CLONING AND CHARACTERIZATION OF AdeMNO RND EFFLUX PUMP OF *Acinetobacter baumannii*

Submitted by

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CERTIFICATE OF APPROVAL

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The undersigned certify that the student has presented her thesis, that the thesis is acceptable in form and content and that a satisfactory knowledge of the field covered by the thesis was demonstrated by the candidate through an oral examination. They recommend this thesis to the Office of Graduate Studies for acceptance.

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ABSTRACT

Acinetobacter baumannii is an opportunistic pathogen which has been implicated in a variety of nosocomial infections among immunocompromised patients worldwide. Recently, Multi-drug resistant (MDR) isolates of *A. baumannii* have been isolated from military personnel returning from service in Iraq and Afghanistan. Antibiotic resistance of *A. baumannii* has limited the number of active antibacterial, making very difficult to treat these types of infections. This work investigated the role of Resistance-Nodulation-cell Division (RND) efflux pumps in the antibiotic resistance mechanism of *A. baumannii*. Expression of six different RND pumps was analyzed in clinical isolates of *A. baumannii*. A novel RND family pump, AdeMNO, was found to be present in a majority of isolates. The *adeMNO* operon was cloned, sequenced, and characterized using the single copy gene expression system in an efflux sensitized surrogate *Pseudomonas aeruginosa* strain. Antibiotics, trimethoprim, chloramphenicol, and clindamicin were identified as the substrates of this pump. In order to understand the mechanisms of regulation of *adeMNO* operon, a putative regulator belonging to the *lysR*-family was identified, cloned, and sequenced from the upstream region of the operon. Promoter regions of the *adeMNO* operon were also sequenced from various clinical isolates and sequence polymorphisms identified that could be implicated in the regulation of *adeMNO* expression.

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To my friends, you made my life at the school fun and I certainly believe that you were a good part of my learning.

I dedicate this work

to my family,

Marco, Isabel and Francisco

To Marco for the enormous opportunity to have you in my life

To Isabel and Francisco this project is an example that with effort dreams do come true.

To my far away family

Juana, Hernan, Lilibeth and Ivan

Thanks for keeping the family together in heart and spirit.

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LIST OF ABBREVIATIONS

*aacC*1	acetyltransferase 3-1 encoding gene conferring Gm-resistance
A/C	amoxicillin/Clavulanic acid
AHL	N-Acyl-Homoserine Lactone
AMK	amikacin
Amp	ampicillin
ATCC	American Type Culture Collection
β-gal	*β*-galactosidase
BLAST	Basic Local Alignment Search Tool
bla	gene encoding *β*-lactamase
bp	base pair(s)
CAN-ICU	Canadian National Intensive Care Unit
CAZ	ceftazidime
Cb	carbenicillin
CEF	cefepime
CFZ	cefazolin
CIP	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CTX	cefotaxime

CXM	Cefuroxime
CXM-AX	cefuroxime-axetil
Δ	Deletion
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleoside triphosphate(s)
Flp	*S. cerevisiae* recombinase
FRT	Flp Recombinase Target
Gm	gentamicin
IPM	imipenem
IPTG	Isopropyl-β-D-Thiogalactoside
Kb	Kilobase(s) or 1000 bp
Km	kanamycin
Kv	Kilovolts
LB	Luria–Bertani (medium)
laqIq	*lacI* gene with q promoter-up mutation
lacZα	*β*-Gal *α* peptide-encoding gene
MF	Membrane Fusion
MHB	Muller Hilton Broth
min	minute(s)
µF	Microfarad
µL	microliter(s)
mL	millilitre(s)
mM	Millimolar
NCCLS	National Committee on Clinical Laboratory Standards

NF	nitrofurantoin
ng	nanograms
OD_{600}	Optical Density at 600nm
oligo	oligodeoxyribonucleotide
OM	Outer Membrane
ori	origin of replication
oriT	origin of transfer
PCR	Polymerase Chain Reaction
PIP	piperacillin
r	resistance/resistant
RNA	Ribonucleic acid
RND	Resistance Nodulation Cell Division
RT	room temperature
sacB	*B. subtilis* levansucrase-encoding gene
sec	second(s)
spp	specie
SXT	Trimethoprim-sulfamethoxazole
TCN	Tetracycline
TIG	Tigecycline
TOB	Tobramycin
TZP	Piperacillin-tazobactam
X-gal	5-bromo-4-chloro-3-indolyl-*β*-D -galactopyranoside

CHAPTER 1

INTRODUCTION

1.1. *Acinetobacter baumannii*

Acinetobacter baumannii is a non fermentative, Gram-negative, non-motile rod-shaped bacterium that has become a recurrent microorganism present on the skin of healthy individuals, especially healthcare personnel. There is a great diversity within the genus *Acinetobacter* making differentiation of species difficult; however, *A. baumannii* is the specie most commonly isolated from health care facilities and it is responsible for 80% of the infections reported in Intensive Care Units (Cisneros *et al*., 1996; Fournier and Richet, 2006; French, 1980).

For many years the Genus *Acinetobacter* spp. received little or no attention from the health care community; these species were considered low risk bacteria until the 1970s-1980s, when its clinical significance began to increase (Abrutyn *et al*., 1978; French *et al*., 1980). This change in the pathogen behavior has been connected to the introduction of potent broad-spectrum antibacterial used after complex invasive procedures that confined patients to ICUs (Bergogne-Berezin and Towner, 1996). This modification has caused an increase in the selection pressure on bacteria like *A. baumannii,* which has resulted in a notable raise in the number of bacteria resistant to the most commonly use antibacterials. Since the 1970s, the spread of multidrug-resistant (MDR) *Acinetobacter* strains have become a concern among the medical community worldwide (Dijkshoom *et al*., 2007).

