide (Figure 4.3). Growth kinetics of the two strains in the presence of chlorhexidine and benzalkonium chloride were also determined to investigate the

Figure 4.3 Effect of *adeRS* Deletion on Growth of AYE in the Presence of Surface Biocides.

AYE AYEΔ*adeRS*



Data are the mean of two independent experiments, each carried out in duplicate. H_2O_2, hydrogen peroxide.

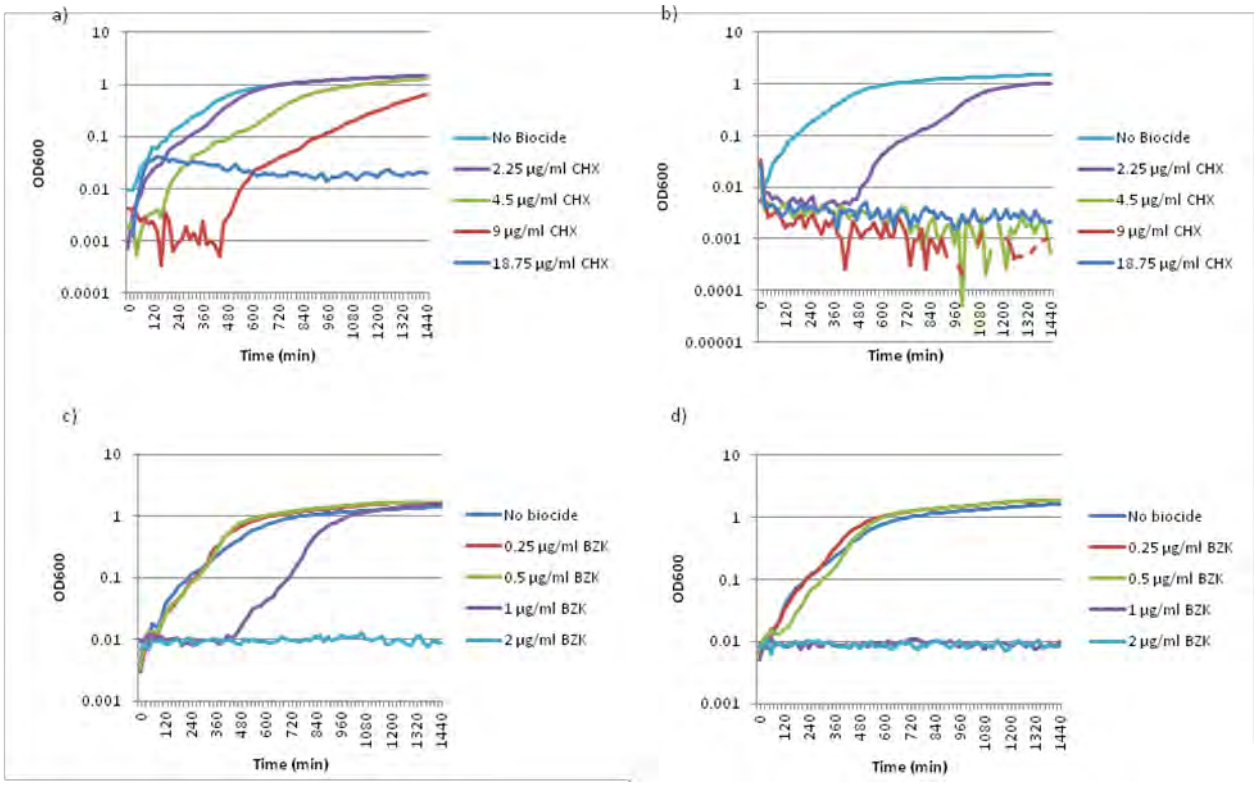
susceptibility difference in greater detail (Figure 5.4). Growth of AYE was impaired in the presence of sub-inhibitory concentrations of chlorhexidine and benzalkonium chloride and growth of AYEΔ*adeRS* was impaired in the presence of a sub-inhibitory concentration of chlorhexidine compared with growth in the absence of biocide (Figure 4.4).

4.5. Effect of *adeRS* Deletion on Accumulation of Dyes and Fluoroquinolones

Hoechst 33342 is a membrane-permeable molecule which fluoresces when bound to DNA, or in a hydrophobic environment in the cell, such as the cell membrane. Therefore, Hoechst 33342 can be used to determine relative differences in efflux between strains (Richmond *et al.*, 2012). There was no significant difference in the level of accumulation of Hoechst 33342 between AYE and AYEΔ*adeRS* at steady state (Figure 4.5). There was also no difference in the accumulation of Hoechst 33342 in the presence of efflux inhibitors PAβN or CCCP, compared with the accumulation in the absence of efflux inhibitors for AYE or AYEΔ*adeRS*. To determine whether deletion of *adeRS* affected fluoroquinolone accumulation, accumulation of ciprofloxacin was measured. The values obtained after five min showed great variation (Figure 4.6). It was possible that there was a molecule present in the bacterial supernatant, which fluoresced at the excitation wavelength used to measure ciprofloxacin and this gave rise to the large differences in values for different experiments. To investigate this, the emission profile for the AYE and AYEΔ*adeRS* supernatants at excitation wavelength 279 nm was compared with that for ciprofloxacin (10 μg/ml). The supernatants did not emit at the same wavelength as ciprofloxacin; therefore this was not the reason for the variation observed. Since there was a large variation in the accumulation of ciprofloxacin between.

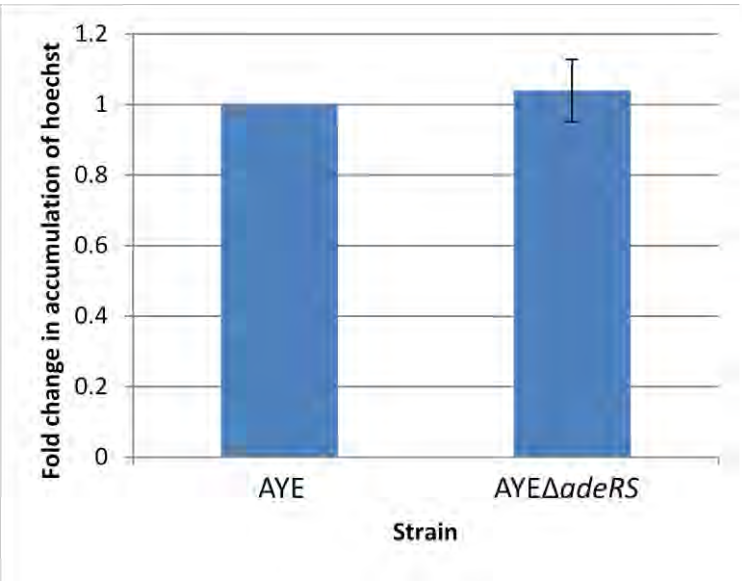
Figure 4.4 Effect of *adeRS* Deletion on Growth of AYE in the Presence of Antiseptics.

AYE AYEΔ*adeRS*



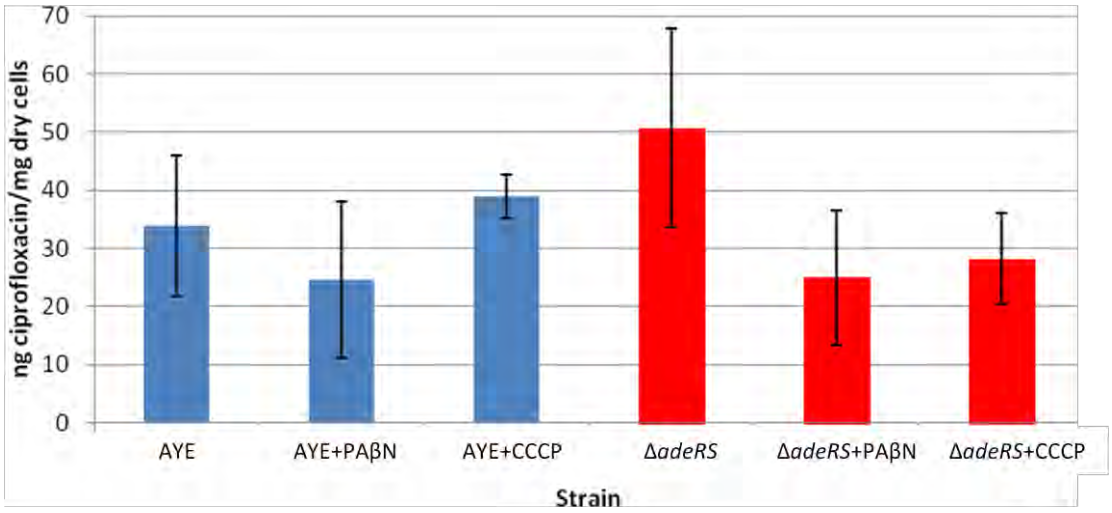
Data are the result of one experiment carried out in duplicate. CHX, chlorhexidine; BZK, benzalkonium chloride.

Figure 4.5 Effect of *adeRS* Deletion on the Accumulation of Hoechst H33342 (Bis-benzimide).



Data are the mean of four separate experiments, each carried out in duplicate. The steady state accumulation for AYEΔ*adeRS* is presented as fold change compared with the steady state value for AYE ± standard deviation. An unpaired, two-tailed Student's *t* test on fold change values generated a *P* value of 0.40.

Figure 4.6 Accumulation of Ciprofloxacin in AYE and AYEΔ*adeRS* at Five Minutes in the Presence and Absence of Efflux Inhibitors.



