CHAPTER 2

MATERIALS AND METHODS

2.1 Buffers and growth media

2.1.1 Solutions and buffers

All reagents listed in Table 2.1 were of molecular grade and obtained from Sigma-Aldrich Australia unless otherwise specified. Powdered compounds were dissolved according to the manufacturers' recommendations or as described in the Merck index (Budavari 1996). Solutions were sterilised by either autoclaving at 121°C for 15 minutes or passaging through a filter with a 0.22 µm pore width.

2.1.2 Bacterial culture media

Typical Luria-Bertani (LB) media contained 1% (w/v) tryptone (Oxoid, Australia), 0.5% (w/v) yeast extract (Oxoid, Australia) and 1% (w/v) NaCl; the pH was adjusted to 7.5. Mueller-Hinton (MH) media were prepared according to the manufacturer's recommendations (BD, Bacto Laboratories Pty Ltd, Australia). A stock solution of 5X M9 media was prepared using 200 mM Na_2HPO_4 (Chem-Supply, Australia), 110 mM KH_2PO_4 (Chem-Supply), 43 mM NaCl (Chem-Supply) and 93 mM NH_4Cl (Chem-Supply). The M9 stock solution was diluted (1:5) and supplemented with 2 mM $MgSO_4$ (Chem-Supply), 0.4% (w/v) glucose and 0.1 mM $CaCl_2$ (Chem-Supply) (final concentrations) on the day of use. For solid media, 1.0% (w/v) J3 grade agar (Gelita, Australia) was added. The agar concentration used in motility assays has been specified in Section 2.3.4. The media were sterilised by autoclaving at 121°C for 15 minutes.

2.2 Bacterial strains and growth conditions

Both *E. coli* and *A. baumannii* were incubated at 37°C, LB media were used for standard overnight (ON) culturing unless stated otherwise. Tubes with broth cultures were incubated at a 45° angle with shaking (200 rpm) for appropriate aeration. The bacterial strains and plasmids used in this study have been listed in Table 2.2 and Table 2.3, respectively. For antibiotic selection, the media were allowed to cool to approximately 50°C after autoclaving. In experiments with *E. coli* this was followed by the addition of; 100 µg/ml of ampicillin, 50 µg/ml kanamycin, 35 µg/ml chloramphenicol, 12.5 µg/ml gentamicin or 12.5 µg/ml tetracycline where appropriate. The following concentrations were used in experiments with *A. baumannii* strain ATCC 17978; 100 µg/ml of ampicillin, 25 µg/ml kanamycin, 35 µg/ml chloramphenicol, 12.5 µg/ml gentamicin or 12.5 µg/ml tetracycline.

Table 2.1: Buffers and solutions

Buffer/solution	Constituents
TAE buffer (50X)	24.2% (w/v) Tris-base, 50 mM ethylenediaminetetra acitic acid (pH 8.0), 5.71% (v/v) glacial acetic acid
Blocking buffer	10% (w/v) skim milk powder in TTBS buffer
Chemiluminescence detection solution	Solution 1; 2% (225 mM 4-aminopthalhydrazide, 42.6 mM Coumaric acid in DMSO) in 0.1 M Tris buffer (pH 9.35)
	Solution 2; 0.036% H_2O_2 in 0.1 M Tris buffer (pH 9.35)
Coomassie stain	30% (v/v) methanol, 10% glacial acetic acid, 0.15% Coomassie Brilliant Blue R-250
Crushing buffer	50 mM Tris-HCl (pH 7.5), 10% glycerol, 300 mM NaCl
Destain solution	30% (v/v) methanol, 10% glacial acetic acid
Electrophoresis loading buffer	50% sucrose (w/v), 50 mM ethylenediaminetetra acitic acid (pH 7.0), 0.05% (w/v) bromophenol blue
Membrane resuspension buffer	20 mM Tris-HCl (pH 7.5), 10% glycerol, 1% n-dodecyl-β-D-maltoside
Phosphate buffered saline	136 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4, 8.1 mM $(Na_2HPO_4)2H_2O$
Potassium urea magnesium buffer	127 mM K_2HPO_4, 53 mM KH_2PO_4, 29.97 mM urea, 0.8 mM $(MgSO_4)7H_2O$
Running buffer for GelPage pre-cast gels (20X)	0.8 M tricine, 1.2 M Tris, 2% SDS, 50 mM sodium bisulphite (pH 8.2 - 8.3)
SDS-PAGE sample buffer (2X)	0.05 M Tris-HCl (pH 6.8), 5% β-Mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% bromophenol blue
Transfer Buffer	25 mM Tris, 192 mM glycine, 10% MeOH (pH 8.3 - 8.8)
TTBS buffer	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% tween

Table 2.2: Bacterial strains used in this study

Strain	Genotype or description [a]	Reference/ source
***Acinetobacter* strains**		
A. baumannii 04117201	International clone II; Tracheal aspirate isolate	[b]
A. baumannii 04145027	Non-international clone; Vaginal isolate	[c]
A. baumannii 04202856	Non-international clone; Urine isolate	[c]
A. baumannii 05293224	Non-international clone; Tracheal aspirate isolate	[b]
A. baumannii 05299446	International clone II; Tracheal aspirate isolate	[b]
A. baumannii 06345143	Non-international clone; Tracheal aspirate isolate	[b]
A. baumannii 08315000	International clone II; Wound isolate	[b]
A. baumannii 08317005	International clone II; Tracheal aspirate isolate	[b]
A. baumannii 08325850	International clone II; Tracheal aspirate isolate	[b]
A. baumannii 1077697	International clone II; Sputum isolate	[d]
A. baumannii 1172312	International clone II; Sputum isolate	[d]
A. baumannii 1182372	International clone II; Sputum isolate	[d]
A. baumannii 1182468	International clone II; Sputum isolate	[d]
A. baumannii 1198673	International clone II; Sputum isolate	[d]
A. baumannii 11986751	International clone II; Pus isolate	[d]
A. baumannii 11986752	Non-international clone; Pus isolate	[d]
A. baumannii 1279683	International clone II; Pus isolate	[d]
A. baumannii 17978hm	Hyper-motile variant of *A. baumannii* ATCC 17978, A1S_0268 disruption	This study
A. baumannii 2320495	Non-international clone; Pus isolate	[d]
A. baumannii 4397670	Non-international clone; Unknown origin	[d]
A. baumannii 6772166	International clone I; Pus isolate	[d]
A. baumannii 6856390	International clone II; Sputum isolate	[d]

Strain	Genotype or description [a]	Reference/ source
A. baumannii 6856775	International clone I; Catheter isolate	[d]
A. baumannii 6870155	International clone I; Sputum isolate	[d]
A. baumannii 6877889	International clone I; Sputum isolate	[d]
A. baumannii 6896168	Non-international clone; Unknown origin	[d]
A. baumannii 6913054	International clone II; Blood isolate	[d]
A. baumannii 6954775	International clone II; Sputum isolate	[d]
A. baumannii 9028181	Non-international clone; Pus isolate	[d]
A. baumannii 9030751	International clone II; Pus isolate	[d]
A. baumannii 9030759	International clone II; Sputum isolate	[d]
A. baumannii 9034499	International clone II; Sputum isolate	[d]
A. baumannii 9038266	International clone II; Sputum isolate	[d]
A. baumannii 9078336	International clone II; Sputum isolate	[d]
A. baumannii 9137982	International clone II; Pus isolate	[d]
A. baumannii AB0057	International clone I; Blood isolate	(Adams *et al.* 2008) CP001182 [e]
A. baumannii ACICU	International clone II; Cerebrospinal fluid isolate	(Iacono *et al.* 2008) CP000863 [e]
A. baumannii ATCC 17978	Non-international clone; Meningitis isolate	(Smith *et al.* 2007) CP000521 [e]
A. baumannii ATCC 17978_abeM4::Gm	Insertion disruption of *abeM4* in *A. baumannii* ATCC 17978	This study
A. baumannii ATCC 19606	Non-international clone; Urine isolate	(Bouvet and Grimont 1986) ACQB00000000 [g]
A. baumannii AYE	International clone I; Urinary tract isolate	(Fournier *et al.* 2006) CU459141 [e]
A. baumannii B23	ATCC 17978 derivative, A1S_0268 disruption, unknown position of mini - Tn*10*::Kan^{R}-GFP	This study

