CHAPTER 6

DEVELOPMENT OF A HIGH-THROUGHPUT CLONING STRATEGY FOR CHARACTERISATION OF *ACINETOBACTER BAUMANNII* DRUG TRANSPORTER PROTEINS

6.1 Introduction

It is common to determine the function of a protein in a recombinant host, such as *E. coli*. Additionally, heterologous expression in *E. coli* is widely used where a large amount of pure protein is required for functional and structural characterisation, which may not be available from the native host. A common problem related to overexpression of recombinant membrane proteins, such as drug transporters (Section 1.3.4), is compromised membrane integrity, which could lead to toxicity of the overexpressed protein to the host cell (Montigny *et al.* 2004; Noirclerc-Savoye *et al.* 2003). Many different expression systems have been developed throughout the years (Ward *et al.* 2001). However, there does not appear to be an expression vector or *E. coli* host that is suitable for all membrane proteins. Therefore, the optimal expression level of a given transport protein, typically requires a number of different expression systems to be tested, which is a time-consuming process.

Several different resistance mechanisms have been studied in *A. baumannii* (Gordon and Wareham 2010) of which active efflux is a known contributor to the development of multidrug resistant *A. baumannii* (Section 1.3.4). Many different *Acinetobacter* efflux systems have been identified (www.membranetransport.org), however, only a fraction of these have been functionally characterised (Section 1.3.4). Gateway technology (Invitrogen) is suitable for high-throughput examination of proteins, as the gene of interest can be shuffled between different expression vectors without the need for cloning. This methodology is very time-efficient when optimising protein expression levels. The Gateway features facilitating this include; *att*R1, *att*R2, *cat* and *ccdB*, and affinity tags, such as His_6 and the V5-epitope. The *att*R sites are the sequences that undergo recombination during the LR-reaction, in which the *att*L sites from the entry clone recombine with the *att*R sites from the destination clone, generating an expression clone. During this process, the gene of interest is transferred from the entry clone to the destination clone. The product of the *ccdB* gene initially located between the *att*R1 and *att*R2 sites is lethal to most *E. coli* strains, therefore, the *ccdB* Survival *E. coli* strain (Invitrogen) needs to be used for propagation of destination clones. Additional negative selection can be achieved by incubation with chloramphenicol, as a *cat* gene is also present between the *att*R sites. The two affinity tags and a stop codon will be introduced in-frame when the gene of interest is being transferred from the entry clone to the destination clone.

Here, the presence of putative drug efflux systems was investigated by PCR in 54 clinical *A. baumannii* isolates. A high-throughput Gateway-based cloning strategy suitable for heterologous expression of *A. baumannii* efflux systems in *E. coli* was developed and expression vectors were tested with transport proteins from different families and used for the functional characterisation of five putative efflux proteins. The cloning strategy was extended to generate an *A. baumannii* knockout system, and was shown to be successful for the disruption of a gene encoding a MATE family protein.

6.2 Results and Discussion

6.2.1 The prevalence of drug transporters in clinical *A. baumannii* isolates

The *A. baumannii* ATCC 17978 genome contains 52 genes encoding putative drug transporters, which was determined using TransAAP (Section 2.7.2; Table 6.1). The presence of these 52 putative transporters was examined in 54 clinical *A. baumannii* isolates, of which the clonal relationship has been determined (Section 4.2.1; Table 2.2). The oligonucleotides for PCR detection were designed to homologous regions of the genes encoding these predicted drug transporters. The homology was determined by genome alignments of fully sequenced *A. baumannii* strains (Section 2.4.14). The clinical strains were randomly selected from different parts of Australia and displayed major variation regarding their clonal relationship and phenotypic characteristics (Section 4.2).

Resistance markers, such as efflux systems, can often be found on mobile genetic elements, such as plasmids and transposons. Indeed, the AbaR-like resistance islands of *A. baumannii* contain many genes functioning in resistance (Section 1.4.1). However, most transporters found in *A. baumannii* strain ATCC 17978 were also identified in the clinical isolates (Table 2.2). Therefore, the majority of genes encoding drug transporters appear to be part of the *A. baumannii* core genome, corroborating previous data (Adams *et al.* 2008). Out of the 52 putative transporters, 23 were found in all strains and an additional 25 were found in more than 50% of the clinical isolates. Two genes found only in strain ATCC 17978, A1S_2562 (*abeM2*; Section 6.2.4) and A1S_2561, were located in a siderophore biosynthesis cluster which appears to have been acquired by horizontal gene transfer (Section 3.2.6). Analysis using Mauve (Section 2.7.2) showed that a TetA-like transporter

Table 6.1: Characteristics and prevalence of the *A. baumannii* ATCC 17978 drug transporters