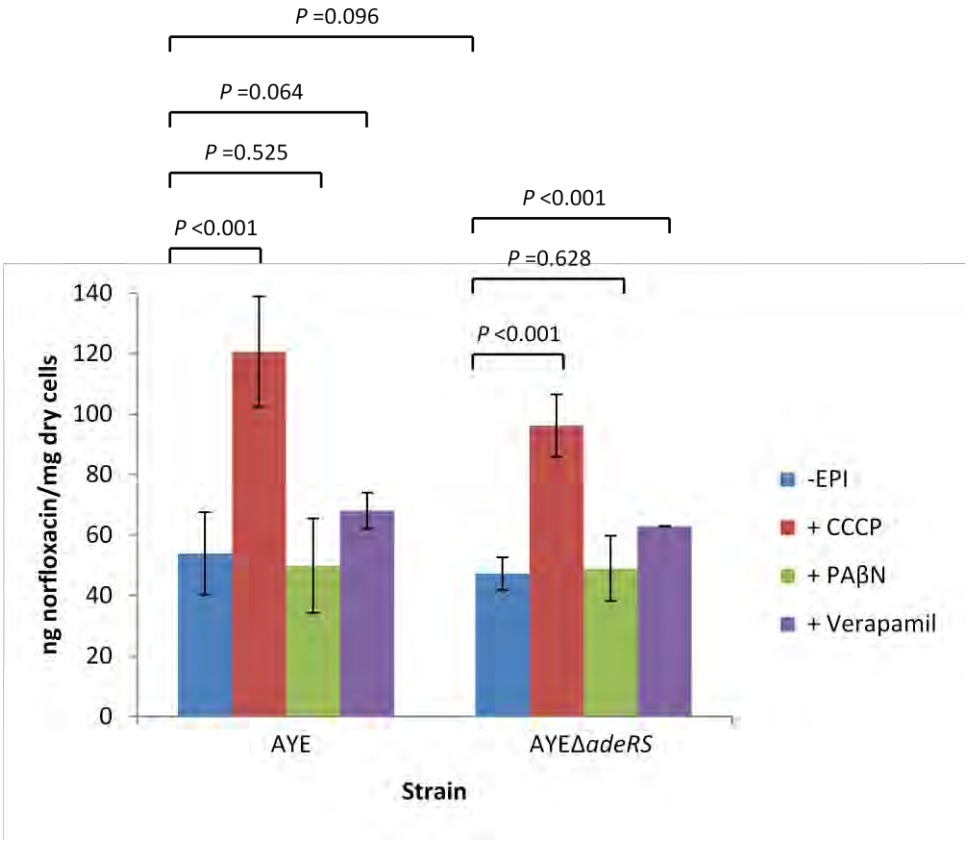
PAβN was added to a final concentration of 50 µg/ml; CCCP was added to a final concentration of 50 µM. The results are all data obtained from two experiments, each carried out in duplicate, except for AYEΔ*adeRS* in the presence of PAβN, which are the data from one experiment, carried out in duplicate.

experiments, norfloxacin was used for further experiments. Norfloxacin accumulates to higher levels (Mortimer and Piddock, 1991) and higher fluorescence levels are measured More consistent results were obtained when norfloxacin was used. However, there was no significant difference in the concentration of norfloxacin accumulated by AYE and AYEΔ*adeRS* in the absence of efflux inhibitors after five min (Figure 4.7).

An increase in accumulation in the presence of an efflux inhibitor indicates that the target classes of efflux pump are active in the absence of efflux inhibitor. To investigate whether efflux pumps were active in AYE and AYEΔ*adeRS*, CCCP, which targets pumps that use the proton motive force (such as RND pumps AdeABC) was used. There was a significant increase in the concentration of norfloxacin accumulated in both strains in the presence of CCCP (50 mM) (Figure 4.7). Since AdeABC was predicted to be down-regulated in AYEΔ*adeRS*, PAβN was used to specifically investigate whether other RND efflux pumps could compensate for down-regulation of AdeABC in AYEΔ*adeRS*. There was no significant difference in the accumulation of norfloxacin for AYE or AYEΔ*adeRS* in the presence or absence of PAβN (50 μg/ml) (Figure 4.7). However, in the presence of verapamil, there was a significant increase in the concentration of norfloxacin accumulated for AYEΔ*adeRS*, but no difference for AYE (Figure 4.7).

The concentration of efflux inhibitors and norfloxacin used in the norfloxacin uptake assay were confirmed to have no effect on growth of AYE and AYEΔ*adeRS.* Comparison of viable count at time 0 min with the same strain after addition of norfloxacin, both in the presence and absence of efflux inhibitor, generated a *P* value of greater than 0.05 (Figure 4.8).

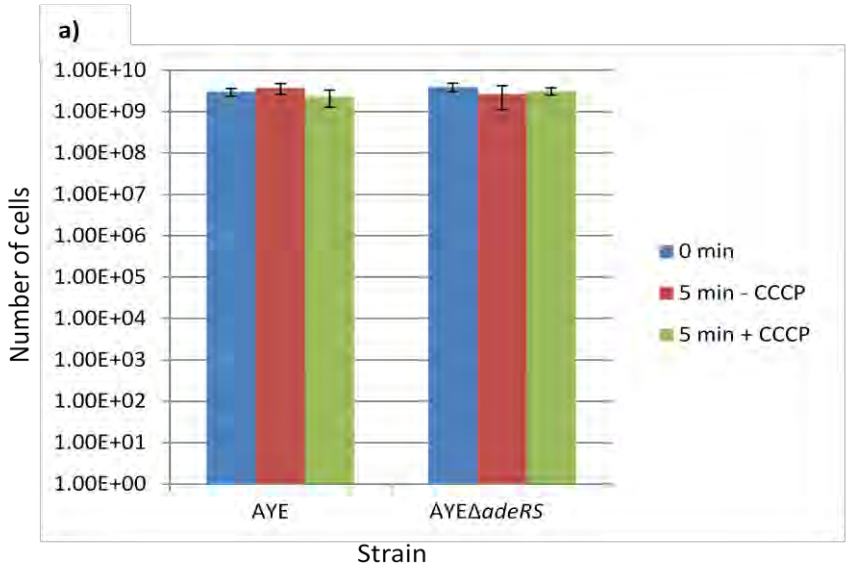
Figure 4.7 Accumulation of Norfloxacin in AYE and AYEΔ*adeRS* at Five Minutes in the Presence and Absence of Efflux Inhibitors.

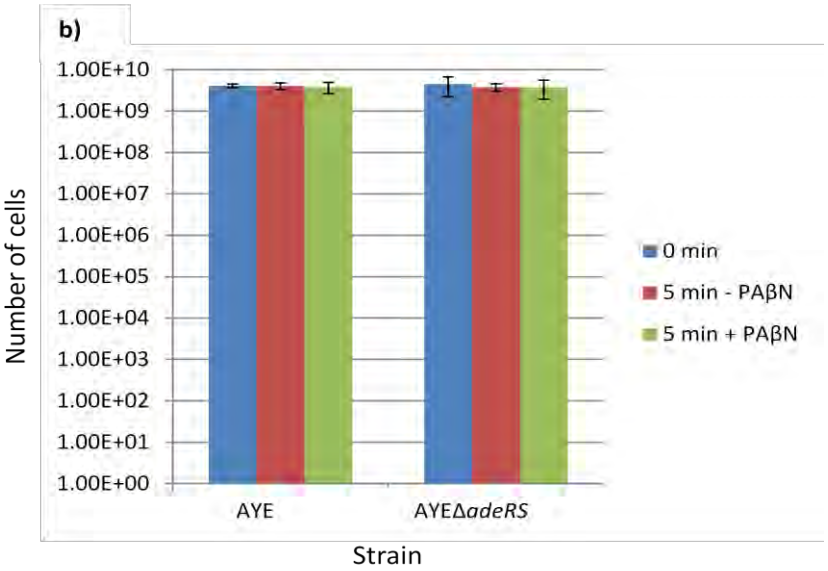


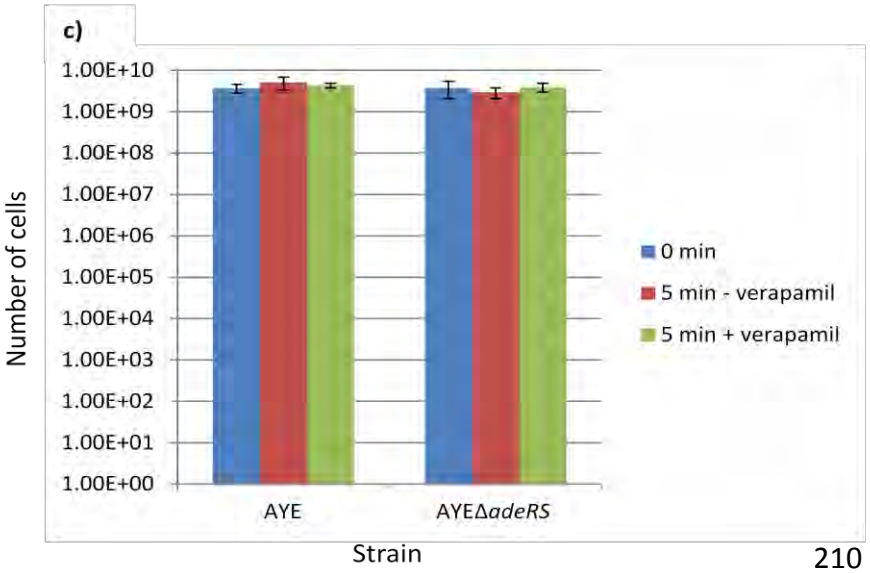
PAβN and verapamil were added to a final concentration of 50 µg/ml; CCCP was added to a final concentration of 50 µM.

An unpaired, two-tailed Student's *t* test was carried out to compare the accumulation of norfloxacin in presence of efflux inhibitor with accumulation in the same strain in the absence of efflux inhibitor. A significant difference is one which generated a *P* value of <0.05.

Figure 4.8 Viable Count of AYE and AYEΔ*adeRS* in the Presence and Absence of Efflux Inhibitors.