A. baumannii has been linked to a variety of nosocomial infections including pneumonia, bacteremia, meningitis, urinary tract infections, as well as skin and soft tissue infections (Fournier and Richet, 2006; Dijkshoom *et al*., 2007). The mortality associated with these pathogens is notably high among immune compromised individuals (Jain, 2004). Most recently, military members injured in Afghanistan and Iraq, have returned to

Canada and United States infected with MDR *A. baumannii* (Tien *et al*., 2007). This has triggered an increase in the number of nosocomial infections among civilian patients exposed to the pathogen in healthcare facilities (Centers for Disease Control and Prevention (CDC), 2004; Hujer *et al*., 2006). Perez *et al*. (2007) reported the existence of a notable increase in community-acquired infections linked to MDR *A. baumannii* during the last decade revealing the severity of the problem and the urgent necessity of finding a solution.

1.2. EPIDEMIOLOGY AND ANTIBIOTIC RESISTANCE

Epidemiological studies have reported the presence of this pathogen among a variety of surfaces, from human skin to inert elements like bed railing and health care equipment (Cisneros *et al*., 1996; Corbella *et al.,* 1996; Houang *et al.*, 2001; Dent *et al*., 2010). Berlau *et al* (1999) observed that 40% of the 192 healthy volunteers participating in their study carried *Acinetobacter* spp. This pathogen has also been reported among body lice obtained from homeless individuals in France (La Scola and Raoult, 2004), and blood of patients treated in hospitals tending military personnel injured in Afghanistan and Iraq-Kuwait (Centers for Disease Control and Prevention (CDC, 2004). Furthermore, epidemiological studies found that *Acinetobacte*r species grew in 17% of fruit and vegetables collected from hospital suppliers, from this group 56% of the isolates corresponded to *A. baumannii* (Berlau *et al*., 1999 a, b). *Acinetobacter* spp. has been recognized for its extraordinary capacity to survive for long periods of time, up to 5 months, on inanimate elements in health care facilities (Bergogne-Berezin and Towner, 1996; Getchell-White *et al*., 1989; Schreckenberger *et al*., 2007). A study published by

Dent *et al*. (2010) described the colonization of a large variety of hospital equipment and room paraphernalia by *A. baumannii*, these observations resulted in a direct correlation with the number of colonized and infected patients in these institutions (Dent *et al*., 2010).

Finally, one of the most important factors that have made of *A. baumannii* a significant threat to hospitalized patients is its ability to acquire resistance to clinical relevant antimicrobial agents. MDR *A. baumannii* has become an emergent cause of outbreaks worldwide (Juni, 1972; Fournier *et al*., 2006; Iacono *et al.*, 2008). Several researchers have reported a higher number of outbreaks involving resistant *A. baumannii* in comparison with other species of *Acinetobacter,* like *Acinetobacter lwoffii, Acinetobacter johnsonii, and Acinetobacter junii,* which are sporadically implicated in these medical incidents (Dijkshoorn *et al.,* 1996; Jawad *et al.,* 1998; Heinemann *et al*., 2000). Infections caused by *A. baumannnii* have become very difficult to treat due to the growing number of MDR strains showing low susceptibility to β-lactams, aminoglycosides, fluoroquinolones, and more recently carbapenem (Afzal-Shah *et al*., 2001; Valenzuela *et al*., 2007; Mark *et al*., 2009).

Drug resistance in *A. baumannii* has been attributed to intrinsic mechanisms present in Gram-negative species, and to its extraordinary capacity to acquire genetic information horizontally by accumulating resistance factors (Magnet *et al*., 2001; Lopez-Hernandez *et al.,* 2001; Mammeri *et al*., 2003; Heritier *et al*., 2005; Poirel *et al*., 2010). In addition, a number of intrinsic mechanisms have been identified in *A. baumannii* including β–lactam resistance, reduced membrane permeability, drug bypass and presence of strong efflux systems, which in concert confer an effective resistance mechanism (Magnet *et al* 2001; Nordmann *et al*. 2005, Ribera *et al* 2003; Spence et Towner*,* 2003; Turton *et al*. 2004; Huys *et al*. 2005; Mussi *et al* 2005; Damier-Piolle *et al*. 2008). Recent studies have demonstrated that the presence of an active efflux system, many with a

broad specificity, plays a central role in the innate resistance of Gram-negative bacteria (Coyne *et al*., 2010; Damier-Piolle *et al*., 2008; Lin *et al*., 2009). These reports emphasize the possibility of using these structures as a potential drug target to develop a more effective treatment against MDR bacteria.

1.3. RESISTANCE-NODULATION CELL-DIVISION (RND) EFFLUX PUMPS

Multidrug efflux pumps belonging to the Resistance-Nodulation cell-Division (RND) family has been identified as the most common and important efflux system among MDR Gram-negative bacteria (Poole *et al.,* 1993; Ma *et al*., 1995; Li *et al.,* 2002; Lin *et al*., 2009). Recent data suggests that RND efflux pumps are also important in pathogenicity and/or survival of these bacteria in a particular ecological niche. Some of the possible roles are cell detoxification by eliminating toxic metabolic by-products from the cytoplasm (Helling *et al*., 2002), host colonization and secretion of virulence factors increasing pathogenicity of these strains (Brown *et al.*, 2007; Lin *et a*l., 2005; Hirakata *et al*., 2002), and cell-cell communication which facilitate a coordinated response of the bacterial colony (Chan et al., 2007). Even though the natural function of the RND efflux pumps in Gram-negative bacteria is a fascinating topic, it is undeniable that their contribution to the resistance mechanism is a more urgent matter.

Three RND pumps have been characterized in *A. baumannii*, AdeABC, AdeDE, and AdeIJK. AdeIJK contributes to resistance to β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, safranin, pyronine, and sodium dodecyl sulphate (Darmier-Piolle *et al*., 2008). AdeABC has been reported to be responsible of aminoglycoside resistance

and is involved in the decrease of the level of susceptibility to drugs including fluoroquinolones, tetracyclines, amikacin, chloramphenicol, cefotaxime, erythromycin, gentamicin, kanamycin, norfloxacin, netilmicin, ofloxacin, perfloxacin, sparfloxacin, tobramycin, trimethoprim, and ethidium bromide, and more recently, tigecycline (Magnet *et al*., 2001; Ruzin *et al*., 2007; Hornsey *et al*., 2010). In addition, AdeDE is responsible for reduced susceptibility to amikacin, ceftazidime, ciprofloxacin, meropenem, and rifampin (Chau *et al.,* 2004; Magnet *et al.,* 2001).