Family [a]	Locus-tag	IC I / IC II / non IC (total) [b]	Substrates	Reference
ABC	A1S_1242	8/33/13 (54)		
	A1S_1535	8/33/13 (54)		
	A1S_1555	8/33/13 (54)		
	A1S_1671	0/32/12 (44)		
	A1S_1672	0/32/12 (44)		
	A1S_1673	0/32/11 (43)		
	A1S_2311	8/33/13 (54)		
	A1S_2312	8/33/13 (54)		
	A1S_2375	8/33/12 (53)		
	A1S_2376/7/8	8/33/12 (53)		
	A1S_2561	0/0/1 (1)		
	A1S_2621	8/33/13 (54)		
	A1S_2622	8/33/13 (54)		
SMR	A1S_2502	8/33/13 (54)		
	A1S_0710	8/33/13 (54)		
	A1S_2298 (*abeS*)	8/33/10 (51)	Erythromycin, novobiocin, deoxycholate, SDS, acriflavin, benzalkonium, ethidium	(Srinivasan *et al.* 2009)
	A1S_2844	8/33/13 (54)		
MATE	A1S_0395 (*abeM*) [c]	8/33/13 (54)	Norfloxacin, ciprofloxacin, DAPI, acriflavine, hoechst 33342, daunorubicin, doxorubicin, ethidium, chlorhexidine, trimethoprim	This study; (Su *et al.* 2005)
	A1S_2562 (*abeM2*) [c]	0/0/1 (1)	Ciprofloxacin	This study
	A1S_3371 (*abeM3*) [c]	8/33/13 (54)	nd [d]	This study
	A1S_3420 (*abeM4*) [c]	8/33/12 (53)	Ciprofloxacin	This study

Family [a]	**Locus-tag**	**IC I / IC II / non IC** (total) [b]	**Substrates**	**Reference**
MFS	A1S_0188 (*aedA*)	8/33/12 (53)	nd [d]	(Hassan *et al.* 2011)
	A1S_0563	8/33/13 (54)		
	A1S_0596	8/30/13 (51)		
	A1S_0775 (*aedB*)	8/30/12 (50)	nd [d]	(Hassan *et al.* 2011)
	A1S_0801/2	7/33/13 (53)		
	A1S_0909 (*aedC*)	8/33/13 (54)	Chloramphenicol, tetracycline	(Hassan *et al.* 2011)
	A1S_0964	8/33/11 (52)		
	A1S_1231	8/33/12 (53)		
	A1S_1418	8/33/13 (54)		
	A1S_1772 (*aedE*)	8/33/12 (53)	nd [d]	(Hassan *et al.* 2011)
	A1S_1799	8/32/12 (52)		
	A1S_2057 (*aedF*/*amvA*)	8/33/13 (54)	Ethidium, DAPI, chlorhexidine, acriflavine, methyl viologen	(Hassan *et al.* 2011; Rajamohan *et al.* 2010)
	A1S_2124	8/33/12 (53)		
	A1S_2198	8/33/12 (53)		
	A1S_2474	8/33/13 (54)		
	A1S_2584	8/33/12 (53)		
	A1S_2795	8/33/13 (54)		
	A1S_3117	8/1/8 (17)		
	A1S_3146 (*craA*)	8/33/12 (53)	Chloramphenicol, tetracycline	(Roca *et al.* 2009)
	A1S_3272	8/31/13 (52)		
	A1S_3288	8/33/13 (54)		
	A1S_3440	8/33/13 (54)		
	A1S_0116	8/8/7 (23)		
	A1S_0519	0/31/5 (36)		
	A1S_1649 (*aedD*)	8/33/12 (53)	nd [d]	(Hassan *et al.* 2011)

Family [a]	Locus-tag	IC I / IC II / non IC (total) [b]	Substrates	Reference
RND	A1S_1750 (*adeB*)	8/33/11 (52)	Gentamicin, cefotaxime, erythromycin, tetracycline, chloramphenicol, trimethoprim, norfloxacin	(Magnet *et al.* 2001)
	A1S_2305 (*adeG*)	8/33/13 (54)	Chloramphenicol, norfloxacin, sulfamethoxazole, tetracycline	(Coyne *et al.* 2010b)
	A1S_2660	8/33/13 (54)		
	A1S_2736 (*adeJ*)	4/21/12 (37)	Ticarcillin, cefotaxime, tetracycline, clindamycin	(Damier-Piolle *et al.* 2008)
	A1S_2818	8/33/13 (54)		
	A1S_3445/6 (*adeM*) [c]	7/31/13 (51)	Norfloxacin, chlorhexidine	This study

[a] Families of drug transporters; ATP-binding cassette superfamily (ABC), small multidrug resistance proteins (SMR), multidrug and toxic compound extrusion proteins (MATE), major facilitator superfamily (MFS) and resistance-nodulation cell division family (RND).

[b] Transporter of interest identified in number of; international clone I (IC I) isolates / international clone II (IC II) isolates / non-international clone (non IC) isolates (total number of isolates containing the transporter).