Data are the mean of at least two independent experiments, each carried out in duplicate. An unpaired, two-tailed Student's *t* test was used to determine any significant difference between strains and for each strain in the presence and absence of norfloxacin (5 min) and efflux inhibitor (indicated).

4.6. Discussion

AdeRS was not essential for growth of AYE cells as a planktonic culture or as a biofilm in the absence of antimicrobials, or in the presence of surface biocides. However, compared with AYE, growth of AYEΔ*adeRS* was impaired in the presence of chlorhexidine and benzalkonium chloride.

So far, the only reported regulatory system of AdeABC expression is the AdeRS two component system. If there are no others, the absence of *adeRS* in AYEΔ*adeRS* should result in down-regulation of AdeABC, which would be expected to result in decreased efflux. This would have an impact on susceptibility to antimicrobials that are substrates for this pump. AYEΔ*adeRS* was more susceptible to five of the sixteen antibiotics tested, compared with AYE (Table 4.2).

AYEΔ*adeRS* was significantly more susceptible than AYE to gentamicin, tobramycin, tigecycline, kanamycin and amikacin. Increased resistance to these antibiotics in strains in which *adeS, adeR* or *adeB* have been disrupted have been reported previously (Table 4.5; Magnet *et al.*, 2001; Marchand *et al.*, 2004; Ruzin *et al.*, 2007; Wong *et al.*, 2009). Although AYEΔ*adeRS* was more susceptible to gentamicin than AYE, the MIC for AYEΔ*adeRS* did not fall below the BSAC breakpoint, so this result is not clinically significant. There is no recommended breakpoint concentration for tigecycline for *Acinetobacter spp*. since there are insufficient clinical data. According to the BSAC non-specific recommended breakpoint concentrations for tigecycline, AYE was resistant (MIC > 0.5 μg/ml), whilst AYEΔ*adeRS* was susceptible (MIC ≤ 0.25 μg/ml). AYE was resistant (MIC > 4 μg/ml) to tobramycin, whilst AYEΔ*adeRS* was susceptible (MIC ≤ 4 μg/ml) (EUCAST, 2012). Therefore, *adeRS* are important

Table 4.5 Comparison of MICs of Antibiotics for AYE and other Strains in which *adeRS* or *adeB* Genes are Inactivated or Overexpressed.

a)

Strain/isolate	Antibiotic							Reference
	CIP	AMK	GEN	KAN	TIG	TET	COL	
AYE	64	16	256	512	1	128	1	This study
AYEΔ*adeRS*	32	8	8	256	0.25	128	1	
BM4454 (MDR clinical isolate)		8	8	4		64		(Magnet *et al.*, 2001)
BM4454 *adeB*::pAT794		1	≤0.25	1		8		
BM4454 (MDR clinical isolate)			12	4				(Marchand *et al.*, 2004)
BM4454 *adeB*::pAT794			0.25	0.25				
BM4454 *adeR*::pAT799			0.25	0.5				
BM4454 *adeS*::pAT800			0.5	1				
CIP 70-10 $AdeS_{T153M}$ (over-expresses *adeABC*)			12	4				
CIP70-12 $AdeR_{P116L}$ (over-expresses *adeABC*)			12	4				

a) continued

Strain	Antibiotic							Reference
	CIP	**AMK**	**GEN**	**KAN**	**TIG**	**TET**	**COL**	
G5140 (one of two clinical isolates studied)			48		4			(Ruzin *et al.*, 2007)
G5140 *adeB*::pCLL3469			0.75		0.5			
15 (one of three carbapenem-resistant clinical isolates studied)	64	8	128					(Wong *et al.*, 2009)
15 *adeB*::pAT801-RA	4	0.5	16					
15 *adeS*::pAT801-RA	2	1	32					
15 *adeR*:: pAT801-RA	2	1	32					
AB210 (Tig-susceptible clinical isolate)	>8				0.5		≤0.5	(Hornsey *et al.*, 2010)
AB210-6 (Tig^R laboratory mutant selected on Tig; derived from AB210)	>8				64		≤0.5	
AB211 (Tig^R clinical isolate isolated from same site as AB210)	>8				16		≤0.5	
AB2B211 *adeB*::pBK-5	>8				0.5		≤0.5	

b)

Strain/isolate	Antibiotic							Reference
	AMP	**CAZ**	**CTX**	**IMP**	**MER**	**CHL**	**ERY**	
AYE	>1024	>1024	1024	0.38	1.5	256	128	This study
AYEΔ*adeRS*	>1024	>1024	512	0.38	1.5	256	128	
BM4454 (MDR clinical isolate)			16			512	64	(Magnet *et al.*, 2001)
BM4454 *adeB*::pAT794			4			128	8	
15 (one of three carbapenem-resistant clinical isolates studied)	>512	> 512	32	32	64			(Wong *et al.*, 2009)
15 *adeB*::pAT801-RA	256	256	2	32	16			
15 *adeS*::pAT801-RA	256	256	2	32	16			
15 *adeR*:: pAT801-RA	256	256	2	32	16			
AB210 (Tig-susceptible clinical isolate)		64		>32	>32			(Hornsey *et al.*, 2010)
AB210-6 (Tig^R laboratory mutant selected on Tig; derived from AB210)		16		8	4			
AB211 (Tig^R clinical isolate isolated from same site as AB210)		64		>32	>32			
AB2B211 *adeB*::pBK-5		128		>32	>32			

for resistance to these compounds, and thus far, there are no resistance mechanisms against these agents that can compensate for loss of *adeRS.*

An MIC of 8 μg/ml (susceptible ≤ 8 μg/ml) was obtained five times for AYEΔ*adeRS*, whilst the MIC for AYE was always either 16 or 32 μg/ml (above the BSAC resistance breakpoint). Therefore, *adeRS* has a role in amikacin susceptibility in AYE. This is consistent with a previous study in which disruption of *adeS* in three amikacin-susceptible *A. baumannii* clinical isolates resulted in at least a four-fold increase in susceptibility to this antibiotic (Table 4.5a ; Wong *et al.*, 2009).

Likewise, AYEΔ*adeRS* was consistently more susceptible to kanamycin. This is consistent with that reported previously for a strain in which *adeS* had been disrupted (Table 4.5a; Marchand *et al.* 2004).

Both AYE and AYEΔ*adeRS* were susceptible to colistin (MIC ≤ 2 μg/ml), which remains one of the last available antibiotics for treatment of *A. baumannii* infections.

There was no difference in the MICs of imipenem or meropenem for both AYE and AYEΔ*adeRS* and both strains were susceptible to these carbapenems (MIC < 2 μg/ml). This suggests that AdeRS (and AdeABC) does not have any effect on susceptibility to these agents. There was also no difference in susceptibility to imipenem in three carbapenem-resistant isolates in which *adeS* and *adeB* had been disrupted, although a four-fold decrease in the MIC of meropenem was reported (Table 4.5; Wong *et al.*, 2009). The unchanged susceptibility of AYEΔ*adeRS* to meropenem may be because AYE was already susceptible to meropenem, in contrast to the isolates studied by Wong *et al.* (2009) which were resistant.

In this study, there was no difference in the MIC of ciprofloxacin for AYEΔ*adeRS* or AYE (MIC values of 64 and 32 μg/ml were obtained for both). This is in contrast to a study by Wong *et al.* (2009) in which the MIC of ciprofloxacin decreased at least four-fold in three ciprofloxacin-resistant clinical isolates when *adeS* was insertionally inactivated (Table 4.5).

In contrast to the results of this study, there was a decrease in the MIC of tetracycline and chloramphenicol for BM4454-1 (Table 4.5; Magnet *et al.*, 2001). AYE contains *cmlA* (MFS efflux pump) and a *cat* gene (encoding a chloramphenicol acetyl transferase) (Fournier *et al.*, 2006), which could mask any change in susceptibility to chloramphenicol conferred by lack of AdeRS. TetA, an MFS family efflux pump, which exports tetracycline (Fournier *et al.*, 2006) has also been identified in AYE and this could mask the effect of the *adeRS* deletion on tetracycline susceptibility. In contrast to previously published data (Magnet *et al.*, 2001; Marchand *et al.*, 2004), lack of AdeRS had no effect on susceptibility to erythromycin, or cefotaxime (Table 4.5b) (MICs of 512 μg/ml and 1024 μg/ml cefotaxime were recorded for both AYE and AYEΔ*adeRS*). Many β-lactamases are present in *A. baumannii*; those present in AYE include VEB-1, OXA-10, AmpC and OXA-69, which could mask any effect of the *adeRS* deletion on cefotaxime susceptibility (Fournier *et al.*, 2006).

The results of this study suggest that *adeRS* are involved in tolerance to chlorhexidine and benzalkonium chloride, since the MIC of these antiseptics decreased at least two-fold when *adeRS* were deleted. Rajamohan *et al.* (2010b) observed an 8-fold decrease in the MIC of chlorhexidine and a 4-fold decrease for benzalkonium chloride when *adeB* was deleted in a MDR clinical isolate. This suggests that the increased susceptibility of AYEΔ*adeRS* is likely to be due to down-regulation of *adeABC.* The susceptibility of AYE to the surface biocides and cetylpyridinium chloride did not increase when *adeRS* were deleted, suggesting that *adeRS*

are not involved in suseptibilty to these agents, or that other mechanisms can compensate for loss of *adeRS.* For example, *qac* genes have been identified in AYE and could retain tolerance against the quaternary ammonium compounds, TBQ and cetylpyridinium chloride (Fournier *et al.*, 2006).