Strain	Genotype or description [a]	Reference/ source
A. baumannii D1279779	Community-acquired Non-international clone; Blood isolate	AERZ00000000 [h]
A. baumannii PW01a	International clone II; Unknown origin	[i]
A. baumannii PW01b	International clone II; Unknown origin	[i]
A. baumannii PW01c	International clone I; Unknown origin	[i]
A. baumannii RB01	International clone II; Blood isolate	[j]
A. baumannii RB02	International clone II; Rectum isolate	[j]
A. baumannii RB02b	International clone II; Sputum isolate	[j]
A. baumannii RB02c	International clone II; Rectum isolate	[j]
A. baumannii RB02d1	International clone II; Tracheal aspirate isolate	[j]
A. baumannii RB02e	International clone II; Rectum isolate	[j]
A. baumannii SDF	Non-international clone; Human body louse isolate (non-pathogenic)	(Fournier *et al.* 2006) CU468230 [k]
A. baumannii WM00	International clone II; Bronchial isolate	[l]
A. baumannii WM97a	Non-international clone; Sputum isolate	[l]
A. baumannii WM98	International clone I; Wound isolate	[l]
A. baumannii WM98c	Non-international clone; Unknown origin	[l]
A. baumannii WM99a	International clone II; Unknown origin	[l]
A. baumannii WM99c	International clone II; Sputum isolate	AERY00000000 [l]
A. baylyi ADP1	Soil isolate	(Barbe *et al.* 2004) CR543861 [l]
Acinetobacter genomic species 13TU WM98b	Sputum isolate	[l]
Acinetobacter genomic species 3 WM97b	Unknown origin	[l]

Strain	Genotype or description [a]	Reference/ source
***E. coli* strains**		
E. coli DH5α	*fhuA*2 Δ(*argF*-*lacZ*)U169 *phoA* *glnV*44 Φ80 Δ(*lacZ*)M15 *gyrA*96 *recA*1 *relA*1 *endA*1 *thi*-1 *hsdR*17	(Hanahan 1983)
E. coli AG100A	*E. coli* K-12 Δ*acrAB* (hyper-susceptible)	(Okusu *et al.* 1996) [m]
E. coli BL21(DE3)	B *dcm ompT hsdS*($r_B^- m_B^-$) *gal* λ(DE3)	(Studier and Moffatt 1986)
E. coli TOP10	*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Δ*lac*X74, *deo*R, *rec*A1, *ara*D139Δ(*ara-leu*)7697, *gal*K, *rps*L, *end*A1, *nup*G	Invitrogen
E. coli ccdB Survival	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *ara*Δ139 Δ(*ara-leu*)7697 *galU galK rpsL* (*StrR*) *endA*1 *nupG fhuA*::IS2	Invitrogen

[a] The international clone determination was assessed as described in Section 2.3.1.

[b] Flinders Private Hospital, Adelaide, Australia.

[c] Flinders Medical Centre, Adelaide, Australia.

[d] Royal Adelaide Hospital, Adelaide, Australia.

[e] Department Bacteriologie-Virologie, Hopital de Bicetre, Le-Kremlin-Bicetre, France.

[f] Department of Infectious, Parasitic and Immune-Mediated Diseases, Instituto Superiore di Sanita, Rome, Italy.

[g] American Type Culture Collection, Manassas, USA.

[h] The Menzies School of Health Research, Darwin, Australia.

[i] Prince of Wales Hospital, Sydney, Australia.

[j] Royal Brisbane and Women's Hospital, Brisbane, Australia.

[k] Collection de Souches de l'Unité des Rickettsies, Marseille, France.

[l] Westmead Millennium Institute, Sydney, Australia.

[m] School of Medical and Molecular Sciences, University of Technology, Sydney, Australia.

Table 2.3: Plasmids used in this study

Plasmids	**Description** [a]	**Reference / source**
pENTR™/DS/D-TOPO	Kan^R; Gateway entry vector	Invitrogen
pENTRabeM	Kan^R; pENTR™/DS/D-TOPO containing PCR product using abeM_TOPO primers	This study
pENTRabeM2	Kan^R; pENTR™/DS/D-TOPO containing PCR product using abeM2_TOPO primers	This study
pENTRabeM3	Kan^R; pENTR™/DS/D-TOPO containing PCR product using abeM3_TOPO primers	This study
pENTRabeM4	Kan^R; pENTR™/DS/D-TOPO containing PCR product using abeM4_TOPO primers	This study
pENTRabeM4::Gm	Kan^R Gm^R; pENTR_abeM4 containing the *Xba*I fragment from pPS856	This study
pENTR_adeM	Kan^R; pENTR™/DS/D-TOPO containing PCR product using adeM_TOPO primers	KA Hassan
pET-DEST42	Amp^R Cm^R *ccdB*; Gateway destination vector	Invitrogen
pETabeM4	Amp^R; generated by recombination of the insert of pENTR_abeM4 into pET-DEST42 using the Gateway LR-reaction	This study
pETadeM	Amp^R; generated by recombination of the insert of pENTR_adeM into pET-DEST42 using the Gateway LR-reaction	This study
pBAD30	Amp^R; Expression vector	(Guzman *et al.* 1995)
pBADgw	Amp^R Cm^R *ccdB*; pBAD30 containing PCR product from pET-DEST42 using Gateway2 primers (*Kpn*I - *Xba*I)	This study
pBADgw_abeM4	Amp^R; generated by recombination of the insert of pENTR_abeM4 into pBADgw using the Gateway LR-reaction	This study

Plasmids	**Description** [a]	**Reference / source**
pBADgw_adeM	Amp^R; generated by recombination of the insert of pENTR_adeM into pBADgw using the Gateway LR-reaction	This study
pBluescriptII	Amp^R; Expression vector	(Alting-Mees and Short 1989)
pBSgwP$_{T7}$	Amp^R Cm^R *ccdB*; pBluescriptII containing PCR product from pET-DEST42 using Gateway2 primers (*Kpn*I - *Xba*I)	This study
pBSgwP$_{T7}$_abeM	Amp^R; generated by recombination of the insert of pENTR_abeM into pBSgwP$_{T7}$ using the Gateway LR-reaction	This study
pBSgwP$_{T7}$_abeM2	Amp^R; generated by recombination of the insert of pENTR_abeM2 into pBSgwP$_{T7}$ using the Gateway LR-reaction	This study
pBSgwP$_{T7}$_abeM3	Amp^R; generated by recombination of the insert of pENTR_abeM3 into pBSgwP$_{T7}$ using the Gateway LR-reaction	This study
pBSgwP$_{T7}$_abeM4	Amp^R; LR-reaction pBSgwP$_{T7}$ and pENTR_abeM4	This study
pBSgwP$_{T7}$_adeM	Amp^R; generated by recombination of the insert of pENTR_abeM4 into pBSgwP$_{T7}$ using the Gateway LR-reaction	This study
pBSgwP$_{lac}$	Amp^R Cm^R *ccdB*; pBluescriptII containing PCR product from pET-DEST42 using Gateway1 primers (*Xba*I - *Xho*I)	This study
pBSgwP$_{lac}$_abeM	Amp^R; generated by recombination of the insert of pENTR_abeM into pBSgwP$_{lac}$ using the Gateway LR-reaction	This study