1.4. STRUCTURE OF RND EFFLUX PUMPS

AcrAB-TolC identified in *E. coli* is the most thoroughly studied RND efflux pump, and its structure is commonly used to explain the general efflux mechanism of RND pumps. First reported in 1993 (Ma e*t al*., 1993), this pump has a broad variety of substrates including acriflavine, β-lactams, fluoroquinolones, chloramphenicol, bile salts, crystal violet, ethidium bromide, fatty acids, macrolides, organic solvents, and SDS (Lin *et al*., 2009; Hornsey *et al*., 2010; Ruzin *et al*., 2007; Ma e*t al*., 1993).

Studies of AcrAB-TolC efflux pump structure revealed the AcrB protein is embedded in the inner membrane, associated with an outer-membrane channel and a periplasmic adaptor protein, TolC and AcrA respectively (Fig. 1.1) (Ma *et al.,* 1993). The *E. coli* outer-membrane protein, TolC, has been identified as a trimer of two joined barrel-like structures that serves as a channel through the outer-membrane during the extrusion of the substrates (Koronakis *et al*., 2000). This protein contains a 12-strand β-barrel configuration which forms a 30Å pore, attached to these strands of β-structures there is an unusual α-helical barrel that extends through the periplasm allowing TolC to reach the

docking domain of AcrB (Murakami *et al.,* 2002). AcrB, which is inserted into the inner membrane, has been described as a homotrimer with three-fold rotational symmetry (Murakami *et al.,* 2002). This protein is composed of 1,100 amino-acids, and it possesses a large periplasmic domain of at least the same size as the transmembrane (TM) domain, the last one divided into 12 segments. The top portion of the periplasmic domain of AcrB has similar dimensions to the tip of TolC binding domain, supporting the idea of a close assembly between AcrB and TolC inside the periplasm (Touze *et al.,* 2004; Koronakis *et al.,* 2000; Murakami *et al.,* 2002). The coordinated operation between AcrB and TolC appears to be mediated by AcrA, this periplasmic protein has been identified as a member of the membrane fusion protein (MFP) family (Tikhonova and Zgurskaya, 2004). The AcrA fragment is composed of three domains: β-barrel, lipoyl, and α-helical hairpin. According to current research, this last structure possesses extraordinary flexibility, relevant characteristic in the coupling mechanism associated with the functionality of the efflux pump tripartite structure (Touze *et al.,* 2004; Tikhonova and Zgurskaya, 2004; Mikolosko e*t al.,* 2006).

The analysis of the recently available genome sequence of *A. baumannii* ATCC17978 reveals the presence of three other RND complex-encoding operons, namely *A1S_2304-A1S_2305-A1S_2306*, *A1S_2817-A1S_2818 and A1S_3219-A1S_3218-A1S_3217-A1S_3214* in addition to the previously described AdeABC (Magnet *et al,* 2001), AdeIJK (Damier-Piolle *et al*., 2007), and AdeDE (Chau *et al*., 2004) pumps (Fig. 1.2). Indeed, while some of the recent studies have shown that AdeABC, AdeIJK, and AdeDE pumps are expressed extensively in the clinical isolates of *A. baumannii* (Chu *et al*., 2006; Lin *et al*., 2009), there are also reports of multidrug resistant strains of *A. baumannii* that do not express any of these three previously characterized

RND pumps (Lin *et al*., 2009). These observations suggest that yet uncharacterized pumps may be involved in the antibiotic resistance mechanism of these microorganisms.

In summary, RND efflux pumps are a great example of adaptation mechanism present in Gram-negative bacteria. The knowledge obtained during this study will contribute to the understanding of RND efflux pumps, as well as their participation in the global mechanism used by this microorganism to overcome adverse conditions in clinical setting. Finally, this research will supply additional information to create an appropriate approach to the MDR phenomenon.

Figure 1.1. Structures of the proteins constituting the tripartite AcrAB-TolC efflux machinery. TolC (OMP) forms a pore in the outer-membrane which is extended by a long periplasmic channel facilitating the compounds extrusion from the cell. AcrA (MFP) mediates the contact between AcrB and TolC. AcrB (RND efflux pump) inserted into the inner membrane, is responsible for substrate recognition/selection as well as for energy transduction. Drug molecules are captured from the periplasm side of the inner membrane, then transferred through the periplasmic porter (pore) domain towards the funnel of TolC and extruded into the external media (Eswaran *et al*., 2004) (Reproduced with permission from the publisher, Appendix 4).

Figure 1.1.