Collaborators at the HPA obtained chlorhexidine MBC values of 64 µg/ml and 512 µg/ml for AYE, supporting the results of the present study. These suggest that the MBC of chlorhexidine is variable for AYE (the experimental parameters were identical each time) and that chlorhexidine may not be reliable for eradication of *A. baumannii* from the skin of infected patients. *A. baumannii* AYE is not inhibited by the working concentrations of chlorhexidine (2-4%) (Borer *et al.*, 2007; Soma *et al.*, 2012). This is of concern because chlorhexidine is a widely used antiseptic in hospitals for whole body washing and in hospital disinfectants (Fuangthong *et al.*, 2011) and it has been suggested that the tolerance of *A. baumannii* clinical isolates to chlorhexidine can increase after repeated exposure to sub-inhibitory concentrations (Kawamura-Sato *et al.*, 2008).

Rajamohan *et al.* (2009) reported that biofilm formation can increase in the presence of biocides (specific agents not reported). None of the surface biocides investigated induced biofilm formation. However, when biofilms were formed in the absence of selective pressure, these were significantly more tolerant of TBQ, Spor-Klenz and H_2O_2. Biofilms are known to be more tolerant of antimicrobials than planktonic cells (Maillard, 2007). Factors accounting for this increased tolerance include the presence of dormant cells within the biofilm, which are able to persist in concentrations that eradicate more peripheral cells and the presence of organic material around a biofilm, which can reduce the activity of biocides.

There have been several reports of H_2O_2 being used to eradicate Gram negative bacteria in the event of an outbreak. It is concerning that in these cases, concentrations less than the MBC (>20,000 ppm) for AYE in biofilm have been used. Hardy *et al.* (2007) reported the use of 280 ppm and Chmielarczyk *et al.* (2012) reported the use of up to 400 ppm of vapourised H_2O_2. A dry mist-generated H_2O_2 system contained 5% H_2O_2; this may be expected to kill AYE biofilms, although the final concentration on the hospital surfaces was not reported (Piskin *et al.*, 2011). Therefore, unless a high enough concentration of H_2O_2 is used, this agent may not be an effective biocide for removal of *A. baumannii.*

There was no difference in the concentration of norfloxacin accumulated by AYE and AYEΔ*adeRS*, despite the hypothesised down-regulation of AdeABC in the mutant. This could be due to up-regulation of other efflux pumps in AYEΔ*adeRS.* The significant increase in accumulation of norfloxacin for AYE and AYEΔ*adeRS* in the presence of CCCP indicates that efflux pumps that use the proton motive force are active in both strains. PAβN is an inhibitor of MexB and AcrB, RND efflux pumps in *P. aeruginosa* and *E. coli*, respectively (Lomovskaya *et al.*, 2001; Yu *et al.*, 2005) PAβN has been shown to result in increased accumulation of Hoechst 33342 in AYE (Richmond *et al.*, 2012). Furthermore, it has been shown to inhibit the *A. baumannii* RND efflux pump AdeFGH (Cortez-Cordova and Kumar, 2011). It is hypothesised that PAβN may also inhibit other RNA pumps in *A. baumannii* such as AdeABC. Based on this hypothesis, further investigation with PAβN suggested that AdeABC is not solely responsible for norfloxacin efflux because there was no difference in the concentration of norfloxacin accumulated by AYE or AYEΔ*adeRS* when PAβN was used. It is likely that other families of efflux pump can compensate for loss of RND pumps in AYE. Verapamil, which targets ABC efflux pumps, caused a significant increase in the

concentration of norfloxacin accumulated by AYEΔ*adeRS*, but not for AYE, suggesting that an ABC pump may be over-produced in AYEΔ*adeRS* (Figure 4.7). Therefore, loss of AdeABC in AYEΔ*adeRS* may be compensated by an upregulation of ABC efflux pumps.

Data from collaborators Wand and Sutton showed that virulence was significantly impaired in AYEΔ*adeRS* compared with AYE ($P = 0.0053$) (unpublished data). RND efflux pumps can confer innate resistance to host defence molecules such as bile (Piddock, 2006). In *Salmonella enterica* serovar Typhimurium, all components of the AcrAB-TolC efflux pump are required for efficient adhesion to and invasion of cells *in vitro* (Buckley et al., 2006). Also, loss of AcrB or TolC of the RND efflux pump AcrAB-TolC in *S. enterica* serovar Typhimurium resulted in decreased expression of genes involved in pathogenesis (Webber *et al.*, 2009). Therefore, AdeABC may enable AYE to survive more easily in *Galleria mellonella* and to establish a more effective infection. However, little is known about the pathogenicity of *A. baumannii*, including the role of efflux pumps such as AdeABC in the virulence of this bacterium (Wand *et al.*, 2012). AdeRS could regulate other genes, which contribute to virulence.

4.7. Further Work

RNA from AYE and AYEΔ*adeRS* was sent for sequencing (ARK-Genomics, Roslin Institute, Edinburgh) in June 2012 but the data have not yet been received. Analysis of the transcriptomes will confirm whether *adeABC* is down-regulated in AYEΔ*adeRS*. It will also indicate whether AdeRS is the only regulator of *adeABC* expression and confirm whether the phenotypic differences are likely to be at least partly due to decreased expression of this efflux pump. Analysis of the whole transcriptome is of further interest, to investigate whether AdeRS also regulates other genes, which could be involved in the increased

susceptibility to antibiotics and biocides. It would also be interesting to carry out RNA sequencing (RNAseq) for AYE and AYEΔ*adeRS* in the presence and absence of an antimicrobial to which AYEΔ*adeRS* was more susceptible, to investigate whether *adeRS* interact with different genes in the presence and absence of the agent and whether the antimicrobial is likely to be a stimulus for AdeRS.

Coyne *et al.* (2010a) showed that AdeABC was overexpressed in AYE compared with CIP 70-10, a non-MDR reference strain (expression of *adeA, adeB* and *adeC* in AYE was 2.84-, 2.80- and 2.28-fold higher, respectively). Had more time been available, a mutant that does not overexpress *adeABC* would have been constructed. To do this, Δ*adeRS* should be complemented with *adeRS* (*adeRS*^{S}) from an antibiotic susceptible strain. To determine which strain from which to clone *adeRS*^{S}, the sequences of the *adeS* genes in AYE and a susceptible *A. baumannii* clinical isolate, S1 (obtained from Network for Antimicrobial Resistance Surveillance, Singapore) were determined and compared. AYE had the same SNP responsible for the Ala94to Val substitution identified in *A. baumannii* isolate AB211, which overexpressed AdeABC (Hornsey *et al.*, 2010; Hornsey *et al.*, 2011). This SNP was not present in S1. No other reported *adeS* mutations were present in AYE or S1 (Table 1.2). Therefore, it was hypothesised that the Ala94 to Val substitution in *adeS* confers *adeABC* overexpression in AYE and that introduction of *adeS* from S1 into AYEΔ*adeS* would result in down-regulation of genes in the AdeRS regulon. RNAseq of this mutant would also indicate which genes are under control of AdeRS and also whether AdeR is a broad-spectrum or narrow-spectrum regulator. Prior to cloning, RT-PCR of *adeB,* (or analysis of RNAseq data if available) in both strains should be carried out to confirm that the expression level of *adeB* is lower for S1 and that *adeRS* from this strain is suitable for this further work.

The biofilm and biocide susceptibility assays could be amended to replicate hospital conditions more closely. In a hospital, there would be the presence of organic material, which can affect the efficacy of biocides (Kawamura-Sato *et al.*, 2008) and low nutrient availability. These conditions could be simulated by using minimal media and including bovine serum albumin in the biocide susceptibility assays. The susceptibility of biofilms to biocides can depend on the surface to which the biofilm is attached (Smith and Hunter, 2008). Therefore, growing the biofilm on common hospital surfaces such as stainless steel (used for surfaces and equipment) (Smith and Hunter, 2008) and Teflon and silicone (used to coat catheters) (Ostadi *et al.*, 2010) would also provide more insight into the susceptibility of AYE biofilms to biocides in the hospital environment.

Although there was no difference in growth of AYE and AYEΔ*adeRS in vitro*, in the absence of any stress, it would be interesting to investigate whether there is any difference in growth of AYE and AYEΔ*adeRS* in *G. mellonella.* Deletion of *adeRS* could reduce the ability of AYE to survive and cause infection in the presence of antimicrobial peptides and other stressors. This could be investigated by determining viable counts of bacteria extracted from *G. mellonella* after infection, to determine the ability of the two strains to replicate during infection.

4.8. Key Findings

- AdeRS is not essential for growth of AYE as a planktonic culture or as a biofilm *in vitro*
- AdeRS is not essential for efflux in AYE
- AdeRS is involved in antibiotic and biocide susceptibility
- AdeRS is required for full virulence in a *G. mellonella* model of infection

5. Role of *pmrAB* in Growth, Antimicrobial and Biocide Susceptibility and Accumulation

5.1. Background

pmrAB have been disrupted previously in ATCC 19606 (Beceiro *et al.*, 2011), ATCC 17978 (Arroyo *et al.*, 2011) and polymyxin-resistant clinical isolates (Arroyo *et al.*, 2011; Beceiro *et al.*, 2011) by inserting a kanamycin cassette into the gene; this resulted in increased susceptibility to polymyxin antibiotics. The involvement of *pmrAB* in growth, efflux and biocide susceptibility has not been reported to date. The role of *pmrAB* in virulence has not been directly investigated, but a colistin-resistant derivative of ATCC 19606 (Lopez-Rojas *et al.*, 2011) and a clinical isolate (Rolain *et al.*, 2011) were less virulent than their colistin-susceptible counterparts.