Plasmids	**Description** [a]	**Reference / source**
pBSgwP$_{lac}$_abeM2	Amp^R; generated by recombination of the insert of pENTR_abeM2 into pBSgwP$_{lac}$ using the Gateway LR-reaction	This study
pBSgwP$_{lac}$_abeM3	Amp^R; generated by recombination of the insert of pENTR_abeM3 into pBSgwP$_{lac}$ using the Gateway LR-reaction	This study
pBSgwP$_{lac}$_abeM4	Amp^R; generated by recombination of the insert of pENTR_abeM4 into pBSgwP$_{lac}$ using the Gateway LR-reaction	This study
pBSgwP$_{lac}$_adeM	Amp^R; generated by recombination of the insert of pENTR_adeM into pBSgwP$_{lac}$ using the Gateway LR-reaction	This study
pEX18Tc	Tet^R; *sacB*-based suicide vector	(Hoang *et al.* 1998)
pEXgwTc	Tet^R Cm^R *ccdB*; Gateway features cloned from pBSgwP$_{lac}$ into pEX18Tc using *Xba*I and *Xho*I	This study
pEXgwTc_abeM4::Gm	Tet^R Gm^R; generated by recombination of the insert of pENTR_abeM4::Gm into pEXgwTc using the Gateway LR-reaction	This study
pPS856	Amp^R Gm^R; Digested with *Xba*I to isolate *aacCI* (Gm^R) for generating the *A. baumannii* ATCC 17978 *abeM4* insertion disruption construct pENTRabeM4::Gm	(Hoang *et al.* 1998)
pWH1266	Amp^R Tet^R; *Acinetobacter*/*E. coli* shuttle vector, generated by ligation of pBR322 and a cryptic *Acinetobacter* plasmid using *Pvu*II digestions	(Hunger *et al.* 1990) [b]

Plasmids	Description [a]	Reference / source
pWH0268	Amp^R; A1S_0268 and 200 bp upstream region isolated by PCR from *A. baumannii* ATCC 17978 and cloned into the pWH1266 using *Xba*I	This study

[a] Amp^R: confers resistance to 100 µg/ml ampicillin; Cm^R: confers resistance to 25 µg/ml chloramphenicol; Kan^R: confers resistance to 50 µg/ml kanamycin; Tet^R: confers resistance to 12.5 µg/ml tetracycline; *ccdB*: the gene product is lethal to most *E. coli* strains, except for the *E. coli ccdB* Survival strain.

[b] Institut für Mikrobiologie und Biochemie, Friedrich-Alexander Universität Erlangen-Nürnberg, Germany.

2.3 Bacterial characterisation assays

2.3.1 International clone determination

Identification of *ompA*, $bla_{OXA51\text{-}like}$ and *csuE* allelic variants was conducted as described previously (Turton *et al.* 2007). The two multiplex PCRs were performed using purified genomic DNA as a template (Section 2.4.2). The PCR conditions were 94°C for 3 minutes, followed by 30 cycles of 94°C for 45 seconds, 57°C for 45 seconds and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. Strains were assigned to the international clone complex based on the obtained amplification pattern as defined by Turton et *al.* (2007). The oligonucleotides used in the two multiplex PCRs (group 1 and 2) have been listed in Table 2.4.

2.3.2 Minimal inhibitory concentration assays

The MIC of bacterial strains to a range of antimicrobial compounds was investigated using two different methods, both using MH media (Section 2.1.2). First, the MIC was determined in a micro-dilution assay based on a method described previously (Wiegand *et al.* 2008). For MIC assays using recombinant *E. coli* cells, ON cultures were diluted 1:25 in MH broth with appropriate antibiotic selection and, if required, expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) or 0.004% L-arabinose when OD_{600} = 0.5 (Section 6.2.4). The *E. coli* cultures were diluted 1:1000 when OD_{600} = 0.6 before use. For MIC assays with *Acinetobacter*, ON cultures were first adjusted to OD_{600} = 0.6 and then diluted 1:1000 in MH broth. Microtiter trays containing a 2-fold dilution series of the compound of interest were prepared and cultures were added 1:1, with the final volume being in 100 µl MH broth. The microtiter trays were incubated ON at 37°C without shaking and wrapped in foil to minimise evaporation. Bacterial growth was examined by measuring the absorbance at OD_{600} on a Fluostar Omega spectrometer (BMG Labtech, Germany).

The agar-dilution method was employed to investigate the resistance profile of *A. baumannii* ATCC 17978 and 17978hm (Section 5.2.8.1). In this study, the agar concentration in MH media was decreased to 0.25% to allow strain 17978hm to express its motility characteristics (Section 5.2.1.1). Again, resistance to the compound of interest was investigated in a 2-fold dilution series. The inoculums were prepared by adjusting ON cultures to OD_{600} = 0.1. MH agar plates were

inoculated by transferring 5 μl of the cell suspension to the centre of the growth medium.

The MIC assays were performed three times on separate days. A differential resistance level, determined using either microtiter trays or agar plates, of 2-fold or more, was considered significant.

2.3.3 Static biofilm formation assays

The static biofilm formation assay was performed as described previously (O'Toole *et al.* 1999) with minor modifications. MH broth was inoculated with a single bacterial colony and incubated ON at 37°C with shaking. The cultures were subsequently diluted 1:100 in fresh MH broth in polystyrene microtiter trays and incubated ON at 37°C. The OD_{600} of the planktonic growing bacteria was measured in a new microtiter tray to ensure strains did not show a growth defect; the residual broth was tipped off. Adherent cells were washed once with phosphate buffered saline (PBS; Table 2.1), stained by incubation with 0.1% crystal violet for 30 minutes at 4°C, and washed three times with PBS. Dye was released from the cells using ethanol:acetone (4:1) and shaking at 200 rpm for 30 minutes at RT. Absorbance was measured at 595 nm on a Fluostar Omega spectrometer (BMG Labtech, Offenburg, Germany). The concentrations of compounds used to investigate the effect of stress on biofilm formation, such as low-iron conditions or supplementation with colistin, have been described in Sections 3.2.5 and 5.2.8.3. The biofilm data represent the average of at least three independent experiments of triplicate wells.

2.3.4 Motility assays

Motility in the form of migration on semi-solid surfaces (swarming) was investigated using media containing 0.25% agar. LB was used when investigating motility of both strain ATCC 17978 and 17978hm, MH media were used when examining the effect of stress on motility by strain 17978hm (Section 5.2.8.2) and for the transcriptomic analysis (Section 5.2.4). In all experiments, after pouring the plates were dried for 25 minutes at RT without their lids, the media were then used immediately before condensation could occur. The plates were inoculated by transferring one loop of cells from an ON motile culture grown on semi-solid MH media and the plates were incubated ON at 37°C. Motility inhibition assays, as described in Section 5.2.8.2, were performed three times.