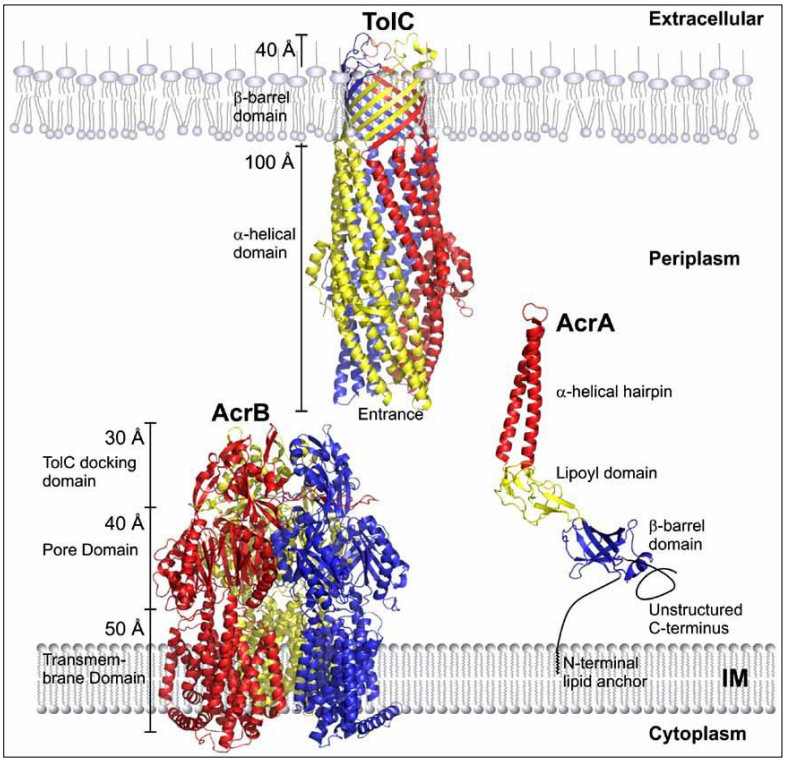
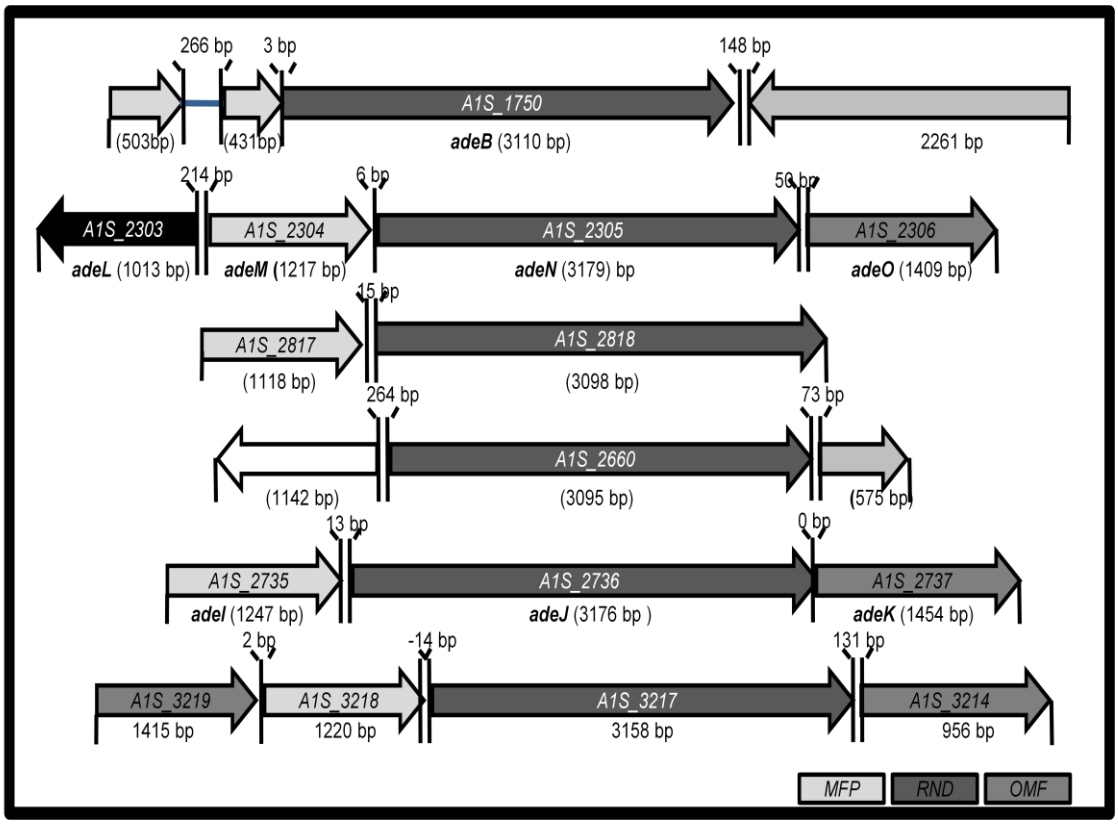

TolC
Extracellular
40 Å
β-barrel domain
100 Å
α-helical domain
Periplasm
AcrA
α-helical hairpin
AcrB
Entrance
30 Å
TolC docking domain
Lipoyl domain
40 Å
Pore Domain
β-barrel domain
Unstructured C-terminus
50 Å
Transmem-brane Domain
N-terminal lipid anchor
IM
Cytoplasm

Figure 1.2. Genetic organization of RND complex-encoding operons in the *A. baumannii* ATCC17978 genome. Genes encoding RND pump components are shown in shaded block arrows, while genes shown in white block arrows do not show any homology to any of the three RND complex-encoding genes.

Figure 1.2.


266 bp
3 bp
148 bp
A1S_1750
(503bp)
(431bp)
adeB (3110 bp)
2261 bp
214 bp
6 bp
50 bp
A1S_2303
A1S_2304
A1S_2305
A1S_2306
adeL (1013 bp)
adeM (1217 bp)
adeN (3179) bp
adeO (1409 bp)
15 bp
A1S_2817
A1S_2818
(1118 bp)
(3098 bp)
264 bp
73 bp
A1S_2660
(1142 bp)
(3095 bp)
(575 bp)
13 bp
0 bp
A1S_2735
A1S_2736
A1S_2737
adeI (1247 bp)
adeJ (3176 bp)
adeK (1454 bp)
2 bp
-14 bp
131 bp
A1S_3219
A1S_3218
A1S_3217
A1S_3214
1415 bp
1220 bp
3158 bp
956 bp
MFP
RND
OMF

1.5. RESEARCH AIMS

This thesis project was divided in three main objectives:

A. Study the expression of RND efflux pumps in clinical isolates of *Acinetobacter baumannii* from Canadian hospitals.

B. Cloning and characterization of AdeMNO, a novel RND efflux pump of *Acinetobacter baumannii*.

C. Study the regulation of the expression of the *adeMNO* operon.

CHAPTER 2

MATERIALS AND METHODS

2.1. BACTERIAL STRAINS, PLASMIDS AND OLIGONUCLEOTIDES

Plasmids, bacterial strains, and oligonucleotides used in this study are listed in Tables 2.1, 2.2, and 2.3 respectively. The clinical isolates of *A. baumannii* used in this study were obtained from Canadian Hospitals all over Canada and were provided by Dr. George Zhanel, University of Manitoba, Canada. *A. baumannii* ATCC19606 was used as the reference strain.