[c] Data in Table 6.2.

[d] No substrates detected.

(A1S_3117), has been replaced by two ORFs encoding proteins of unknown function in *A. baumannii* strains ACICU and WM99c (data not shown). These strains belong to the international clone II group (Table 2.2) and the PCR detection confirmed that A1S_3117 was absent in most, but not all, international clone II isolates (Table 2.2). The last non-conserved transporter is A1S_0116. This gene encodes an RND transporter with a putative function in secretion of secondary metabolites as described in Section 5.2.6.1.

6.2.2 Annotation and isolation of the *A. baumannii* efflux systems

In this study, four transporter proteins classified in the MATE family and two in the RND superfamily from *A. baumannii* strain ATCC 17978 were investigated. Proteins classified within these families of transporters display vastly different structural characteristics and were chosen to test the limits of the expression system developed (Section 1.3.4). First, the ORFs of all predicted MATE genes, A1S_0395, A1S_2562, A1S_3371 and A1S_3420, and two novel RND genes, A1S_3445 and A1S_3446 were manually annotated as differences were observed in multiple sequence alignments with homologues from other fully sequenced *A. baumannii* strains (data not shown). Sequence analysis using Mauve (Section 2.7.2) suggested that A1S_3446 and A1S_3445 of *A. baumannii* ATCC 17978 were actually a single ORF that had been split by a sequencing error. This was confirmed by sequencing of the chromosomal DNA by Sanger sequencing (Section 2.4.7) using oligonucleotides specific to the termini of both ORFs (adeM_TOPO; Table 2.4). Using Glimmer3 (Delcher *et al.* 1999), alternative start codons were identified for all MATE ORFs and A1S_3445/6 (data not shown). The protein sequences encoded by the manually annotated transporter genes were examined using the topology prediction program TMHMM (Krogh *et al.* 2001) and found to adhere to the regular topological organisations of their respective transporter families, i.e., 12 predicted transmembrane helices for both the MATE and RND proteins, as well as long periplasmic loops between helices 1 and 2, and 7 and 8, of the RND family transporter (Section 1.3.4.5). RND proteins are known to have the ability to form tripartite efflux complexes that span both the inner and outer membranes of Gram-negative bacteria. These protein complexes consist of the RND protein, a pore forming OMP and a MFP (Section 1.3.4.5). The ORF of A1S_3445/6 is preceded by a gene encoding a predicted MFP and a divergently-transcribed regulator. This gene cluster does not contain an OMP. However, the RND transport protein and MFP

could form a complex with an OMP transcribed elsewhere on the genome to form the tripartite complex. A similar interaction of an RND with a distally-encoded OMP has been observed for other RND tripartite systems, such as the AcrAB (MFP and RND) and TolC (OMP) proteins that form a major drug efflux system encoded in *E. coli* (Ma *et al.* 1993).

A substrate profile for AbeM (A1S_0395) has been described previously (Su *et al.* 2005). Therefore, this protein was used as a positive control in the assays described below. The three other putative MATE genes were named *abeM2* (A1S_2562), *abeM3* (A1S_3371) and *abeM4* (A1S_3420), and the RND family member *adeM* (A1S_3445/6). All genes were amplified from *A. baumannii* strain ATCC 17978 with a CACC-overhang sequence at the 5'-end and without a stop-codon (Section 2.4.9; Table 2.4). The amplicons containing the 5' CACC overhangs were inserted into the pENTR™/SD/D-TOPO vector (Invitrogen) using the TOPO reaction, generating so called entry clones. During the TOPO reaction, topoisomerase, which is bound to the termini of the linearised pENTR™/DS/D-TOPO vector, facilitates efficient site-specific ligation of the PCR product with the vector (www.invitrogen.com). The pENTR™/DS/D-TOPO vector contains an *E. coli* optimised ribosome binding site, which is positioned correctly upstream of the insert.

6.2.3 Construction of a Gateway-based cloning system for heterologous expression of membrane proteins

In this study, various expression vectors based on Gateway technology were designed to allow 'shuffling' of the gene of interest into different expression systems previously shown to be successful in the overexpression of membrane transport proteins from various transporter classes. The vectors included pET-DEST42, pBAD30 and pBluescriptII, which have differences in their copy-number, the promoter and regulation of transcription. The pET and pBAD plasmids are propagated at a low to medium copy-number in the cell, but expression is driven by strong promoters (Dubendorff and Studier 1991; Guzman *et al.* 1995). Gene expression can be controlled by the LacI/O and AraC regulators for pET and pBAD, respectively, and both plasmids also contain transcriptional terminators. These expression systems are considered suitable for obtaining high levels of recombinant protein for *in vitro* investigation (Hassan *et al.* 2009). In contrast, the lower expression levels obtained by using pBluescriptII (Alting-Mees and Short 1989) behind the T7 promoter (pBS_P_{T7}) in *E. coli* appear more suitable for *in vivo*