Twitching motility was investigated as previously described (Semmler *et al.* 1999). In brief, one ON grown colony was collected with a sterile toothpick and stabbed through MH medium containing 1% agar to the bottom of the Petri dish. Plates were subsequently incubated ON at 37°C. Positive twitchers were defined as those strains that showed a zone of >10 mm around the site of inoculation.

2.3.5 Pellicle formation assays

The pellicle formation assays were based on a method used previously for characterisation of pellicles formed by *Acinetobacter* strains (Marti *et al.* 2011). ON bacterial cultures in LB broth were diluted 1:100 in fresh LB broth containing 100 mM NaCl (Section 2.1.2). The pellicle formation assays were performed in polypropylene tubes with a diameter of 1.4 cm and were incubated at RT without shaking for 72 hours. The pellicle film was separated from the tube and dehydrated by the addition of 500 μl methanol. Pellicles were transferred to a 1.5 ml tube (of which the weight was measured beforehand) using a pipet tip. After pelleting, the supernatant was carefully removed by pipetting and the pellet was dried for 30 minutes at 37°C. The pellicle biomass was measured on an analytical balance and results were expressed in mg. The experiments were performed at least three times.

2.3.6 Eukaryotic cell adherence assays

Adherence of *A. baumannii* strains to A549 cells (human type 2 pneumocytes) (Giard *et al.* 1973) and Detroit 562 cells (human nasopharyngeal cells) (Peterson *et al.* 1968) was determined essentially as described elsewhere (Talbot *et al.* 1996). Cell lines were grown in Dulbecco's Modified Eagle medium (Invitrogen, Australia) supplemented with 10% fetal bovine serum (Bovogen, Australia). Prior to use, the cell monolayer was examined microscopically to ensure >95% coverage of the bottom of the surface of the 24-well culture plate. Washed A549 or Detroit 562 monolayers were subsequently infected with a bacterial inoculum containing ~$1x10^7$ CFU. The inoculum numbers were determined by viable count assays. After incubation at 37°C for 4 hours, culture medium was removed, and the monolayers washed three times with 1 ml of PBS. The cell monolayers were detached from the plate by treatment with 0.25% trypsin and 0.02% ethylenediaminetetraacitic acid. Eukaryotic cells were subsequently lysed using Triton X-100 at a final concentration of 0.025%. A 10-fold dilution series thereof were plated on LB agar to determine the number of CFU of adherent bacteria per well. The collated data for the adherence

assays were obtained from at least three independent experiments and represent the data points for each experiment of quadruplicate wells.

2.3.7 Hydrophobicity test

Cell surface hydrophobicity was examined as described previously (Rosenberg *et al.* 1980). ON cultures grown in LB were diluted 1:10 in fresh LB broth and incubated at 37°C for 2 hours with shaking. The cells were then washed twice in potassium urea magnesium (PUM) buffer (Table 2.1) and incubated at 30°C for 20 minutes. The cell suspension was adjusted to OD_{600} = 0.25 in 5 ml PUM buffer in glass vials and 3 ml of xylene was added. The two phases were allowed to separate for 15 minutes at RT after vortexing for 2 minutes. An aliquot of the aqueous phase was transferred to a 1.5 ml tube using a glass Pasteur pipette. The optical density of the cell suspensions before ($OD_{initial}$) and after addition of xylene (OD_{final}) were measured at 600 nm. Experiments were performed three times. The hydrophobicity index (HI) expressed in percentage was calculated using the following formula:

$$HI (\%) = (OD_{intial} - OD_{final}/OD_{initial})*100$$

2.3.8 Phenotype MicroArray analysis

Cells from ON cultures on LB agars were transferred with a swab to the inoculation fluid (BIOLOG, Inc.). Cells were added until 42% transmittance was achieved on a Turbidimeter (BIOLOG, Inc.). 100 µl of the cell suspensions were transferred to the wells of MicroPlate™ PM1 and PM2A plates. Phenotype MicroArray™ analysis was carried out by Dr. KA Hassan (Macquarie University, Sydney, Australia).

2.3.9 Statistical analyses

Statistical analyses of eukaryotic cell adherence (Section 2.3.6), cell surface hydrophobicity (Section 2.3.7), biofilm formation (Section 2.3.3) and pellicle formation (Section 2.3.5) were performed using a two-tailed student *t*-test.

2.4 DNA and RNA techniques

2.4.1 Plasmid DNA isolation

Between 1 and 5 ml of ON culture was used for isolation of plasmid DNA depending on the copy number of the plasmid of interest. The HiYield™ Plasmid Mini Kit (RBC) was used as per manufacturer's recommendations.

2.4.2 Genomic DNA isolation

Up to 5 ml ON culture was used for isolation of *Acinetobacter* genomic DNA. The Wizard® genomic DNA purification kit (Promega) was used and the manufacturer's protocol for DNA isolation form Gram-negative bacteria was followed.

2.4.3 RNA isolation

Cells for analysis of mRNA levels were grown as described in the relevant sections. Typically, cells were harvested from (semi-)solid media using pre-chilled PBS. When in broth, cultures were grown until OD_{600} = ~0.6. The cells were pelleted by centrifugation (15,700 x *g* for 2 minutes at 4°C) and lysed in TRIzol® reagent (Invitrogen, Australia) and chloroform. Following phase separation by centrifugation (full speed for 15 minutes at 4°C), RNA was extracted from the aqueous phase using the PureLink™ Micro-to-Midi Total RNA Purification kit (Invitrogen), incorporating an on-column DNaseI (Invitrogen) digestion, as per the manufacturer's recommendations. RNAse free disposables were used during all steps of the RNA isolation. Diethyl pyrocarbonate (DEPC)-treated mQ water was used for elution and preparation of 70% ethanol solutions.

2.4.4 DNA agarose gel electrophoresis

DNA fragments were electrophoresed on an agarose gel following standard molecular biology methods (Sambrook and Russell 2001). Agarose (Bioline) was dissolved in 0.5X TAE buffer (Table 2.1) at the following concentrations; 2% agarose for DNA fragments of <1 Kb, 1% agarose for DNA fragments between 1 and 5 Kb and as low as 0.5% agarose for DNA fragments >5 Kb. 0.2 volumes of electrophoresis loading buffer (Table 2.1) were added to the DNA sample prior to electrophoresis in 0.5X TAE buffer (Table 2.1) at 100 V until the dye front reached the bottom half of the gel. HyperLadder I (BioLine) was used as a molecular weight marker, which allowed estimation of the DNA concentration of the electrophoresed samples as per the manufacturer's description. Gels were stained in 10 µg/ml solution of ethidium bromide for approximately 15 minutes and destained using dH_2O for at least 15 minutes. The DNA fragments were visualised and photographed using a DigiDoc (Bio-Rad).

2.4.5 Purification of DNA fragments

PCR products, excised DNA fragments from an agarose gel (Section 2.4.4) and DNA fragments treated with endonucleases or shrimp alkaline phosphatase (Section 2.4.8) were purified using the HiYield Gel/PCR DNA Mini Kit (RBC) as per manufacturer's recommendations.

2.4.6 Quantitation and quality assessment

Quantitation and assessment of quality of either DNA or RNA was performed using a Thermo Scientific NanoDrop™ 1000 spectrophotometer. A >1.8-fold purity ratio for both OD_{260}/OD_{280} (protein contamination) and OD_{260}/OD_{230} (salts and/or detergent contamination) was ensured for sensitive down-stream applications, such as microarray or genome sequencing. Alternatively, the quantity and integrity of DNA or RNA was investigated by electrophoresis using HyperLadder™ I (Bioline).