2.2. MEDIA AND GROWTH CONDITIONS

All bacterial strains were routinely grown in Luria-Bertani (LB) medium (Bioshop Canada Inc, Burlington, ON, Canada) and incubated at 37˚C. The following concentrations of antibiotics were used in order to maintain respective plasmids, for selection or counterselection of *E. coli* strains, 100 µg/mL ampicillin (Ap) (Bioshop, Burlington, ON, Canada), 10 µg/mL gentamycin (Gm) (Bioshop, Burlington, ON, Canada), 15 ug/mL streptomycin (Sigma Aldrich Co., St Louis, MO, USA). Concentrations of antibiotics used for *Pseudomonas aeruginosa* and *A. baumannii* are listed in the following sections. LB media supplemented with 5% sucrose (Bioshop, Burlington, ON, Canada) was used for curing plasmids containing the *sacB* counterselection marker. Mueller-Hinton broth (BD-Canada, Mississauga, ON, Canada) was used in order to perform the antibiotic susceptibility assays. Induction of gene expression was achieved by supplementing the media with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Biobasic, Markham, ON, Canada).

2.3. FREEZER STOCKS

Freezer stocks were prepared by mixing 1.5 mL of saturated overnight culture with sterile 80% glycerol solution to a final concentration of 15% glycerol. The cryovials were immediately placed into the -80˚C freezer for storage.

2.4. DNA EXTRACTION

Genomic DNA was extracted using the DNeasy Kit from Qiagen (Qiagen, Mississauga, ON, Canada) according to manufacturer's instruction. Cells from 1.5 mL of overnight culture were harvested and directly lysed by incubating with proteinase K at 56˚C for 10 min, buffer containing 51% of 96-100% alcohol was added and the mix was transferred to a silica base column. The column was washed twice with ethanol solution and the DNA was extracted from the column using 20 to 50 µL of elution buffer.

Plasmid DNA was extracted using EZ-10 Spin Column Plasmid DNA Miniprep Kit from BioBasic (BioBasic Inc., Markham, ON, Canada) by following the manufacturer's instructions. The DNA concentration was measured using Eppendorf BioPhotometer Model AG (Barkhausenweg, Hamburg, Germany) and the samples were stored either at 4˚C or -20˚C.

2.5. EXTRACTION OF DNA FRAGMENTS FROM AGAROSE GELS

The EZ-10 Spin Column Gel Extraction Kit from BioBasic (Bio Basic Inc., Markham, ON, Canada) was used to purify DNA from agarose gels following the manufacturer's instructions. Briefly, gel containing the DNA was dissolved in Binding

Buffer by incubating at 56˚C for 10 min. The mix was transferred to a column containing a silica membrane and washed twice with ethanol solution. The DNA was extracted using between 25 to 50 µL of Elution Buffer. Samples were stored at -20˚C until use.

2.6. RNA EXTRACTION AND cDNA SYNTHESIS

RNA extractions were performed using the RNeasy Kit (Qiagen, Mississauga, ON, Canada). Overnight cultures grown in LB was subcultured in fresh LB, 1 mL of late log phase (A600nm ≈0.8) culture was harvested, and the pellet was frozen at -80ºC for 15 min to facilitate cell lysis. RNA extraction was carried out following the manufacturer's instructions, 1 x 10^9 bacteria were disrupted using a guanidine-thiocyanate–containing lysis buffer. After adding ethanol to the mix, the samples were loaded into the RNeasy Mini Spin Column. Total RNA binded to the RNeasy silica membrane, and impurities were washed away using alcohol base solution. Total RNA was extracted from the spin column using RNase-free water. Synthesis of the cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions. Briefly, RNA samples were treated with gDNA Wipe-out buffer for 10 minutes to eliminate any trace of genomic DNA. After DNA elimination, cDNA was obtained from 0.5 µg of total RNA using Reverse Transcriptase.

2.7. DNA RESTRICTION ENZYME DIGESTION AND LIGATION

Restriction enzymes were used to confirm the identity of the plasmids, as well as for cloning of DNA. Restriction digestions were performed according to manufacturer's

instructions. Ligations were accomplished using T4 DNA ligase (New England Biolabs Ltd., Pickering, ON, Canada) using varying molar ratios of the insert and vector. Ligation reactions were incubated for 1 hr at 37˚C or overnight at room temperature. Blunt end ligation reactions were incubated overnight in a 25˚C water bath.

2.8. POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

Plasmids, genomic DNA, and cDNA prepared as described above were used as template for PCR amplification. Occasionally the template for PCR was also prepared from cells taken from a culture plate. Cells scraped from a bacterial colony using a sterile toothpick were suspended in 20 µL of sterile millipore water and the suspension boiled for 10 min. Cell debris was removed by microcentrifugation at 13,300 rpm for 2 min; 2 µL of the supernatant were used as template in a 25 µL PCR reaction.

Depending on the reaction conditions and the size of the product, PCR reactions were performed using a variety of polymerases. Taq DNA polymerase (New England Biolabs, Pickering, ON, Canada) was used routinely for the screening procedures, Bio-X-Act (Bioline, Randolf, MA, USA) was successfully used in the amplification of *adeMNO* operon, Phire (Finnzymes, Espoo, Finland) was used in the final stage of the Splicing by Overlapping Extension PCR described bellow and Go Taq® DNA polymerase (Promega, Madison, WI, USA) was regularly used in the amplification of any other template. All these polymerases were utilized following the manufacturer's instructions.

DNA amplification was carried out for either 25 or 30 cycles using 50 ng of genomic DNA from *A. baumannii* isolates or 1µL of cDNA obtained from 0.5 µg of total RNA in each reaction. The annealing temperature (T_a) for each PCR primer was

calculated using the following formula: 4(G+C) + 2 (A+T) – 5. For RT-PCR reactions, primers were designed using the OligoPerfect primer designing tool from Invitrogen (http://www.invitrogen.com) to anneal to the RND component-encoding gene of each operon. Ribosomal 23S rRNA gene was used as the housekeeping control, while *A. baumannii* ATCC19606 was used as the reference strain.