characterisation of membrane transporters. Uninduced expression from pBluescriptII in *E. coli* strain DH5α has previously been used for *in vivo* characterisation of heterologously expressed membrane transporters (Hassan *et al.* 2006; Xu *et al.* 2006). However, in this study, leaky expression of T7 RNA polymerase in *E. coli* strain BL21(DE3) may be responsible for transcription of the gene of interest from pBluescriptII. Cloning the gene in the opposite direction allows transcription to be directed by the lac promoter (pBS_P_{lac}). This is a native promoter site for *E. coli* RNA polymerases. Therefore, this expression system allows the user to select any *E. coli* host background, e.g. the hypersusceptible *E. coli* strain AG100A (Table 2.2) when performing drug resistance studies.

The pET-DEST42 Gateway vector was obtained commercially (Invitrogen) and the Gateway features from this plasmid were isolated by PCR using the Gateway1 and Gateway2 oligonucleotides listed in Table 2.4. The purified fragments obtained by PCR using the Gateway2 oligonucleotides were subsequently cloned into pBAD30, generating pBADgw, and in pBluescriptII generating pBSgwP_{T7}, the purified fragment obtained by PCR using the Gateway1 oligonucleotides were also cloned into pBluescriptII generating pBSgwP_{lac} (Section 2.4.8; Figure 6.1; Table 2.3). Functionality of the Gateway features (Section 6.1), such as chloramphenicol resistance, activity of the *ccdB* gene and recombination using the LR-reaction, was confirmed. Furthermore, the DNA sequence of the insert was examined by Sanger sequencing (Section 2.4.7).

Genes encoding representatives of the MATE and RND family, *abeM4* and *adeM*, respectively, were recombined into all four destination vectors to investigate protein expression. The expression clones obtained were then transformed into appropriate *E. coli* host strains; the pET clones into BL21(DE3), pBAD into TOP10, pBS_P_{T7} into BL21(DE3) and pBS_P_{lac} into DH5α or AG100A (for drug resistance assays). ON cultures in LB medium were diluted and expression was induced at OD_{600} = 0.5 for the pET and pBAD expression systems, with 0.5 mM IPTG and 0.004% L-arabinose, respectively. All cultures were harvested when OD_{600} = 0.7. The membrane fraction of the cultured cells was isolated (Section 2.6.1) and equal amounts of total protein, 60 µg, were loaded onto 12% SDS-PAGE gels (Section 2.6.3). Western blot analysis using anti-X6 HIS epitope tag antibodies (Rockland) was employed to detect recombinant protein in the isolated membrane fraction