2.4.7 DNA sequencing

All Sanger sequencing reactions were performed by the Australian Genome Research Facility (Brisbane and Adelaide nodes). Typically, 500 ng of template DNA and 1 µM of primer in a volume of 12 µl mQ water was submitted. Illumina BeadArray technology was used for sequencing of strain *A. baumannii* ATCC 17978 and 17978hm (unpublished data), and 454-pyrosequencing for strains WM99c (AERY00000000) and D1279779 (AERZ00000000). Genome sequencing was performed by the Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Australia. The whole genome shotgun sequence reads were assembled by Dr. LDH Elbourne (Macquarie University, Sydney, Australia) using Velvet 1.1 (Zerbino and Birney 2008).

2.4.8 Conventional cloning

Restriction digestions were performed with endonucleases incubated as per manufacturer's recommendations (New England Biolabs, Genesearch, Australia). Shrimp alkaline phosphatase (Promega) treatment was carried out prior to ligation using single-digested vectors or blunt-ended vectors. Endonucleases and alkaline phosphatase were heat-inactivated if possible as per manufacturer's recommendations, alternatively, DNA fragments were purified, as described in Section 2.4.5. DNA fragments for cloning were excised from a gel (Section 2.4.4) and column purified if required for isolation of the fragment of interest for the

ligation. Ligations were performed in a 1:3 ratio (vector: insert) ON at 4°C or for 8 hours at 16°C using T4 DNA ligase (Promega). T4 DNA ligase was heat-inactivated by incubation at 70°C for 10 minutes prior to transformation (Sections 2.4.11 and 2.4.13).

2.4.9 Gateway cloning

The oligonucleotides specific to the 5'-end of the target sequence were fitted with a CACC overhang (Table 2.4), allowing insertion into the Gateway entry vector (pENTR™/DS/D-TOPO; Table 2.3). The oligonucleotides specific to the 3'-end of the target sequence were designed to exclude amplification of the stop codon to allow transcriptional coupling with affinity tags introduced when recombined into the Gateway expression vectors (Table 2.3). The TOPO reaction of the purified amplicons with the Gateway entry vector was performed in a 1:1 ratio, as per the manufacturer's recommendations (Invitrogen). The insert of interest in the Gateway entry vector was recombined into a Gateway expression vector using the LR-reaction with a 1:1 ratio of the vectors included. The LR-reaction was performed as per the manufacturer's recommendations (Invitrogen).

2.4.10 Preparation of chemically competent *E. coli* cells

An ON culture of the *E. coli* strain of interest was grown from a single colony and diluted 1:100 in fresh LB media. Cultures were grown with shaking at 200 rpm until they reached early log-phase (OD_{600} = 0.45). Cells were washed once and the pelleted cells (4000 X *g* for 10 minutes at 4°C) were carefully resuspended in 0.15 volumes of cold 0.1 M $CaCl_2$ and incubated on ice for approximately 1.5 hours. The cells were then pelleted by centrifugation (4000 X *g* for 10 minutes at 4°C) and resuspended in 0.03 volumes (of the initial culture volume) of cold 0.1 M $CaCl_2$ containing 15% glycerol. Single aliquots of 50 µl were stored at -80°C.

2.4.11 Transformation of chemically competent *E. coli* cells

An aliquot of chemically competent *E. coli* cells (Section 2.4.10) was thawed on ice prior to use. Typically, 1 ng of purified plasmid DNA or up to 10 µl of ligation mix was incubated with cells on ice for 30 minutes. The cells were then heat-shocked by rapidly heating at 42°C for 45 seconds. The cells were then allowed to recover on ice for 2 minutes, followed by 5 minutes at RT. 950 µl of LB broth was added and cells were incubated at 37°C with shaking for at least 1 hour before plating on LB

agar containing appropriated antibiotic selection. Plates were incubated ON at 37°C and single colonies were purified for further analysis.

2.4.12 Preparation of electrocompetent *A. baumannii* cells

Competent *A. baumannii* cells were prepared freshly on the day of use. ON cultures grown from a single colony from solid LB media were diluted 1:100 in LB broth and grown at 37°C with shaking. Cells were grown until the culture reached OD_{600} = 0.4. These early log-phase cells were pelleted (4000 X *g* for 10 minutes at 4°C), resuspended in cold sterile mQ water and incubated on ice for approximately 1 hour. After pelleting (4000 X *g* for 10 minutes at 4°C), the supernatant was removed by decanting and cells were resuspended in the residual liquid.

2.4.13 Transformation of electrocompetent *A. baumannii* cells

Up to 2 µg of purified plasmid DNA (Section 2.4.1) was used for transformations. The competent cells (Section 2.4.12) and DNA were mixed and incubated on ice for 5 minutes followed by electroporation using a MicroPulser (Bio-Rad) at 2.5 kV, 200Ω and 25 µF. The cells were then allowed to recover on ice for 2 minutes, followed by 5 minutes at RT. 950 µl of LB was added and cells were incubated at 37°C with shaking for at least 1 hour before plating on LB containing appropriate antibiotic selection. Plates were incubated ON at 37°C and single colonies were purified for further analysis.

2.4.14 Polymerase chain reaction

Oligonucleotides (Table 2.4) used for PCR were synthesised by either GeneWorks (Adelaide, Australia) or Integrated DNA Technologies (USA). Standard PCRs were performed on a MultiGene (Labnet International inc.). Standard cycling conditions using purified DNA were; 2 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1 minutes/Kb at 72°C, followed by 72°C for 5 minutes. Initial denaturation was extended to 5 minutes when performing a colony screening PCR. For colony screening, a single colony was transferred to 500 µl mQ water. The suspension was vortexed and 5 µl was used in the PCR. MangoTaq (Bioline, Australia) or GoTaq (Promega) DNA polymerases were used for PCR screening purposes. Amplification of fragments used for cloning (Sections 2.4.8 and 2.4.9) or Sanger sequencing (Section 2.4.7) was performed using Velocity (Bioline, Australia)

Table 2.4: Oligonucleotides used in this study

Name [a]	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Reference / source**
Oligonucleotides for qRT-PCR analysis			
A1S_0109 (*abaI*)	TCTGGTGAGCAGGGA ATAGG	TGTCGTGGCTCAAG ACAGAG	This study
A1S_0329 (*pilB*)	AGAAGGAACCCCCAT ATTGC	CGTGACTTGGAAAC AGCAGA	This study
A1S_0395 (*abeM*)	TCATGCTCTTGTTCAG TGGC	GCATTGCCAATACC CCTAGA	This study
A1S_0895 (*FUR*)	GCGCAAAGCTGGACT TAAAG	CGGTAAACTGTCGC AAGTCC	This study
A1S_0897 (*pilT*)	CCGCGAGCGACTAAG C	TGTCGCAGCCCATG AA	This study
A1S_1292	ACGCAACGCGTAATA AAGTG	TAAAGGGTCAAAA GGCGAAC	This study
A1S_1336 (*paaA*)	CGTGCGATGGTACGT ATTTG	ACGGTTCACTGCAT CTTGTG	This study
A1S_1509	CCAAGGAAGGCGCTG T	TTGGGGAATGGCTT GC	This study
A1S_1647	GGACGCCATCGTCTCG	GCGTCCCGGCTTTG TA	This study
A1S_1925	GGTGGCGCGCTATTTG	GTTGCGCCATTGGG TA	This study
A1S_2080	GGTCGATGGCGTTCCA	CAGCCGCTTTCGTG GT	This study
A1S_2501 (*gapdh*)	CAACACTGGTAAATG GCGTG	ACAACGTTTTTCAT TTCGCC	This study
A1S_2562 (*abeM2*)	TTGCCATCAGTAGTGC AACC	TCCTGCAATCACAA CACCAT	This study
A1S_2565	TGGCTCGATATTCAAC GTCA	TAACAGCAAACCAC CACCAA	This study
A1S_3168 (*pilW*)	CTTACCGTATCACCTG CACC	CAACAGCAACAAA TGCCAAC	This study
A1S_3177 (*pilA*)	GTTTTCCCTGGGCTGA TTTC	GATCGTGGTTGCCA TTATCG	This study