5.2. Aims and hypotheses

pmrAB are involved in outer membrane modification (Beceiro *et al.*, 2011). It was hypothesised that deletion of *pmrAB* would result in a difference in biocide and antibiotic susceptibility for AYEΔ*pmrAB* compared with AYE, since a change in the outer membrane is one of the contributing resistance mechanisms against these compounds (Nikaido, 1994; McDonnell and Russell, 1999). In particular, AYEΔ*pmrAB* was hypothesised to be more susceptible to polymyxin antibiotics than AYE, since PmrAB are involved in resistance to these agents (Adams *et al.*, 2009).

pmrAB have been reported to be involved in virulence in *Salmonella enterica* serovar Typhimurium (Tamayo *et al.*, 2005) and in *Pseudomonas aeruginosa,* PmrAB is activated by

antimicrobial peptides (McPhee *et al.*, 2003). In light of these publications, AYEΔ*pmrAB* was hypothesised to be less virulent than AYE in a *G. mellonella* model of infection.

The aims were to investigate whether *pmrAB* have a role in growth, antibiotic and biocide susceptibility, accumulation and virulence in AYE.

5.3. Effect of *pmrAB* Deletion on Growth and Antibiotic and Biocide Susceptibility *in vitro*.

There was no significant difference in generation time or final optical density at 600 nm between AYE and AYEΔ*pmrAB* grown in LB broth (Figure 5.1; Table 5.1).

There was no difference in susceptibility of AYE or AYEΔ*pmrAB* to any of the antibiotics or biocides tested including polymyxin B (Table 5.2; Table 5.3).

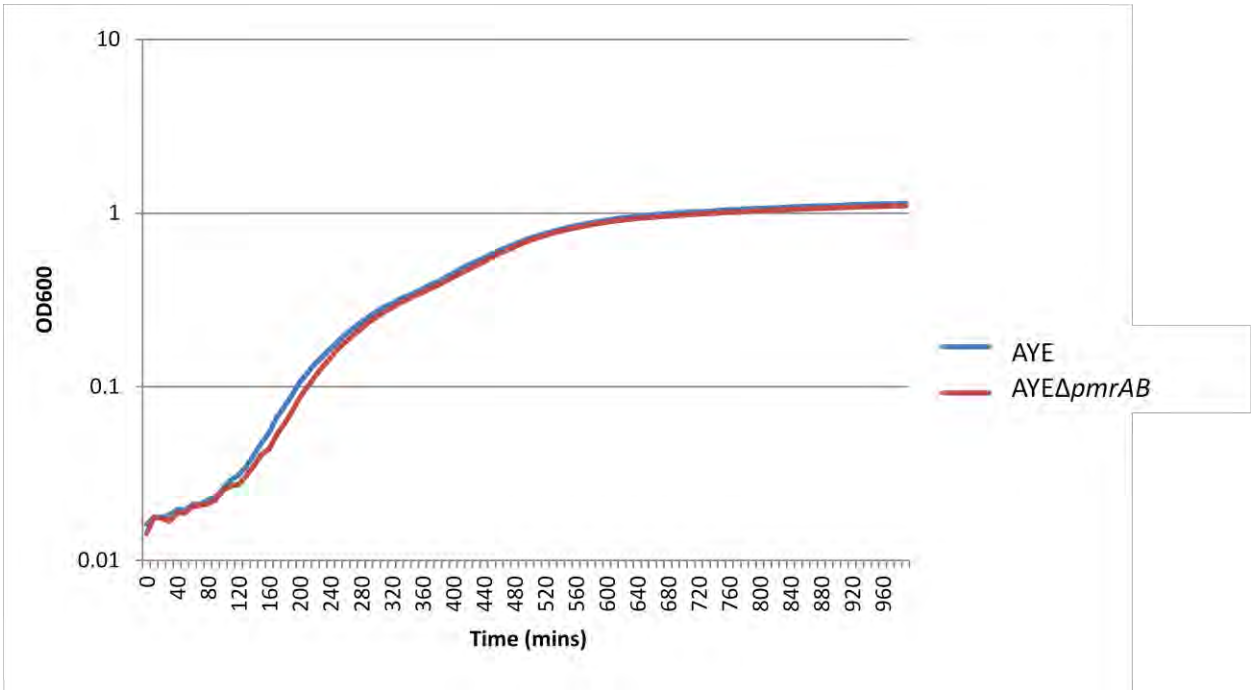
5.4. Effect of *pmrAB* Deletion on Accumulation of Dyes and Fluoroquinolones

There was no significant difference in accumulation of Hoechst 33342 between AYE and AYEΔ*pmrAB* (Figure 5.2).

As in the experiments in section 4.4, great variation was observed when ciprofloxacin was used to compare accumulation in AYE and AYEΔ*pmrAB* (Figure 5.3). The supernatant of AYEΔ*pmrAB* was scanned to ensure that there was not a molecule present in the supernatant, which could account for this variation (section 4.5). No fluorescence was measured for the supernatant of AYEΔ*pmrAB*.

There was a significant difference in the concentration of norfloxacin accumulated by AYE and AYEΔ*pmrAB* in the absence of efflux inhibitors, with a higher concentration accumulated in the mutant (53.96 ± 13.60 ng/mg dry cells vs. 73.43 ± 7.14 ng/mg dry cells) (Figure 5.4).

Figure 5.1 Growth of AYE and AYEΔ*pmrAB* in LB broth.



Data are the mean of three separate experiments, each carried out in duplicate. Data for AYE are the same as those presented in Figure 4.1**.**

Table 5.1 Mean Generation Times and Optical Density (± Standard Deviation).

Strain	Mean generation time (min)	T-test (*P*)	Mean OD_{600} at stationary phase	T-test (*P*)
AYE	163.76	---	1.19	---
AYEΔ*pmrAB*	163.50	0.995	1.11	0.45

Data are the mean of three separate experiments, each carried out in duplicate. The generation times were determined from the steepest part of the exponential curve. A two-tailed Student's *t* test was used to determine any significant difference ($P < 0.05$) between strains. Data for AYE are the same as those included in Table 4.1.

Table 5.2 Minimum inhibitory concentrations of antibiotics for AYE and AYEΔ*pmrAB*.

	CIP	AMK	GEN	KAN[a]	TOB	TIG	TET[a]	COL	PMX[a]	AMP[a]
AYE	**64**	**16[I]**	**256**	512	**16**	**1**	128	1	0.5	>1024
AYEΔ*pmrAB*	**64**	**16[I]**	**128**	1024	**16**	**1**	128	1	0.5	>1024

	CAZ[a]	CTX[a]	IMP	MER	CHL[a]	ERY[a]	AMP/SUL[a]	EtBr[a]
AYE	>1024	1024	0.38	1.5	256	128	8	512
AYEΔ*pmrAB*	>1024	512	0.5	1.5	256	128	8	512

CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; TIG, tigecycline; TET, tetracycline ; COL, colistin; PMX, polymyxin B; AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; IMP, imipenem; MER, meropenem; CHL, chloramphenicol; ERY, erythromycin; AMP/SUL, ampicillin/sulbactam combination; EtBr, ethidium bromide.

The minimum inhibitory concentration (MIC) values are the mode value of at least three agar-dilution experiments, each carried out in triplicate. The MIC of IMP and MER were determined three times by E-test.
MICs which correspond to resistance ([I] intermediate resistance) to that agent are in bold ([a]breakpoint concentration not available). Clinically relevant antibiotics (used to treat *Acinetobacter* infection) are highlighted in yellow.
Data for AYE are the same as those in Table 4.2

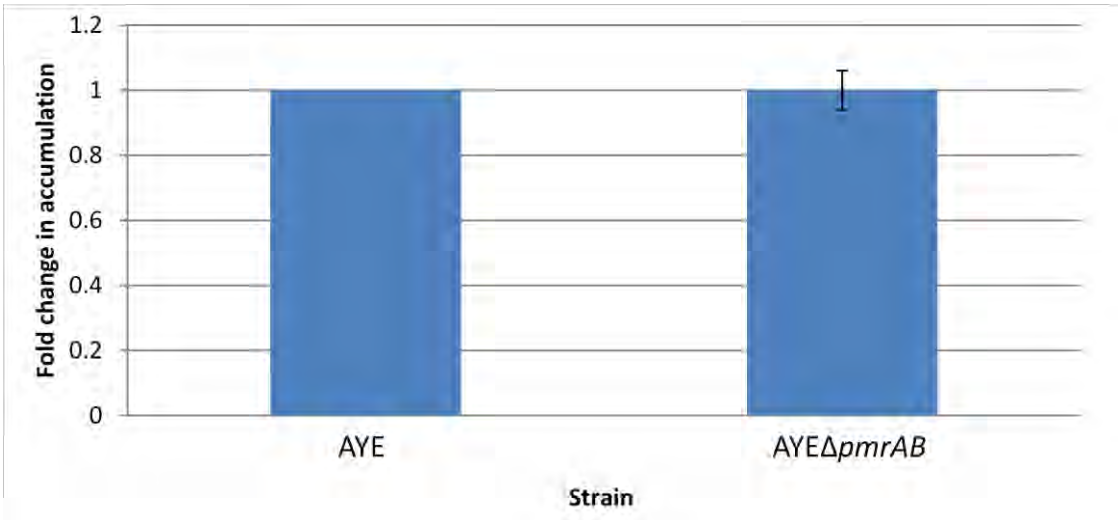
Table 5.3 Minimum Inhibitory and Bactericidal Concentrations of Biocides for AYE and AYEΔ*pmrAB*.

	Peracetic acid %		TBQ %		Hydrogen peroxide %		SporKlenz %		Ethanol %	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
AYE	0.08	0.08/0.16	0.375	0.75	0.03/0.06	0.03/0.06	3.125	3.125/6.25	3.125/6.25	25
Δ*pmrAB*	0.08	0.08	0.375	0.75	0.03/0.06	0.03/0.06	3.125	3.125	3.125/6.25	25

Minimum inhibitory concentrations and minimum bactericidal concentrations were each determined in duplicate on two separate occasions.