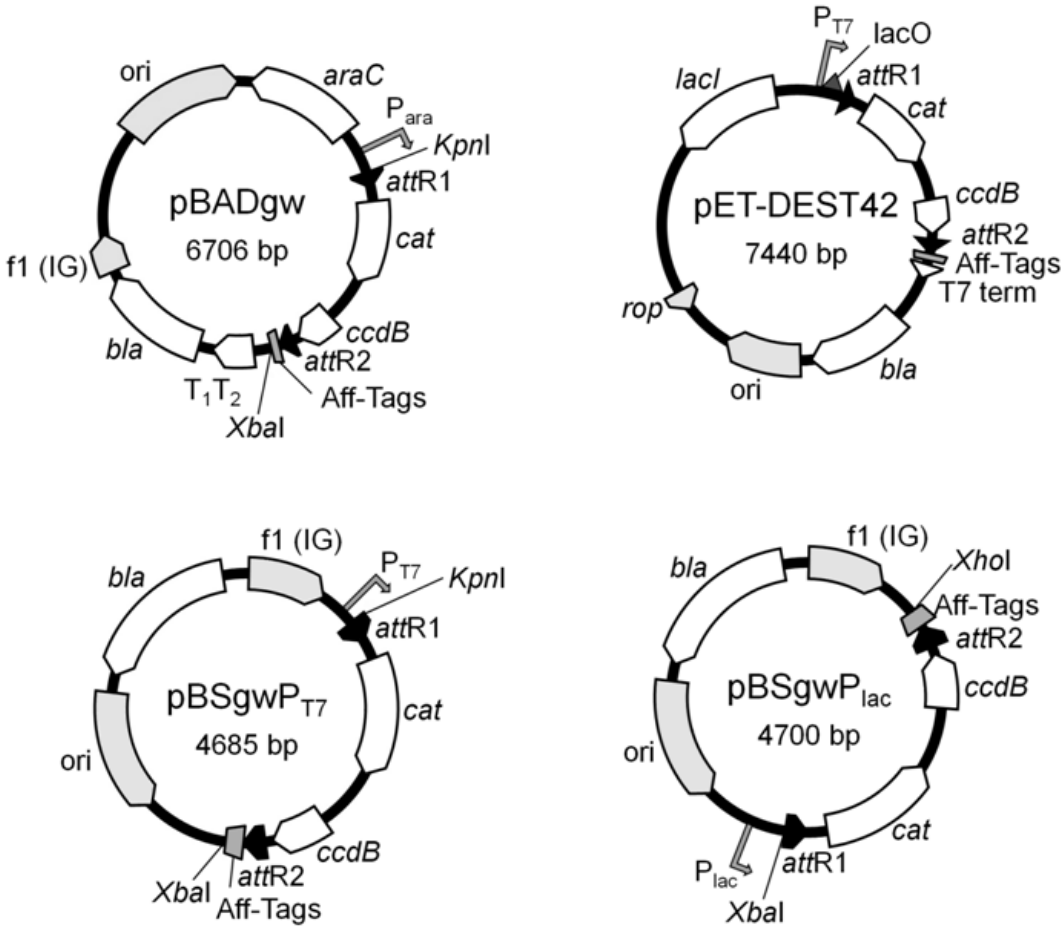

Figure 6.1: The Gateway-based expression systems used for expression of *A. baumannii* efflux proteins

The Gateway features from pET-DEST42 were cloned into pBAD30 downstream of P_{ara} and into pBluescriptII behind both P_{T7} and P_{lac}, generating pBADgw, pBSgwP_{T7} and pBSgwP_{lac}, respectively. All plasmid backbones contain the *bla* gene for ampicillin selection. The replication features, including the origin of replication (ori), the intergenic region of phage f1 [f1(IG)] and the copy-number repression site (*rop*) of the plasmids are represented in light-grey. The promoter and direction of transcription are indicated by a narrow arrow (P_{T7}, P_{ara} or P_{lac}). The *lacI* gene encodes a product that binds to the *lacO* site for transcriptional inhibition from P_{T7} in pET-DEST42. Both pET-DEST42 and pBADgw contain transcription termination sites, T7 term and T_1T_2, respectively. The *att*R1 and *att*R2 sites form the site specific recombination sequences, which allow the gene of interest to be inserted in-frame with the His_6 and V5-epitope tags (Aff-Tags) and a stop codon. Two negative selection genes are located between the *att*R sites, *cat* for chloramphenicol selection and the *ccdB* gene that encodes a lethal product to most *E. coli* strains.

(Sections 2.6.5 and 2.6.6; Figure 6.2). Both AdeM and AbeM4 were detected at their expected sizes, ~110 kDa and ~50 kDa, respectively (Figure 6.2). AdeM synthesis was at similar levels when using pBADgw_adeM, pBSgwP$_{T7}$_adeM or pBSgwP$_{lac}$_adeM, however, the levels appeared higher in the sample from pET_adeM. AbeM4 was detected when using pET_abeM4, pBADgw_abeM4 and pBSgwP$_{T7}$_abeM4 expression systems, however no recombinant protein could be detected with pBSgwP$_{lac}$ _abeM4 as an expression vector. Expression levels of AbeM4 obtained with pBSgwP$_{T7}$_abeM4 were lower than those observed using the pET_abeM4 and pBADgw_abeM4 expression clones. The respective negative controls for the four different expression systems showed no reactive signal using the anti-X6 HIS antibody (data not shown).

6.2.4 Functional characterisation of *A. baumannii* efflux systems

In order to identify substrates of the efflux systems, MIC assays were carried out on *E. coli* cells expressing the putative efflux pump genes from *A. baumannii*. The micro-dilution method was performed at least three times using freshly transformed cells (Section 2.3.2). All four MATE proteins, AbeM, AbeM2, AbeM3 and AbeM4, and the RND member AdeM were investigated in this study. Resistance to a wide range of compounds was tested, including various antibiotics (tetracycline, ciprofloxacin, norfloxacin, kanamycin, colistin, erythromycin, trimethoprim, gentamicin, chloramphenicol and rifampicin), antiseptics (triclosan, sodium dodecyl sulphate, chlorhexidine, benzalkonium and tetraphenylphosphonium) and dyes (ethidium, DAPI and rhodamine 6G) (Table 6.2). The pBSgwP$_{T7}$ and pBSgwP$_{lac}$ destination clones were used for the *in vivo* functional characterisation of the putative efflux proteins, as protein expression from the pET-DEST42 and pBADgw vectors was significantly detrimental to cell growth, possibly as a result of the high expression levels obtained from these vectors (Section 6.1; Figure 6.2). Western blot analyses indicated that all recombinant proteins were present in the membrane fraction using either pBSgwP$_{T7}$ or pBSgwP$_{lac}$ (data not shown), except for AbeM4 when using pBSgwP$_{lac}$ as described above. Interestingly, the relative resistance observed for AbeM was the same when using pBSgwP$_{T7}$_*abeM* in *E. coli* BL21(DE3) or pBSgwP$_{lac}$_*abeM* in the hypersusceptible *E. coli* strain AG100A (Table 6.2). Therefore, the resistance of the other transporters was investigated using the pBSgwP$_{T7}$ clones in *E. coli* BL21(DE3) cells only. The relative resistance was