Name [a]	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Reference / source**
A1S_3195 (*pilM*)	GCGCTCAACCGCGTAA	TGCCGGATCGTCTTGC	This study
A1S_3371 (*abeM3*)	CAGATCCAACTGTGGTGGTG	TCAGCATCGGTACGGTTACA	This study
A1S_3420 (*abeM4*)	GCCTTGCTTTACTTGTTCCG	GCATCAGTAAATGGGCAGGT	This study
A1S_r01 (*16SrDNA*)	CAGCTCGTGTCGTGAGATGT	CGTAAGGGCCATGATGACTT	(Higgins *et al.* 2004)
ABAYE1887	CCCTTTTGATGATTTTACGG	CAAGGCTTAAGCGCGGTA	This study
ABAYE1888	CCAGCGCATCACCACA	TCCGCTCGAACAACTCA	This study
ABAYE1889	GGGGCGATTTCAAGTGC	TCGCGATCAGCCAACA	This study
Oligonucleotides for cloning purposes			
A1S_0268	GAGAGGATCCATAAATATTAAGAAAATATATTAC	GAGAGGATCCTTAGATTAAGAAATCTTCAAG	This study
abeM_TOPO	CACCATGTCGAATGTCACGTCGTT	GGTTTGACTTAAACGTTTGGTATT	This study
abeM2_TOPO	CACCATGAATATGCTCAAAGACAT	GGTTGAAATGGTCTCACCAACTGG	This study
abeM3_TOPO	CACCATGGGCGATGGAGAATACAT	AATAAGAACTTTGATCTTCTTTTT	This study
abeM4_TOPO	CACCATGAACCAGATTTTTAAATT	AAAACCTCTTAACTGCTTTCTAAA	This study
adeM_TOPO	CACCATGAAATTTAATCTCTCTGAATG	TGCTGTTTTTTTCACCTTAAACC	KA Hassan
Gateway1	GAGATCTAGAACAAGTTTGTACAAAAAAGC	GAGACTCGAGTCAATGGTGATGGTGATGAT	This study
Gateway2	GAGAGGTACCACAAGTTTGTACAAAAAAGC	GAGATCTAGATCAATGGTGATGGTGATGAT	This study

Name [a]	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Reference / source**
Oligonucleotides for screening drug transporters in clinical *A. baumannii* isolates			
A1S_0116	AAGCATTGGCTCAGAAGG	CAAGACAATACGGCGGTG	KA Hassan
A1S_0188	CATTCCTGCAATGGCACAC	AAACGGTCAGCCAACCATC	KA Hassan
A1S_0395	ATTGCGGTAGGTGTAGGC	TGGCGGACAATCACTGG	KA Hassan
A1S_0519	TGTGGTACGTGCAGCATC	TCTGCCAATGGTGGAATC	KA Hassan
A1S_0563	CGGCACATCATTTACCCG	AATAGCCCGCAACCCATC	KA Hassan
A1S_0596	CATCTGCATGGGTTGGGTC	TTGCATAAAGCTCACGTGG	KA Hassan
A1S_0710	TGTCTGAAGGTTTTACACGC	ATTGCACCAATGCCTGTCC	KA Hassan
A1S_0775	ATTTGGTGGCCTTGTTGGC	GTGATGGCTGCACTACG	KA Hassan
A1S_0801	GCTGTGGCGACCATACTC	TTCCACCACAACTGCTTG	KA Hassan
A1S_0802	AATTGTCGCTGCTCTTGC	CGTCCGACACGAGAGAAC	KA Hassan
A1S_0909	TATACCGCGACGTGGG	TTAAACCAAAGCTCGCCAG	KA Hassan
A1S_0964	AAAATTGTAGGCGCCGTG	AATCAGGCCAACCTCCG	KA Hassan
A1S_1231	ACCGTTAAGTGCACCACCC	ATTAACAGTGCTGCCAAGC	KA Hassan
A1S_1242	CGAGCTGTTCTTGAACTG	CCCCTTGGCTCACTTG	KA Hassan
A1S_1418	CTATGTGTCGGGGCCTATC	GCTGCAACAATTCCACCTG	KA Hassan
A1S_1535	CCATTGCTCAAATGGACCG	AGAAAACCGCCCGTTTGAC	KA Hassan
A1S_1555	AAGAGTCAGAACAAGAGCG	TAGAGCCATTGCACAAGC	KA Hassan

Name [a]	Forward primer (5'-3')	Reverse primer (5'-3')	Reference / source
A1S_1649	TTCCTAAGCCAGCGTTTTG	ATCCGCTACAAAAGCCTTG	KA Hassan
A1S_1671	TCGGATTGTACAAGCCCAG	GTCGCCTATTTGCCACTAG	KA Hassan
A1S_1672	TGCCTGAAGCAGAACAG	AGCAGTTGCTATGGTGGTC	KA Hassan
A1S_1673	TCAGCTTTAACGTGGCTG	ACAGGTGTCAGTAAGCCTG	KA Hassan
A1S_1750	GTCATGGGTTCAAGCGGTC	TTCACCCGATGACGTATCG	KA Hassan
A1S_1772	ATGGCACTGGATGTTCCTG	CCCACCCAAAAACATCGCC	KA Hassan
A1S_1799	GCTTTAAGTGCTGCGTTAG	GCATGGTACTAAGGCTTGC	KA Hassan
A1S_2057	CCATGATTGCTTTGGTTGGC	ACCTGCTAATGGACCACCT	KA Hassan
A1S_2124	TACAGACACGCCTCATGG	GGCTCAGTACCCAACCACC	KA Hassan
A1S_2198	TGCTGTTTCAGCTATAGGC	CAAGCTGTAAGGGGTGGG	KA Hassan
A1S_2298	TGCGATTGCTTGTGAAGTT	CCTGACCAAATGGCATA	KA Hassan
A1S_2305	GCTTAAACCCAAGGGAGGC	TTGCCCTGTTAAGCCTGTC	KA Hassan
A1S_2311	GCTGAACCTATTGCCCCAC	GCTGCGAACACGAATACC	KA Hassan
A1S_2312	AATGCTTTCCGTTTCGGC	TCACGCATTCCTGAACCAC	KA Hassan
A1S_2375	TTAATTGGACGTGCAGCAG	GGGTGTAATGGCTCAGGC	KA Hassan
A1S_2376	AGATGAGCCAACGAGTGC	CTGTTGTCGGCATGAGC	KA Hassan
A1S_2378	CGGCATTCGTGATAATCG	AACAGCAGCGAGTGTTGC	KA Hassan
A1S_2474	CATGCTACTGCTTTGCTC	GGCAAACCCAATGGTGGC	KA Hassan