2.9. SPLICING BY OVERLAP EXTENSION (SOEing) PCR

The splicing Overlap Extension (SOEing) reaction was used to generate deletion fragments for *adeMNO* and *adeL* with the purpose of creating gene knock-outs using Choi and Schweizer method (Choi and Schweizer, 2005) (Fig 2.1.). This method consists of two rounds of PCR reaction. The first round involved amplifying the gentamycin-resistant (Gm^r) marker and the flanking regions of the target gene/operon using Go Taq® DNA polymerase (Promega, Madison, WI, USA). Gm^r marker was obtained by PCR amplification of the *aacC1* gene from 50 ng of the plasmid pPS856 (Hoang *et al*, 1998). Flanking regions of the target gene/operon were amplified using the genomic DNA of *A. baumannii* ATCC19606 and adding engineered overlapping regions to the *aacC1* gene in each of the fragments. The sequences of the primers used in this work are described in Table 2.3. The resulting fragments Gm^r (1,053 bp), the 5'- and 3'-ends of the target gene/operon were purified from agarose gel using the method described in section 2.5. and its concentration determined spectrophotometrically ($Absorbance_{260\ nm}$) using the Eppendorf BioPhotometer Model AG.

Forty nanograms of each of the three fragments were used as template for the second round of the PCR reaction. The reaction proceeded without any primers for 5

cycles (annealing temperature, 55˚C), following which, the forward and reverse primers of 3'- and 5'-ends of the target gene were added and the reaction continued under the same conditions for 35 cycles. Phire (Finnzymes, Espoo, Finland) Taq® DNA polymerase was used for SOEing reactions following the manufacturer's instructions. The resulting PCR product was cloned into the pUC18 cloning vector, followed by sub-cloning into pEX-Km5 suicide plasmid for further applications. The creation of Δ*adeMNO*::Gm-*FRT* fragment is illustrated in the Fig. 2.1.

2.10. PREPARATION OF COMPETENT CELLS AND TRANSFORMATION

Transformations were performed using chemically prepared competent cell (Samshcokbrook and Russell, 2001) or commercial competent cells *E. coli* JM109 (Promega, Madison, WI, USA). Transformations were performed using the heat shock method following standard laboratory protocols (Sambrook and Russell, 2001). Transformation reactions were incubated at 37˚C for 1 hr with shaking and plated on LB agar selective media containing appropriate concentration of antibiotic(s). For blue and white colony screening, the transformants were plated on LB agar supplemented with 40 µg/mL X-gal, 1 mM IPTG and the appropriate antibiotic. Competent cells JM109 purchased from commercial suppliers were used according to manufacturer's instructions. Briefly, 100 µL cells were retrieved from -70˚ C freezer and thawed on ice for 5 min. Fifty to one hundred nanograms of the plasmid were added into the cell suspension and the mix incubated for 20 min on ice. Cell were then heat shocked at 42˚C for 90 sec, incubated immediately on ice for other 2 min, followed by the addition of 1 mL

of room temperature LB media. After incubating the transformed cells at 37 ˚C for 1 hr, they were plated into the appropriated selection media and incubate at 37˚C overnight.

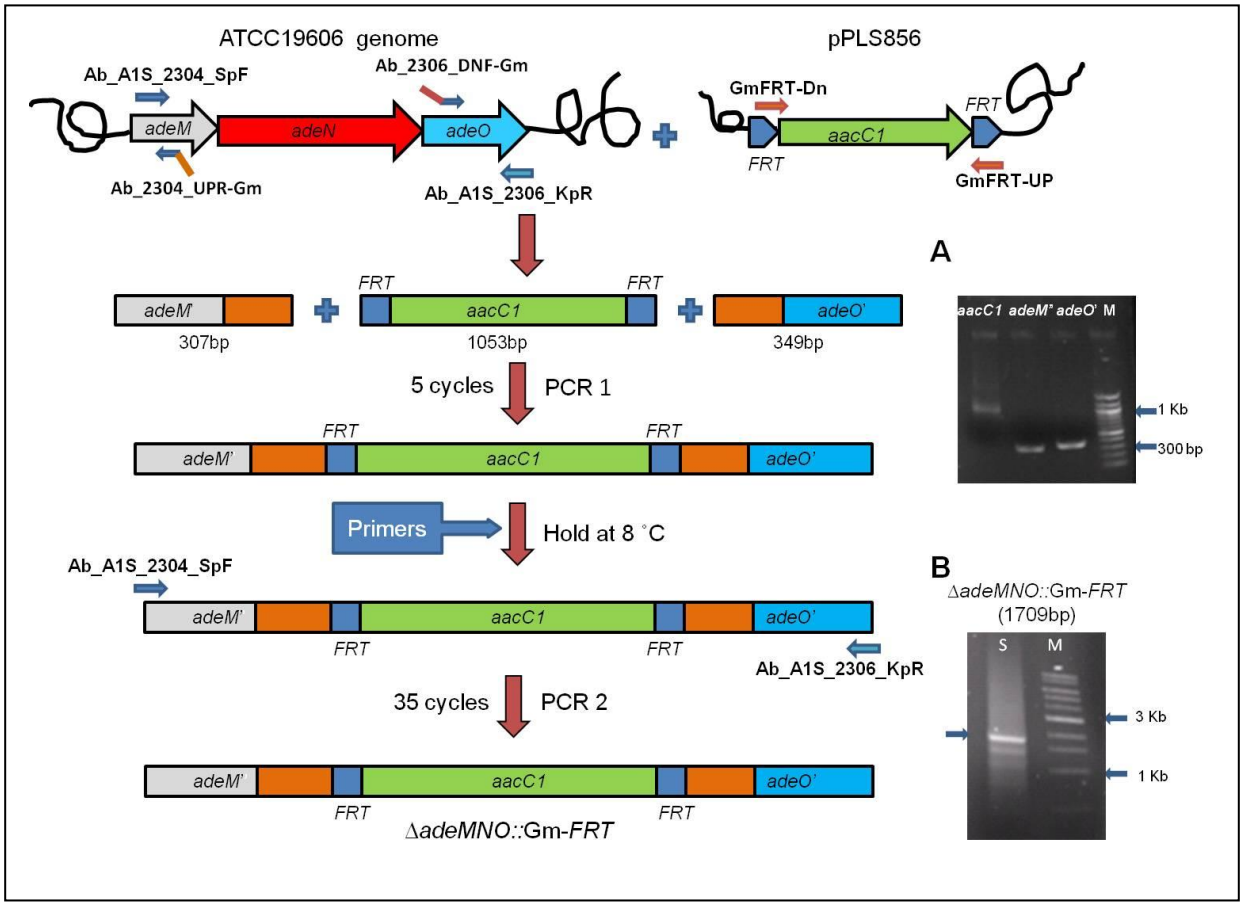
2.11. PREPARATION OF ELECTROCOMPETENT CELLS AND TRANSFORMATION BY ELECTROPORATION