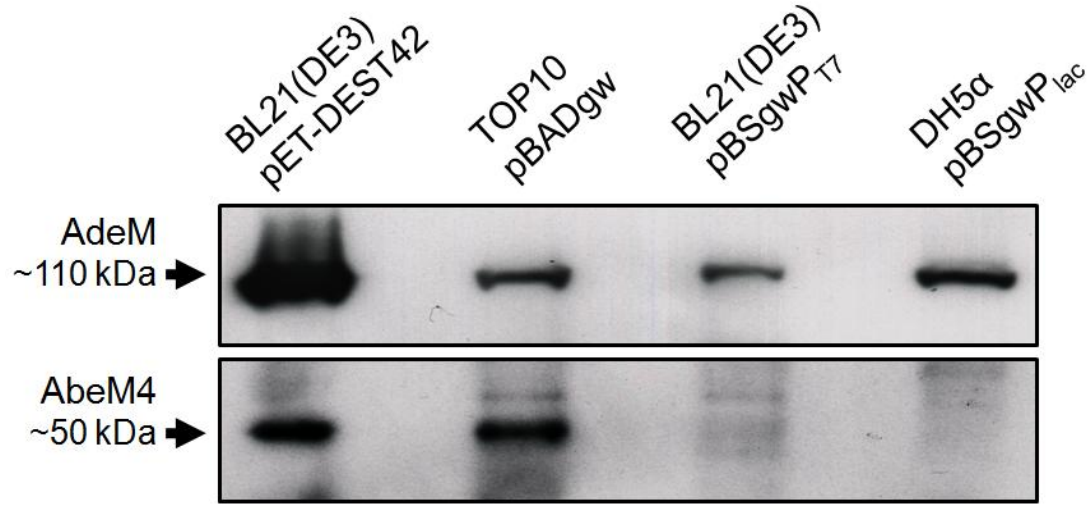

Figure 6.2: Western blot detection of heterologously expressed *A. baumannii* efflux proteins in *E. coli*

Expression of AdeM and AbeM4 in the four different expression systems was investigated by Western blot analysis (Section 2.6). The membrane fraction of *E. coli* cells expressing each construct was isolated and an equal amount of total protein (60 µg) was loaded onto a 12% SDS-PAGE gel. Recombinant protein was detected using an anti-X6 HIS antibody.

Table 6.2: Substrate profile of heterologously expressed *A. baumannii* efflux proteins in *E. coli*

Efflux protein	**Destination clone /*E. coli* host**	**Substrates** [a,b] (Increase in times-fold over vector control)
AdeM	pBSgwP$_{T7}$ / BL21(DE3)	Norfloxacin (2), chlorhexidine (2)
AbeM	pBSgwP$_{T7}$ / BL21(DE3)	Norfloxacin (4), ciprofloxacin (4), trimethoprim (2), chlorhexidine (4), ethidium (2), DAPI (2)
	pBSgwP$_{lac}$ / AG100A	Norfloxacin (4), ciprofloxacin (4), trimethoprim (2), chlorhexidine (4), ethidium (2), DAPI (2)
AbeM2	pBSgwP$_{T7}$ / BL21(DE3)	Ciprofloxacin (2)
AbeM3	pBSgwP$_{T7}$ / BL21(DE3)	-
	pBSgwP$_{lac}$ / AG100A	-
AbeM4	pBSgwP$_{T7}$ / BL21(DE3)	Ciprofloxacin (2)

[a] MIC assays were performed at least three times.

[b] Compounds tested: tetracycline, ciprofloxacin, norfloxacin, kanamycin, colistin, erythromycin, trimethoprim, gentamicin, chloramphenicol, rifampicin, triclosan, benzalkonium, chlorhexidine, sodium dodecyl sulfate, tetraphenylphosphonium, ethidium, 4',6-diamidino-2-phenylindole (DAPI), rhodamine 6G.