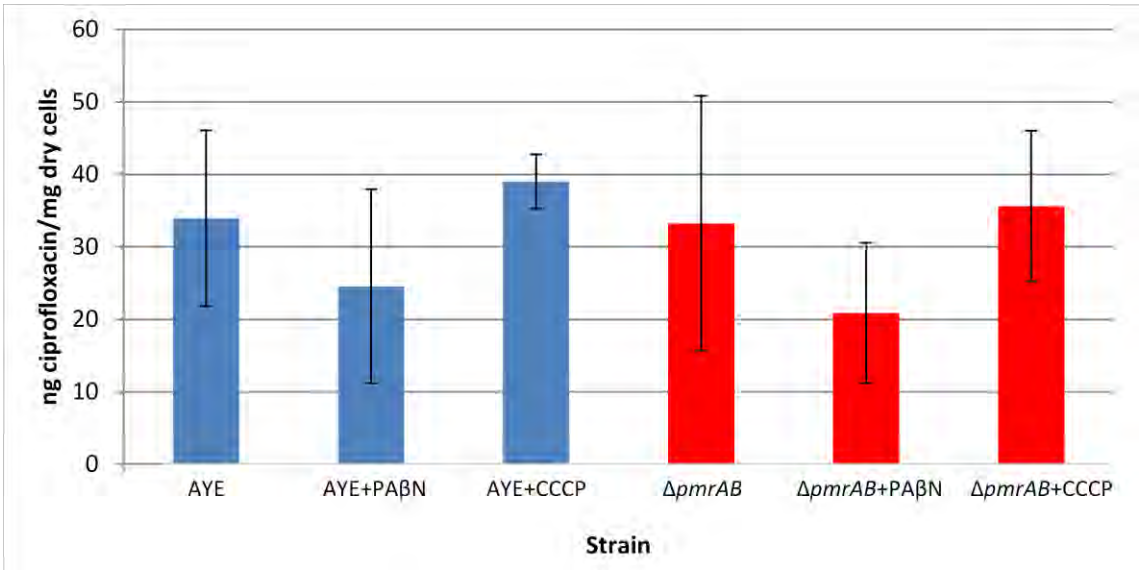
Data for AYE are the same as those in Table 4.3.

Figure 5.2 Effect of *pmrAB* Deletion on the Accumulation of Hoechst H33342 (Bis-benzimide).



Data are the mean of three separate experiments, each carried out in duplicate. The steady state accumulation for AYEΔ*pmrAB* is presented as fold change compared with the steady state value for AYE ± standard deviation. Student's *t* test on fold change values returned a *P* value of 0.98. Data for AYE are the same as those in Figure 4.3.

Figure 5.3 Accumulation of Ciprofloxacin in AYE and AYEΔ*pmrAB* at Five Minutes in the Presence and Absence of Efflux Inhibitors.



PAβN was added to a final concentration of 50 µg/ml; CCCP was added to a final concentration of 50 µM. The results are all data from two experiments, each carried out in duplicate. Data for AYE are the same as those in Figure 4.4.

To investigate whether efflux pumps were active in the two strains, CCCP was used. In the presence of CCCP, there was a significant difference in the concentration of norfloxacin accumulated by both AYE and AYEΔ*pmrAB*. In the presence of PAβN, there was no difference in the concentration of norfloxacin accumulated for either strain. In the presence of verapamil, there was a significant increase in norfloxacin accumulation for AYEΔ*pmrAB*, but not for AYE (Figure 5.4).

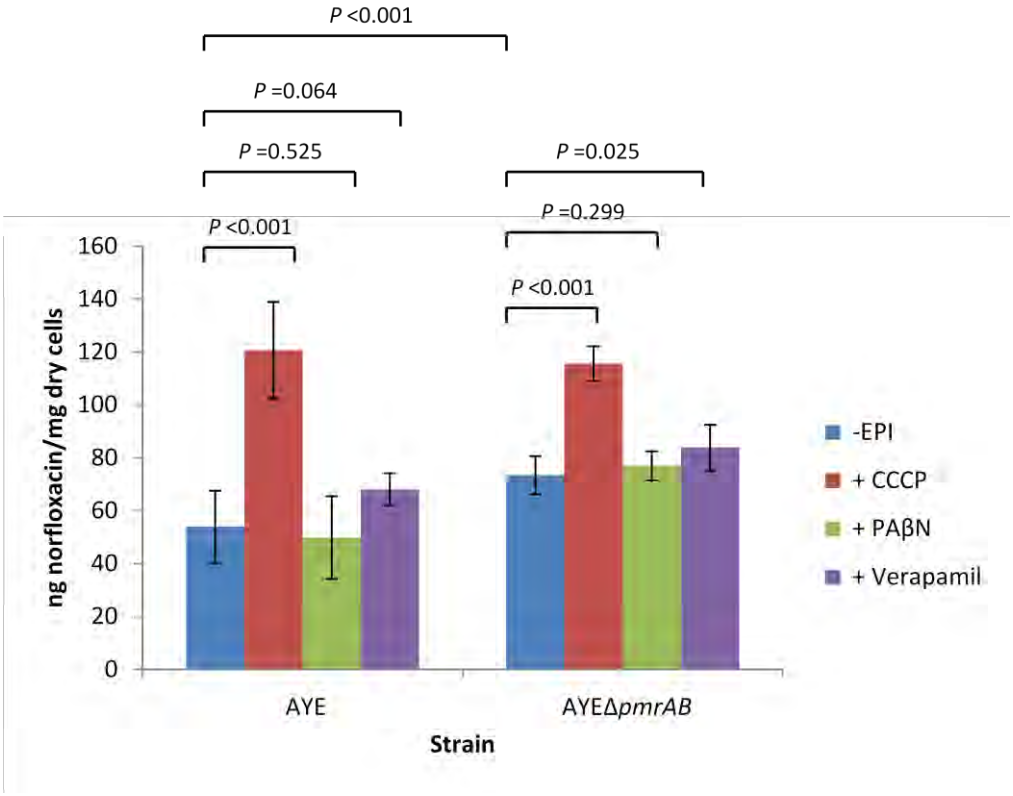
The concentration of efflux inhibitors and norfloxacin used in the norfloxacin uptake assay were confirmed to have no effect on growth of AYE and AYEΔ*pmrAB.* Comparison of viable count at time 0 min with the same strain after addition of norfloxacin, both in the presence and absence of efflux inhibitor generated a *P* value of greater than 0.05 (Figure 5.5).

5.5. Discussion

There was no difference in growth of AYE and AYEΔ*pmrAB* suggesting that *pmrAB* are not essential for the growth of AYE.

In AYE, *pmrAB* were also not involved in susceptibility to any of the antibiotics and biocides tested. PmrAB has been implicated in resistance to polymyxin B and colistin and disruption of *pmrAB* in colistin or polymyxin B-resistant strains and clinical isolates has been shown to cause loss of resistance to these agents (Arroyo *et al.*, 2011; Beceiro *et al.*, 2011). Therefore, it was hypothesised that AYEΔ*pmrAB* would be more susceptible to the polymyxins. Arroyo *et al.* (2011) reported that when *pmrAB* were disrupted in polymyxin-resistant derivatives of ATCC 17978, the MIC of the antibiotic decreased by 8-16-fold. However, there was no significant change in MIC of polymyxin B for wild-type ATCC 17978, which is sensitive to

Figure 5.4 Accumulation of Norfloxacin in AYE and AYEΔ*pmrAB* at Five Minutes in the Presence and Absence of Efflux Inhibitors.