Name [a]	Forward primer (5'-3')	Reverse primer (5'-3')	Reference / source
A1S_2502	TTAGGGGGCTTTTGGTGGG	CGCAATAACCAACCCGAG	KA Hassan
A1S_2561	TACGCGGTTATTCGTTGG	TAACCACGTTGCTTGTCG	KA Hassan
A1S_2562	AACAGGACTTGCCACTGC	CCAATCGGTAGACCTTGC	KA Hassan
A1S_2584	GTTGCCTGCTTTAGGTGAG	TCCAATCGCATCGGAAAG	KA Hassan
A1S_2621	TTTAGCAGACTATGCTGGC	TACGGCTGGAACCATGAAG	KA Hassan
A1S_2622	CCCCTATTCGTTTTGCAGG	GAACAGCGACTAGCAAACC	KA Hassan
A1S_2660	ATTGCCAGAGTTGAGCTAG	TGGTGGATGAGCTTAGAGG	KA Hassan
A1S_2795	TGTACTTACCTGCGTTGCC	ACGGTCGCTTAAAGGTCC	KA Hassan
A1S_2818	GCTGATGGGGCTGGTAAC	TGGGCGTAAACGGGTTTTG	KA Hassan
A1S_2844	ATTCAGCTAAAGGCAATGG	CCACCCCAGACCACAACAC	KA Hassan
A1S_3117	ATTTCACTGGCTGGGTCAG	ATAAGCACTTGCAACAGCC	KA Hassan
A1S_3146	TTCAGCAGTGGGATATGCC	AAAGCTCCAAGTACAGGCC	KA Hassan
A1S_3272	GTACCCGCAGTGACTATGG	CCATACCGACACGGCC	KA Hassan
A1S_3288	TCCAATCCAGTCGAAAGCC	AGACACTCCATCCAATCGC	KA Hassan
A1S_3371	GTTTCGGAGCTCATGCC	AACACGTGCGAGTAGGTC	KA Hassan
A1S_3420	CGGAAGCTTATGGTTGCC	CGACATGTTCAGATGCACC	KA Hassan
A1S_3440	TGCACCGTTTATGACCCTG	GGCTTGTAATGAGACGTGC	KA Hassan

Name [a]	Forward primer (5'-3')	Reverse primer (5'-3')	Reference / source
A1S_3445	CCGTCCGATCATTCTCAC	ACCACGCTGCATACAATG	KA Hassan
A1S_3446	TACCTATTGCCGGCTCAG	CCAAGGTGCTTGATGACC	KA Hassan
Oligonucleotides for sequencing purposes			
A1S_0268	GAGAGGATCCATAAATATTAAGAAAATATATTAC	GAGAGGATCCTTAGATTAAGAAATCTTCAAG	This study
A1S_0628	AGCAACTGAAGCTGAAATTCG	TTGGTTCCGAATTAGACTTGC	This study
A1S_1200	GAGAGGATCCTTATTGCCCAGAACGAATAATG	GAGAGGATCCAATCTTTACAGCCATATAAC	This study
A1S_1893	GAGAGGATCCTTACCCTTGAGTCGCATCAATC	GAGAGGATCCTAGATAATGTTCGATAGTCG	This study
A1S_3222	GAGAGGATCCTTATGATTTCCATCGTACGG	GAGAGGATCCTTATACAGATGAAAGTGCTTG	This study
AB57_1747	CACCCATGATATTTTACCATATGG	TTGATAAATAATCGTGTATTTC	This study
AB57_2007	CACCATGAAAAAACTTGCTTTGATC	TTGATAGATAATTGTGTATTG	This study
AB57_2423	CACCGGGTTAGAATGGGTAATTG	ATAAGTTACTGTGACTGTCAC	This study
M13	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC	Promega
pEX18	CCTCTTCGCTATTACGCCAG	GTTGTGTGGAATTGTGAGCG	This study
Oligonucleotides used for international clone determination			
Group1ompAF306	GATGGCGTAAATCGTGGTA	CAACTTTAGCGATTTCTGG	(Turton *et al.* 2007)
Group1csuEF	CTTTAGCAAACATGACCTACC	TACACCCGGGTTAATCGT	(Turton *et al.* 2007)

Name [a]	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Reference / source**
Gp1OXA66F89	GCGCTTCAAAATCTGATGTA	GCGTATATTTTGTTTCCATTC	(Turton *et al.* 2007)
Group2ompAF378	GACCTTTCTTATCACAACGA	CAACTTTAGCGATTTCTGG	(Turton *et al.* 2007)
Group2csuEF	GGCGAACATGACCTATTT	CTTCATGGCTCGTTGGTT	(Turton *et al.* 2007)
Gp2OXA69F169	CATCAAGGTCAAACTCAA	TAGCCTTTTTTCCCCATC	(Turton *et al.* 2007)

[a] Oligonucleotides are grouped under experimental subheading.

or Pfu (Stratagene, Integrated Sciences, Australia) DNA polymerase. Amplicons were analysed by gel electrophoresis (Section 2.4.4).

2.4.15 Quantitative reverse transcription-PCR

cDNA was synthesised using random hexamers (GeneWorks, Australia) and M-MLV reverse-transcriptase (Promega) according the manufacturer's recommendations. Oligonucleotides used in this study were designed using primer3 (Rozen and Skaletsky 2000) as an integrated part of UGENE v1.6.1 (Unipro) and are listed in Table 2.4. qPCR was performed on a Rotor-Gene RG-3000 (Corbett Life Science) using DyNAmo SYBR® green qPCR kits (Finnzymes, Australia). A typical qPCR run included; 10 minutes at 95°C, 40 cycles of 10 seconds at 95°C, 15 seconds at 55°C and 20 seconds at 72°C. The run was completed with melting temperature analysis using 0.5°C increments from 72°C to 95°C. The results were used for identification of non-specific amplicons. Transcriptional differences were calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). Both *gapdh* (A1S_2501) and *16srDNA* (A1S_r01) were used as reference genes for which the oligonucleotides have been listed in Table 2.4.

2.5 Microarray analyses

2.5.1 Array development

The 8 x 15K custom genomic microarray for *A. baumannii* ATCC 17978 was developed by Dr. KA Hassan (Macquarie University, Sydney, Australia) on the Agilent platform using the Agilent eArray package (http://earray.chem.agilent.com/earray/). At least four 60 mer DNA oligonucleotides with an average GC content of 41.5%, were incorporated into the design for each of the protein coding genes annotated in the ATCC 17978 genome sequence (Smith *et al.* 2007). The array also included a set of intra-array controls; 132 probes replicated at least 10 times in the design, and the Agilent control spots.

2.5.2 cDNA synthesis and microarray hybridisation

For the microarray analyses, the cDNA synthesis, labelling and hybridisations were conducted at the Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Australia. Total RNA was reverse transcribed and labelled with either Cy3 or Cy5 using the Agilent Fairplay Microarray Labelling kit (Stratagene). Labelled cDNA samples were hybridised to a custom designed 8 x 15K two colour

gene expression microarray slide (Section 2.5.1). The results reported are based on three biological and four technical repeats, including one dye-swap experiment. Statistical analysis was performed on Log_2-transformed signal ratios of the replicate spots using the statistical analysis of microarray (SAM) algorithms (Tusher *et al.* 2001). All results described were found to be significant using a false discovery rate of less than 5% unless otherwise indicated. All microarray data presented are in accordance with the Microarray Gene Expression Data Society's minimum information about microarray experiment recommendations (Brazma and Vilo 2001). The iron-limitation quantification data have been deposited into the gene expression omnibus database (http://www.ncbi.nlm.nih.gov/geo/) and can be accessed using the accession number GSE24921.