measured as the difference in the MIC value between the expressing clone and its respective vector control, e.g., for AbeM this was 4-fold in both systems for ciprofloxacin (Table 6.2). The MIC values were consistent across three independent experiments, therefore, a 2-fold increase in the relative resistance was considered to be the result of the recombinantly expressed protein and not due to fluctuating background levels. An extensive substrate profile was obtained for AbeM, as resistance was observed to norfloxacin, ciprofloxacin, trimethoprim, chlorhexidine, ethidium and DAPI. This confirmed that AbeM is a multidrug efflux system, as it has the ability to transport structurally-diverse compounds, correlating with a previous study (Su *et al.* 2005). Therefore, the same approach was applied to characterise the three other MATE proteins and AdeM. AbeM2 and AbeM4 were found to only confer resistance to ciprofloxacin, which appears to be a common substrate of MATE proteins (Section 1.3.4.4). Of note, the *abeM2* gene is located in a putative siderophore biosynthesis cluster and may play a role in iron homeostasis (Section 3.2.4); *abeM2* was only detected in *A. baumannii* strain ATCC 17978 (Table 6.1). Expression of AbeM3 did not result in increased resistance to any of the 18 compounds tested in *E. coli* BL21(DE3) using pBSgwP_{T7}. The same MIC tests for AbeM3 were also performed using the pBSgwP_{lac} expression system in *E. coli* AG100A, however, no increased resistance over the background levels was observed for any of the compounds tested (Table 6.2). Ciprofloxacin and chlorhexidine were positively identified as substrates of the RND transporter AdeM by *in vivo* characterisation of the recombinant protein in *E. coli*. Although it was shown that AdeM is a multidrug transporter, the resistance levels observed in the heterologous expression study may only represent a partial potential of this pump. In its native environment in *A. baumannii* it is likely to form a tripartite complex with an MFP and OMP, like many other RND proteins. Such a fully assembled efflux system is likely to potentiate higher levels of drug resistance than the resistance observed in this study on the heterologously expressed RND protein only.

6.2.5 Constructing directed *A. baumannii* knockout strains

The Gateway-based cloning system was extended from heterologous expression to use in *A. baumannii* knockout construction. The Gateway suicide vector constructed in this study was based on the *P. aeruginosa* pEX vectors, which have shown to be successful in other *A. baumannii* knockout studies (Dorsey *et al.* 2004; Roca *et al.* 2009). A vector for delivery of the knockout construct, pEX18Tc, a

derivative of pEX100T, was selected. In this vector the *bla* gene has been replaced with a *tet* gene and therefore, its use in *A. baumannii* is more widely applicable, since this species is typically resistant to ampicillin (Hoang *et al.* 1998; Schweizer and Hoang 1995). The Gateway features were digested from pBSgwP$_{lac}$ using *Xba*I and *Xho*I and inserted into pEX18Tc behind the lac promoter, generating pEXgwTc (Section 2.4.8; Figure 6.3A).

The existing entry clone harbouring *abeM4*, pENTRabeM4, was used as a template and the gentamicin resistance gene *aacC1* was isolated from pPS856 (Hoang *et al.* 1998) using *Xba*I and was subsequently inserted into a native *Xba*I site in *abeM4*. This site is located close to the middle of the ORF (position 618 of 1344), leaving >600 bp of flanking DNA on both sides for a double cross-over recombination event to occur. The *abeM4::Gm* insertion disruption construct was cloned into pEXgwTc using the LR-reaction (Section 2.4.9). Importantly, the use of Gateway cloning in preparation of a knockout construct is advantageous as multiple cloning steps in the procedure can limit the availability of endonuclease restriction sites. This novel method requires only one conventional cloning step. If desired, conventional cloning can be avoided completely by using an overlap extension PCR to generate a deletion/disruption construct containing a selection marker with flanking regions specific to the gene of interest. The suicide construct generated here, pEXgwTc_abeM4::Gm, was transformed into *A. baumannii* strain ATCC 17978 by electroporation (Section 2.4.13). The transformed cells were then cultured ON with tetracycline and gentamicin to propagate the suicide construct and allow the double cross-over recombination event to take place. The *sacB* gene, located on the suicide vector, allows for selection of cells that have lost the plasmid when growing them on M9 media agar containing sucrose instead of glucose. Colonies were washed in PBS and cultured ON on M9 medium with sucrose (0.5%) and gentamicin (12.5 µg/ml). This selects for clones that have lost the suicide delivery vector, but have the gentamicin resistance gene inserted into their genome. Over 30 colonies were analysed by PCR with the abeM4_TOPO oligonucleotides (Table 2.4), which are located at the termini of the *abeM4* ORF. An increase in the single PCR product from ~1344 to ~2170 bp, indicated that insertion disruption of *abeM4* by *aacC1* had taken place in all 30 screened colonies (Figure 6.3B). Susceptibility to tetracycline indicated that the suicide vector was removed from the clones investigated.