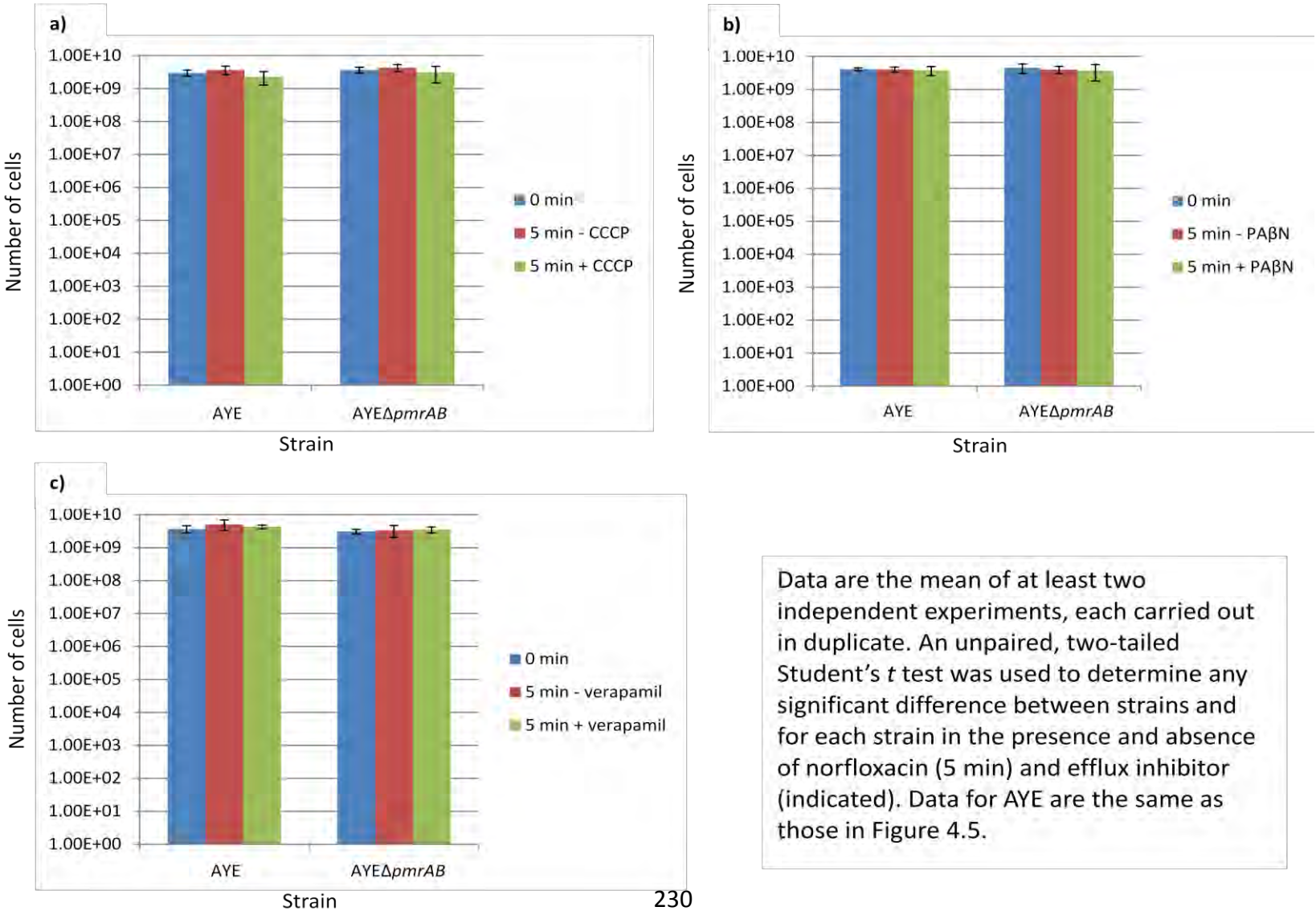


PAβN and verapamil were added to a final concentration of 50 µg/ml; CCCP was added to a final concentration of 50 µM.

A two-tailed Student's *t* test was carried out to compare the accumulation of norfloxacin in presence of efflux inhibitor with accumulation in the same strain in the absence of efflux inhibitor. A significant difference is one which generated a *P* value of <0.05.

Data for AYE are the same as those in Figure 4.5.

Figure 5.5 Viable Count of AYE and AYEΔ*pmrAB* in the Presence and Absence of Efflux Inhibitors.



Data are the mean of at least two independent experiments, each carried out in duplicate. An unpaired, two-tailed Student's *t* test was used to determine any significant difference between strains and for each strain in the presence and absence of norfloxacin (5 min) and efflux inhibitor (indicated). Data for AYE are the same as those in Figure 4.5.

polymyxin B. This result suggests that *pmrAB* can mediate resistance to polymyxins, but that it contributes little to the intrinsic level of resistance in ATCC 17978. These data support the results obtained for AYE. AYE is susceptible to colistin and polymyxin B, which could account for the lack of MIC change when *pmrAB* were deleted.

By contrast, Beceiro *et al.* (2011) did observe increased susceptibility to colistin in both resistant (MIC 32 μg/ml compared with 0.19 μg/ml) and susceptible derivatives (MIC 1 μg/ml compared with 0.125 μg/ml) of ATCC 19606 when *pmrAB* were disrupted. The results of this study may suggest that *pmrAB* are not expressed in wild-type AYE, but that they are expressed in wild-type ATCC 19606.

Other factors, which could compensate for loss of *pmrAB* in AYE are differences in the outer membrane of AYE and ATCC 19606. Soon *et al.* (2012; 2011a; 2011b) investigated the surface hydrophobicity, net charge and rigidity of the outer membrane of colistin-resistant and -susceptible *A. baumannii*. Differences in cell membrane, such as a more hydrophobic cell surface, a less rigid outer membrane and a more positively charged cell surface were identified in resistant bacteria in these studies. Differences in the capsules of the two strains could also provide AYE with more protection than ATCC 19606; increased production of capsule polysaccharide has been implicated in polymyxin B resistance in *K. pneumoniae* (Campos et al., 2004). The MIC of colistin for both AYE in this study and ATCC 19606 (Beceiro *et al.*, 2011) was 1 μg/ml. However, the results of this study suggest that the intrinsic protective mechanisms against colistin differ between the strains, with *pmrAB* being important in ATCC 19606, but not in AYE. Complete loss of LPS production has been observed in colistin-resistant ATCC 19606 derivatives, but there are no published reports of this phenomenon in other strains of *A. baumannii* to date. The outer membrane of ATCC

19606 may have a higher affinity for the polymyxins than other strains, which would account for PmrAB having a more important role in this strain than in AYE and ATCC 17978, and explain why loss of LPS is a more common resistance mechanism in ATCC 19606 than in others.

The increased concentration of norfloxacin accumulated in AYEΔ*pmrAB* compared with AYE suggests that PmrAB is important for the level of accumulation of some antimicrobials in AYE. However, there was no difference in the level of Hoechst 33342 accumulation, so this conclusion cannot be drawn for all agents. PmrAB could be involved in the regulation of efflux pumps, and the deletion of *pmrAB* may result in fewer active efflux pumps. The increased activity of ABC efflux pumps (there was increased norfloxacin accumulation in the presence of verapamil) could be a compensatory mechanism.

Alternatively, the deletion of *pmrAB* may have resulted in an alteration in the cell outer membrane, allowing more norfloxacin into the cell, with no change in the level of efflux. It has been reported that PmrAB is involved in maintaining outer membrane integrity in *Citrobacter rodentium* and *Salmonella enterica* serovar Typhimurium (Viau *et al.*, 2011; Murata *et al.*, 2007). There was no difference in accumulation of the membrane-permeable molecule, Hoechst 33342, which fluoresces when in the lipid membrane (Richmond *et al.*, 2012), which could indicate that there was no difference in the hydrophobicity of the outer membrane of AYEΔ*pmrAB.* However, there was a difference in the concentration of norfloxacin accumulated. Norfloxacin enters the bacterial cell through porins suggesting that the porin composition of the outer membrane could have changed (Hirai *et al.*, 1986). However, the reason for this result may be that norfloxacin is a better indicator of overall

accumulation in AYE. The highly significant (P <0.001) difference in norfloxacin accumulation in the presence of CCCP confirms that efflux pumps are active in both strains.

Data from collaborators Wand and Sutton showed that compared with AYE, the ability of AYEΔ*pmrAB* to kill *G. mellonella* was significantly impaired (P = 0.0007) (unpublished data). The *Galleria mellonella* haemolymph is analogous to the blood in mammals and is important in the clearance of microbial infections. Antimicrobial peptides are released into the haemolymph and these target bacterial cell walls (Kavanagh and Reeves, 2004). The TCS PmrAB has been implicated in mediating antimicrobial peptide resistance and increased virulence in *Salmonella enterica* serovar Typhimurium due to an altered LPS structure (Tamayo *et al.*, 2005). In *Pseudomonas aeruginosa*, PmrAB is activated by antimicrobial peptides and regulates resistance to these proteins (McPhee *et al.*, 2003). However, Arroyo *et al.* (2011) reported that the deletion of *pmrAB* in polymyxin B- resistant mutants of *A. baumannii* ATCC 17978 resulted in an increased susceptibility to polymyxin B, but not to antimicrobial peptides. It is also possible that PmrAB regulates virulence genes required for infection of *G. mellonella* in AYE, rather than the decreased virulence being a result of altered LPS.

5.6. Further Work

RNAseq would provide more information about the genes that PmrAB regulates, and possibly begin to address the question of why more norfloxacin accumulated in AYEΔ*pmrAB* than in AYE. A comparison of the LPS and outer membrane protein profiles of AYE and AYEΔ*pmrAB* could also be carried out to investigate whether the outer membrane is any different between the two strains. Had more time been available, the experiments carried

out to investigate the role of *adeRS* in biofilm formation and biocide susceptibility (Section 4.3) would have been carried out for AYEΔ*pmrAB*.

As for AYEΔ*adeRS* (Section 4.7), it would be interesting to investigate whether there is any difference in growth of AYE and AYEΔ*pmrAB* in *G. mellonella.* Although there was no difference in growth of AYE and AYEΔ*pmrAB in vitro*, deletion of *pmrAB* could reduce the ability of AYE to survive and cause infection in the presence of antimicrobial peptides and other stressors.

5.7. Key Findings

- *pmrAB* are not involved in growth *in vitro*
- *pmrAB* are not involved in susceptibility to any of the antibiotics tested in this study
- *pmrAB* are involved in the level of accumulation of some compounds, but not others
- *pmrAB* are required for full virulence in a *G. mellonella* model of infection

6. Overall Discussion, Key Findings and Conclusions

6.1. Overall Discussion

A. baumannii are often resistant to the antibiotics commonly used to select bacteria during genetic manipulation experiments. A method, which used a tellurite resistance cassette to overcome this problem, was used to delete the genes, *adeRS* and *pmrAB* in *A. baumannii* AYE.

AYE, AYEΔ*adeRS* and AYEΔ*pmrAB* were all able to accept pMo130-Tel^R deletion constructs by conjugation and recombine them into their chromosome. By contrast, none of the strains were able to recombine the complementation constructs. pMo130-TelR/*bfmRS* could not be introduced into AYE, suggesting that there may be regions of the chromosome, which are difficult to introduce new DNA into. However, since the same UP and DOWN fragments were used, this does not explain why the complementation construct could not recombine in the same region as the deletion construct used to delete the genes initially. Sequencing of the UP and DOWN regions in the deletion mutants suggested that the regions were sufficiently conserved after gene deletion for recombination to occur between the chromosome and complementation construct (Figure 3.13; Figure 3.27). It is not known whether the complementation constructs and *bfmRS* deletion construct were accepted by AYE during conjugation, since pMo130-Tel^R is a suicide vector and is lost if recombination does not occur. Therefore, the limiting factor of the method could be either the conjugation or recombination step.