Transformation of *P. aeruginosa* strains was performed using the electroporation method previously described by Choi and Schweizer (2005). Electrocompetent cells were obtained by equally distributing 6 mL of overnight culture in 4 microcentrifuge tubes followed by centrifugation at 13,300 rpm for 2 min. The pellets were pooled and washed twice using 1 mL of 300 mM sucrose solution. After the second wash, the supernatant was discarded and the cells resuspended in a total of 100 µL of 300 mM sucrose solution.

Electroporation was performed by adding 50-250 ng of DNA to the cell suspension. The mix was transferred to a 2 mm electroporation cuvette and an electrical pulse was applied at 2.5 KV with default settings of 10 µF for 5 sec using an Eppendorf electroporator Model 2510 (Eppendorf North America, Inc., Westbury, N.Y., USA). Immediately following the pulse 1 mL of room temperature LB media was added to the cuvette, the cells were transfered to a microcentrifuge tube and incubated at 37˚C for 2 hr. One hundred microliters of the culture was plated on LB agar supplemented with the appropriate antibiotic. The remaining cells were harvested by centrifugation, resuspended in 100 µL LB broth and plated on the same selective media. Plates were incubated at 37^{o}C overnight and observed for colonies the following day.

Figure 2.1. Schematic illustration of mutant fragment generation by SOEing reaction for *adeMNO* operon. A PCR was performed to amplify 307 bp of the 5'-end of the *adeM* gene, designed in the figure as ***adeM'***, and 349bp of the 3'-end of the *adeO* gene, designed as ***adeO'***. These fragments were mixed with Gm^r cassette (*aacC1*) to obtain the *adeMNO* deletion fragment. Quality of the PCR products was accessed on a 0.8% agarose gel (A). These purified products which contained *aacC1* overlapping sequences (orange boxes) were then assembled by SOEing reaction using PCR1 and PCR2 cycles. PCR1 reactions were prepared using equal amounts (40 ng) of each fragment in the absence of the primers, the run was paused after 5 cycles and primers were added to the reaction. Immediately after, PCR2 was run for 35 cycles to allow the amplification of the SOEing product. The presence of Δ*adeMNO*::Gm-*FRT* gene deletion fragment obtained from PCR2 was visualized on a 0.8% agarose gel prior to cloning, indicated by an arrow in B.

Figure 2.1.


ATCC19606 genome
pPLS856
Ab_A1S_2304_SpF
Ab_2306_DNF-Gm
GmFRT-Dn
FRT
adeM
adeN
adeO
aacC1
FRT
Ab_2304_UPR-Gm
Ab_A1S_2306_KpR
GmFRT-UP
A
adeM'
FRT
FRT
adeO'
307bp
1053bp
349bp
aacC1 adeM' adeO' M
5 cycles
PCR 1
1 Kb
300 bp
Primers
Hold at 8 ˚C
B
Ab_A1S_2304_SpF
ΔadeMNO::Gm-FRT
(1709bp)
Ab_A1S_2306_KpR
S
M
35 cycles
PCR 2
3 Kb
1 Kb
ΔadeMNO::Gm-FRT

2.12. INSERTION OF the *adeMNO* OPERON IN SINGLE COPY IN *P. aeruginosa*

A single copy insertion of *adeMNO* operon into *P. aeruginosa* PAO750, PA0200 and PA0397, was perfomed as described by Kumar *et al.* (2006). The insertion was obtained by electroporating *P. aeruginosa* with plasmids pPLS018 and pTNS2 that provided transposes A, B, C, and D in *trans*.

Electroporation mix was plated on LB-agar Gm30 plates and incubated at 37^{o}C overnight. Gentamicin resistant colonies were screened for the insertion of the mini-Tn*7* element by PCR using primers Tn7R and PglmSDN1. The primer Tn7R binds to the miniTn7 plasmid sequence, while PglmSDN1 binds to the *glmS* gene generating an amplicon of 240 bp. Insertion was also confirmed by the presence of *aacC1* gene. Screening PCR was performed using internal primers for the *aacC1* gene, Gm_R and Gm_F; together they amplified a product of 548 bp. The *P. aeruginosa* PAO750 derivative that contained the marked insertions of *adeMNO* operon was named *P. aeruginosa* PA006.

Removal of the Gm^{r} marker was achieved by using the *Flp-FRT* system described by Choi and Schweizer (Choi and Schweizer, 2006). Briefly, *P. aeruginosa* PA006 was electroporated with pFLP2 (Kumar *et al*, 2006) as described above in section 2.11 and cells plated on LB agar media supplemented with 200 µg/mL carbenicillin (Cb200). Screening was performed by patching colonies on Cb200 and Gm30, selecting those that grew on Cb but not on Gm. The plasmid pFLP2 was cured by streaking the patches on LB-agar supplemented with 10% sucrose. Resulting colonies were patched on LB-10% sucrose, LB-Cb200 and LB-Gm30 plates to confirm the loss of pFLP2 plasmid and reconfirm the absence of *aacC1* gene. Patches that were sucrose resistant but Cb- and Gm-susceptible were screened once again for the removal of the *aacC1* gene by PCR

described above and also re-tested for the insertion of the mini-Tn*7* element. The resulting strain was named as *P. aeruginosa* PA008.