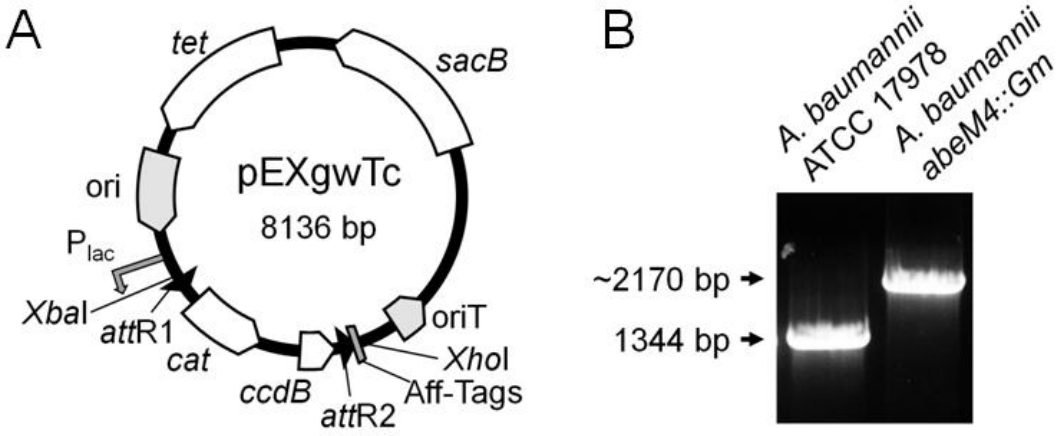

Figure 6.3: The *A. baumannii* insertion disruption strategy developed in this study

Schematic representation of the suicide Gateway construct generated in this study (A). The Gateway features, which include the *att*R recombination sites, two selection markers (*cat* and *ccdB*) and two affinity tags (His_6 and V5-epitope), were inserted into pEX18Tc using *Xba*I and *Xho*I, generating pEXgwTc. The *abeM4* insertion disruption strain *A. baumannii* ATCC 17978_abeM4::Gm showed an amplification product of increased size when using *abeM4* specific primers (B).

Disruption of *abeM4* was confirmed by DNA sequencing (Section 2.4.7) of two representative clones using the abeM4_TOPO oligonucleotides (Table 2.4). The resulting *A. baumannii* mutant strain ATCC 17978_abeM4::Gm was phenotypically characterised. No significant difference was observed in the growth rate in LB broth between the mutant and WT strain, moreover, the colony size and morphology appeared unaffected on non-selective LB agars (data not shown). The drug susceptibility profile, determined by micro-dilution MICs (Section 2.3.2), was compared between ATCC 17978 and ATCC 17978_abeM4::Gm using the 18 compounds described previously (Section 6.2.4; Table 6.2). Interestingly, whereas ciprofloxacin was shown to be a substrate of AbeM4 in the heterologous expression studies (Section 6.2.4), no differences in susceptibility to ciprofloxacin were observed for the *abeM4* disrupted *A. baumannii* mutant strain. In fact, no significant differences in susceptibility were observed to any of the 18 compounds tested (data not shown). Although low, *abeM4* transcription levels in *A. baumannii* grown in MH broth cultures were similar to that found for other genes encoding transporters, such as *abeM* and A1S_2305, a gene encoding a RND transporter (data not shown). The genes encoding efflux proteins may show transcriptional responses upon exposure to its substrates, as observed for *mexXY*, an operon encoding a *P. aeruginosa* RND transporter system (Sobel *et al.* 2003). However, qRT-PCR analysis (Section 2.4.15) showed no transcriptional up-regulation of *abeM4* when ATCC 17978 cells were exposed to sub-inhibitory concentrations of ciprofloxacin (data not shown). In a study on the effect of increased NaCl levels to *A. baumannii*, it was shown that numerous transporter proteins were significantly up-regulated, including *abeM4* (>12-fold), and also an increased resistance to several different antibiotics was observed (Hood *et al.* 2010). Subsequently, in this study, drug resistance of ATCC 17978 and ATCC 17978_abeM4::Gm was investigated under high-salt conditions (MH supplemented with 150 µM NaCl), however, no differences in resistance between the two strains were seen (data not shown). The role of AbeM4 as a transporter of, for example, ciprofloxacin in *A. baumannii*, may be overshadowed by other transporters with similar substrate profiles.

6.3 Conclusions

The PCR analysis of the putative drug transporters identified in *A. baumannii* strain ATCC 17978 showed that, in general, these resistance markers are well conserved between distinct clinical isolates. The relatively high number of transporters may be associated with the diverse origin of *Acinetobacter*, in which it requires a broad selection of detoxification mechanisms, *viz*. in the form of efflux pumps. This highlights that the evolutionary function of transporter proteins is not efflux of antibiotics, as described previously (Paulsen 2003). Instead, drug efflux appears to be of an opportunistic and adaptive nature.

The Gateway-based cloning and expression systems designed in this study were proven successful for heterologous expression of distinct *A. baumannii* membrane transport proteins. Functional characterisation of all putative *A. baumannii* ATCC 17978 MATE proteins and AdeM, a member of the RND family, was carried out and their function as drug transport proteins was confirmed for four of the five investigated. A novel Gateway-based suicide vector was constructed and used to generate an *abeM4* insertion disruption in *A. baumannii* ATCC 17978. The described cloning strategy could also be applied as a versatile tool for characterisation of various other membrane proteins.