It was important to complement AYEΔ*adeRS* and AYEΔ*pmrAB* to confirm that the increased susceptibility of AYEΔ*adeRS* and increased efflux in AYEΔ*pmrAB* were a consequence of the

adeRS and *pmrAB* gene deletions respectively. However, in this research, these mutants could not be complemented by introducing the deleted region directly back onto the chromosome. This method was chosen as some of the response regulator and a downstream gene were deleted in each case (Figure 3.15; Figure 3.31) and it was unknown, when designing the complementation strategy, whether both components of the two component system were needed to complement, or whether only the histidine kinase was required. *adeR* transcripts were detected in both AYE and AYEΔ*adeRS* (as shown in RNAsequencing; Richmond and Piddock, unpublished). However, it was hypothesised that AdeR would not be active in AYEΔ*adeRS* as 126 bp of *adeR* (C-terminus, DNA binding domain; Figure 3.15) was deleted. If this is true, both *adeR* and *adeRS* would be required to complement. However, it was unknown whether the transcript was degraded after translation (Sabate *et al.*, 2010), or whether a truncated protein remained in the cell. If both *adeR* and *adeS* were introduced on a plasmid, a truncated AdeR protein could compete with the normal AdeR for interaction with AdeRS, since the N-terminal receiver domain was unaffected. As only 12 bp of *pmrA* (C-terminus, DNA binding domain, Figure 3.31) was deleted, this gene may have given rise to a functional protein, but again, it was not certain. Ideally, only *adeS* (or all of *adeRS*) and *pmrB* (or all of *pmrAB*) would have been deleted and only *adeS* (or *adeRS*) and *pmrB* (or *pmrAB*) complemented, respectively.

Time did not permit investigation into whether AdeR and PmrAB were produced and functional. Therefore, complementation on the chromosome was considered to be the best strategy and this was pursued for as long as possible. However, a disadvantage of this method was that the genome could have been disrupted as a result of a second genetic manipulation. Had the complemented strains been constructed, the upstream and

downstream regions and the target gene would have been sequenced to ensure that the region was restored without any errors. If more time were available, a better strategy would have been to re-make the mutants, to ensure that *adeRS* and *pmrAB* were deleted without disrupting any flanking genes. To do this, the primers would have been designed to bind inside the genes to be deleted. This would have deleted an internal region of the gene(s), rather than the whole gene(s), but would have ensured that flanking genes were undisrupted. The initial strategy was to delete the whole of *adeS*, but it was not noticed until after the genes were deleted that the location of the primers meant that flanking genes would also be disrupted. However, since the main aim of the research was to investigate the role of TCSs in adaptive responses, disruption of the response regulator did not mean that this aim was not addressed.

AdeRS were not essential for growth of AYE planktonic or biofilm cells in the absence of antimicrobials. However, in the presence of chlorhexidine and benzalkonium chloride, it played a role in growth and susceptibility.

AdeRS are essential for clinical resistance to tigecycline and tobramycin, increased resistance to gentamicin and increased tolerance to chlorhexidine and benzalkonium chloride, suggesting that other efflux pumps cannot completely compensate for loss of AdeABC. AdeRS were also involved in a small difference in susceptibility to kanamycin and amikacin. The involvement of *adeRS* in resistance to carbapenems and polymyxins could not be elucidated, since AYE is susceptible to these compounds. The interpretation of the results obtained for AYEΔ*adeRS* relied upon the assumption that there are no other regulatory mechanisms of *adeABC* transcription and that *adeABC* is down-regulated in AYEΔ*adeRS*.

Biofilm formation protected both AYE and AYEΔ*adeRS* cells against some biocides, but there was no difference in the protective effect of biofilm between the two strains. This may be because *adeRS* had no effect on biofilm formation and there was no difference in susceptibility of the biocides to which cells in biofilm were exposed.

Data suggested efflux via ABC efflux pumps in AYEΔ*adeRS* and AYEΔ*pmrAB* (Figure 4.7 and Figure 5.4, respectively). ABC efflux pumps may compensate for down-regulation of other classes of efflux pump such as the RND pump, AdeABC. More work (for example analysis of the outer membrane protein or LPS profiles of AYEΔ*pmrAB*) must be carried out before conclusions can be drawn about the role of PmrAB in antimicrobial accumulation. However, *pmrAB* may be involved in the regulation of efflux pumps. This is because the concentration of norfloxacin accumulated was higher for AYEΔ*pmrAB* (73.43 ng nor/mg dry cells) than for AYE (53.96 ng nor/mg dry cells) in the absence of efflux inhibitors (Figure 5.4). PmrAB did not appear important in AYE for susceptibility to the antibiotics tested, although in some strains such as ATCC 19606, it may have a more important role in intrinsic polymyxin resistance (Beceiro *et al.*, 2011). This suggests that any efflux pumps that may be regulated by *pmrAB* are not required for antibiotic resistance and that the role of *pmrAB* in antibiotic susceptibility varies between strains.

Both AdeRS and PmrAB are required for wild-type levels of virulence, although they are not essential for growth *in vitro.* However, it is possible that they do play a role in growth during infection in *G. mellonella*, since this was not investigated in this study. It is also possible that *adeRS* and *pmrAB* regulate genes required for virulence in *G. mellonella.*

Analysis of the AYE, AYEΔ*adeRS*, AYEΔ*adeRS*/*adeRS*^{S} (Section 4.7) and AYEΔ*pmrAB* transcriptomes is valuable further work, which would indicate which genes are regulated by

AdeRS and PmrAB and begin to answer the further questions raised by the results of this study.

6.2. Overall Key Findings

In AYE:

- *adeRS* and *pmrAB* were deleted, but could not be complemented; *bfmRS* could not be deleted

- In the absence of antimicrobials, neither AdeRS and PmrAB were essential for growth of planktonic cells and AdeRS was not required for biofilm formation

- AdeRS played a role in antibiotic and biocide susceptibility whilst PmrAB was not involved in susceptibility to any of the antibiotics tested. Cells in biofilm were more tolerant of biocides than their planktonic counterparts

- Efflux pumps were active in all strains. There may be increased activity of ABC efflux pumps in AYEΔ*adeRS* and AYEΔ*pmrAB*.

- AdeRS and PmrAB are required for full virulence

6.3. Overall Conclusions

The TCSs AdeRS and PmrAB are important in AYE, since both are required for full virulence. AdeRS appears to have a more important role than PmrAB in antimicrobial susceptibility.

7. Appendices

Appendix 1. Buffer and Agar Constituents.

Glycine Hydrochloride Buffer (0.1 M, pH 3.0)

Glycine (Sigma-Aldrich, UK, 50046) (3.75 g) was dissolved in dH_2O (400 ml) and the solution was adjusted to pH 3.0 with HCl. dH_2O was added to a final volume of 500 ml. The buffer was sterilised by autoclaving.

Sodium Phosphate Buffer (50mM, pH 7.0)

$Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich, UK, 71643) (2.67 g) was dissolved in dH_2O (300 ml) to produce an alkaline solution. $NaH_2PO_4 \cdot 2H_2O$ (Sigma-Aldrich, UK, 71505) (2.34 g) was dissolved in dH_2O (300 ml) to produce an acidic solution. The acidic solution was added to the alkaline solution until pH 7.0 was achieved. The buffer was filter sterilised (Millipore Corporation; SCHVU05RE).

High Magnesium Agar (MgLB)

Tryptone, 10 g/L; yeast extract, 5g/L, NaCl, 5 g/L; 1mM $MgCl_2$, agar, 15 g/L.

Appendix 2. pMo130-Tel^{R} Sequence, Primers and Restriction Sites.

gacgaaagggcctcgtgatacgcctatttttataggttaatgtcatgataataatggtttcttagacgtcaggtg
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tctgctcccggcatccgcttacagacaagctgtgaccgtctccgggagctgcatgtgtcagaggttttcaccgtc
atcaccgaaacgcgcga

*Not*I *Bam*HI *Sph*I *Nsi*I

Primer sequences

pMo130-Tel^{R}FW
ccatctacttcttcgaccc

pMo130-Tel^{R}RV
tcacagcttgtctgtaagcg

Appendix 3. Spreadsheet used to Calculate ng Ciprofloxacin/mg Dry Cells.

	A	B	C	D	E	F	G	H
1		DATE =	C1					
2		ORGANISM =	I113					
3		ANTIBIOTIC =	C3					
4								
5		INPUT OD 660 =	C5	<-- cells harvested				
6		CONCENTRATION FACTOR =	=20/C5	<-- the culture was concentrated to 20 units				
7		INPUT DRY WEIGHT (mg/ml) =	C7	<-- for OD in C7				
8		CONVERSION FACTOR =	=C6*C7	<-- the weight (mg/ml) of dry cells in the concentrated culture (OD 20 units)				
9								
10		Time (min)	concentration (µg/ml)	Dilution factor (µg/ml)	ng/ml	ng/mg	ng/mg corrected	
11								
12	minus CCCP	0	C12	=C12*100	=D12*1000	=E12/C8	=F12-F12	
13	minus CCCP	5	C13	=C13*100	=D13*1000	=E13/C8	=F13-F12	
14	minus CCCP	5	C14	=C14*100	=D14*1000	=E14/C8	=F14-F12	
15								
16	and CCCP	0	C16	=C16*100	=D16*1000	=E16/C8	=F16-F16	
17	and CCCP	5	C17	=C17*100	=D17*1000	=E17/C8	=F17-F16	
18	and CCCP	5	C18	=C18*100	=D18*1000	=E18/C8	=F18-F16	
19								

Data was entered into the grey cells.

The calculation in column F converts the mass (ng) of fluoroquinolone taken up per milliliter of culture (column E) to the mass of fluoroquinolone taken up per milligram of dry cells. The value obtained in column F was used to analyse differences in fluoroquinolone accumulation.

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