2.6 Protein detection

2.6.1 Sample preparation

Acinetobacter and *E. coli* cells were cultured as described in Sections 5.2.1.1 and 6.2.3, respectively. For *Acinetobacter*, cells were collected from semi-solid MH media by pipetting with 5 ml PBS. After pelleting using a GSA rotor in a RC-5B Superspeed centrifuge (Sorvall) (4000 X *g* for 10 minutes at 4°C), cells were resuspended in 30 ml crushing buffer (Table 2.1). The cell suspension was passed through an EmulsiFlex-C5 homogeniser (Avestin Inc., Ottawa, Canada) at 20,000 psi up to 3 times. Insoluble cell particles were removed by centrifugation using a SS-34 rotor in the RC-5B Superspeed centrifuge (Sorvall) (27,000 x *g* for 15 minutes at 4°C). The supernatant was transferred to a new tube and the membrane fraction was pelleted by ultra-centrifugation using a SW 32Ti rotor in an Optima™ L-100 XP Ultracentrifuge (Beckman-Coulter) (125,000 x *g* for 1 hour at 4°C). The membrane fraction was resuspended in membrane resuspension buffer (Table 2.1).

2.6.2 DC-BCA protein assay

The protein concentration of the sample obtained in Section 2.6.1 was examined using a detergent compatible (DC) bicinchoninic acid (BCA) protein assay (Bio-Rad), which is a modified Lowry assay. The reagents were used according to the manufacturer's recommendations. Assays were performed in microtiter trays and samples of bovine serum albumin at known concentrations were used for reference. Concentrations of the sample were determined as per comparison to the standard curve generated from the bovine serum albumin references.

2.6.3 SDS-PAGE

Protein samples in membrane resuspension buffer (Section 2.6.1; Table 2.1) were incubated at 37°C for 30 minutes with 1 volume of 2X SDS-PAGE sample buffer (Table 2.1) prior to electrophoresis. Precast 12% SDS-PAGE gels (PAGEgel, Inc. San Diego, CA, USA) were loaded with the pre-incubated sample. The precision Plus Dual Color (Bio-Rad) molecular weight marker was included to give an indication of the protein molecular mass. The gels were electrophoresed at 150 V until the dye front had migrated to the bottom of the gel. Gels were stained or the protein content was transferred to a membrane as described in Sections 2.6.4 and 2.6.5, respectively.

2.6.4 Coomassie stain

SDS-PAGE gels (Section 2.6.3) were removed from the holders and placed in Coomassie stain (Table 2.1) for a minimum of 4 hours. The gel was incubated ON in destain solution (Table 2.1) and scanned using a dual-lens flatbed scanner (Epson perfection V700).

2.6.5 Protein transfer to polyvinylidine fluoride membrane

After protein separation using SDS-PAGE (Section 2.6.3), gels were removed from the casket and rinsed in 1X transfer buffer (Table 2.1). A polyvinylidine fluoride (PVDF) membrane (Hybond-P™; Amersham Pharmacia Biotech) was cut to the same size of the gel and soaked in 100% methanol for 10 seconds and placed on top of the acrylamide gel after a brief rinsing with transfer buffer. Two pieces of Whatman paper were soaked in transfer buffer and used to sandwich the gel and PVDF membrane. This assembly was loaded into a transfer system (PAGEgel) with the membrane facing the anode, 30 mA was applied ON to allow all proteins to migrate to the membrane. The membrane was stored in 1X TTBS buffer (Table 2.1) until further use.

2.6.6 Immunological detection of recombinant proteins

The PVDF membrane containing the transferred proteins (Section 2.6.5) was incubated in blocking buffer (Table 2.1) for 1.5 hours. After washing in TTBS, the membrane was incubated in TTBS containing a 1:5000 dilution of rabbit anti-X6 HIS epitope tag antibodies (Rockland, Gilbertsville, USA) for at least 1.5 hours. The membrane was washed for 30 minutes in TTBS and during which the buffer was

replaced several times. The washed membrane was then incubated for 1 hour in a 1X TTBS solution containing a 1:10,000 dilution of goat-anti-rabbit secondary antibodies (Rockland) and washed thoroughly for 30 minutes with at least six buffer replacements. Chemiluminescence detection solutions 1 and 2 (Table 2.1) were mixed (1:1) and pipetted on top of the membrane and incubated for 1 minute. After removal of the chemiluminescence detection solution the membrane was wrapped in a transparent plastic film and inserted into a X-omatic developing cassette (Kodak) with an ECL Hyperfilm™ (GE Healthcare) in a dark room. After a 2 minute exposure, the film was developed using the X-omat processor 1000 (Kodak). The developed film was scanned using a dual-lens flatbed scanner (Epson perfection V700). Cross-reactivity of the goat-anti-rabbit secondary antibodies with the markers, which were included during SDS-PAGE (Section 2.6.3), allowed visualisation of protein weight estimates in kDa.

2.7 Bioinformatic analyses

2.7.1 Alignments and *in silico* manipulations

Alignment and manipulation of DNA sequences of plasmids, inserts for cloning or sequenced fragments were performed with Sequencher™ 4.9 (GeneCodes). Multiple sequence alignments of DNA or amino acid sequences were generated using the on-line version of ClustalW2 and phylogenetic trees were obtained using the Neighbour-joining clustering method (Saitou and Nei 1987). Alignments were visualised by CLC sequence viewer (CLC bio) and phylogenetic trees using TreeView 1.6.6 (Roderic D. M. Page, 2001).

2.7.2 Genomic DNA analyses

Individual GenBank files were visualised and interpreted using UGENE v1.6.1 (Unipro). The scaffold sequences of *A. baumannii* strains ATCC 19606, WM99c, D1279779, ATCC 17978 (sequenced during the course of this study, as described in Section 2.4.7) and 17978hm were tiled to the ATCC 17978 (CP000521) genome using Mauve version 2.3.1 (Darling *et al.* 2004). Mauve was also used to generate multiple genome alignments. More specific searches for nucleotide sequences, proteins or protein domains were performed using Blastn (NCBI), Blastp (NCBI) and the conserved domain database (NCBI) (Marchler-Bauer *et al.* 2011). Identification of genes encoding putative membrane transport proteins in the *A. baumannii* ATCC 17978 genome was performed by Dr. LDH Elbourne (Macquarie University,

Sydney, Australia), using the transautomated annotation pipeline (TransAAP; www.membranetransport.org).

2.7.3 Motif and DNA binding site analyses

For identification of *A. baumannii* FUR binding sites (Section 3.2.3), a scoring matrix was defined from the 48 experimentally determined *E. coli* FUR binding sites (Stojiljkovic *et al.* 1994) using the multiple em for motif elicitation (MEME) tool (Bailey and Elkan 1994). The *A. baumannii* ATCC 17978 genome was analysed with the resulting scoring matrix using the motif alignment and search tool (MAST) (Bailey and Gribskov 1998). Putative FUR binding site sequences that are located within the 200 bp region upstream a start codon, with a probability value (p-value) (Bailey and Gribskov 1998) of less than $10E^{-5}$ and of which the downstream gene showed more than 4-fold up-regulation were further investigated. Subsequent MEME and MAST analyses with the described criteria were performed until no new positive hits were obtained. The resulting 21 putative FUR binding site sequences were aligned using Weblogo 3.0 (Crooks *et al.* 2004) to create the optimised *A. baumannii* FUR motif.

Analysis of AbaR binding sites was performed using the methodology described above. A scoring matrix was created with the *Vibrio fischeri* LuxR binding sites (Antunes *et al.* 2008).