2.13. ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing for the bacterial strains in this study was performed using the Vitek II system (BioMerieux Inc, Montreal, Canada), two fold serial dilution test and the disk diffusion susceptibility assays. All tests were carried out following the recommendations by Clinical and Laboratory Standards Institute (CLSI) (NCCLS 2010). Muller-Hinton broth was used for the susceptibility assays. Induction of the efflux pump-encoding operon was achieved by supplementing the growth medium with 1mM IPTG. Bacterial growth was examined after 18 hrs of incubation at 37°C. Antimicrobial resistance results were interpreted according to CLSI guidelines (NCCLS 2010). Antibiotic tested were chosen according to reference and availability.

2.14. FOUR PARENTAL MATING

Four parental mating was performed following a method previously described (Goldberg and Ohman, 1984) in order to transfer mini-Tn*7* elements in *A. baumannii* strains. This involved transfer of two plasmids required for achieving transposition, namely the mobilizable mini-Tn7 suicide delivery plasmid (*E. coli* strain with the mini-Tn7 suicide delivery plasmid) and the mobilizable transposes-encoding plasmid (*E. coli* strain containing the helper plasmid pTNS2) to the recipient strain (*A. baumannii* ATCC19606) with the help of a plasmid (*E. coli* DH5α strain containing the helper plasmid pRK2013)

encoding the mobilization helper functions. One hundred millilitres of the overnight cultures of the donor, helpers and recipient stains grown in LB broth containing appropriate antibiotic(s) (ampicillin for the *E. coli* strain containing the mini-Tn7 delivery vector and helper plasmid containing pTNS2, kanamycin for the *E. coli* strain containing pRK2013 and no antibiotic for *A. baumannii* recipient strain) were mixed into 1mL of LB broth followed by microcentrifugation at 13,300 rpm for 1 min. The bacterial pellet was washed twice with 1mL of LB and carefully suspended into 50 µL of fresh LB broth. Bacterial suspension was spot plated on a pre-dried LB agar plate and incubated overnight at 37˚C. Following the incubation, the bacterial cells were washed with 1mL of saline (0.85% NaCl), 100 µL of the suspension was plated on LB agar plate supplemented with Gm (100 µg/mL) for the selection of *A. baumannii* transformants containing the mini-Tn*7* insertion and streptomycin (15 µg/mL) for the counterselection of *E. coli* strains. The remaining cells were then centrifuged at 13,300 rpm for 2 min, the supernatant was eliminated, and the pellet resuspended in 100 µL of LB broth. The cells were then plated on a second LB agar plate supplemented with the above antibiotics. The plates were incubated at 37˚C overnight and the colonies screened for insertion using PCR.

Screening of the mini-Tn*7* insertion in *A. baumannii* cells was performed using single colony PCR by determining the presence of *aacC1* gene (internal primers Gm_R and Gm_F) and mini-Tn7 insertion downstream from *A. baumannii glmS* gene (Kumar *et al*, 2010) (primers Tn7R in conjunction with *glmS* primer PglmSDN1). The conditions for the run were as follow: initial denaturation at 95˚C for 5 min; 95˚C for 30 sec, 55˚C for 30 sec, and elongation at 72˚C for 30 sec. Fragments obtained in this amplification were 548 bp long for the Gm^r and 240 bp for the mini-Tn7 insertion.

2.15. N-ACYL HOMOSERINE LACTONE (AHL) BIOASSAY

AHL bioassays were performed using the method described by Andersen *et al*. (Andersen *et al*., 2001) with some modifications. Overnight cultures of *E. coli* MT102 harbouring pJBA132 (grown at 30˚C) and the *P. aeruginosa* strain containing the *adeMNO* operon (grown at 37˚C) were equilibrated in saline using the 0.5 McFarland turbidity standard (~10^8 cells/mL). Five microliters of each equilibrated suspension was streaked close to each other in the form of a 'T' on a LB-agar plate supplemented with- or -without 1 mM IPTG. Plates were incubated at 30˚C, and examined for green fluorescence every 24 hrs for 4 days.

2.16. SEQUENCING AND SEQUENCE ANALYSIS

DNA sequencing was carried out at the Genome Quebec facility at McGill University, Montreal, QC. The sequence analysis was performed using Basic Local Alignment Search Tool (BLAST), Conserved Domains Database (CDD) and Gene Construction Kit Software.

Table 2.1. List of plasmids used in the study

Plasmid	Relevant characteristics	Source
pGEMT-Easy	Ap^r, TA PCR cloning vector	Promega
pTNS2	Ap^r; R6K replicon, helper plasmid encoding the site-specific TnsABCD Tn*7* transposition pathway	(Choi and Schweizer, 2005)
pFLP2	Cb^r; source of *Flp* recombinase; curable by sucrose counterselection	(Hoang *et al*., 1998)
pPLS1464	Ap^r Gm^r; mini-Tn*7* expression vector containing *lacI*^{q} and *tac* promoter	(Choi *et al.*, 2005)
pJBA132	Tc^r; pME6031-*luxR*-P_{luxI}-RBSII-*gfp*(ASV)-T_0-T_1	(Andersen *et al*., 2001)
pPLS012	Ap^r, pGEM®-T-easy containing the *adeL gene* of *A. baumannii* 64797 obtained by PCR amplification	This study
pPLS013	Ap^r, pGEM®-T-easy containing the *adeL gene* of *A. baumannii* 63487 obtained by PCR amplification	This study
pPLS017	Ap^r, pGEM®-T-easy containing the *adeMNO* operon of *A. baumannii* ATCC19606 obtained by PCR amplification	This study
pPLS018	Ap^r, pUC18T-mini-Tn*7*T-Gm-LAC containing the *adeMNO* operon of *A. baumannii* ATCC19606	This study
pPLS019	pEX-Km5 contains the *sacB gene* that can be used as counter-selectable marker. Also contains the *gusA* gene	(Lopez *et al*, 2009)
pUC18	Ap^r, pUC18	Lab. collection
pPLS022	Ap^r, pGEM®-T-easy containing the *adeMNO promoter region* of *A. baumannii* 64797	This study

pPLS023	Ap^r, pGEM®-T-easy containing the *adeMNO promoter region of A. baumannii* 63487	This study
pPLS024	Ap^r, pGEM®-T-easy *adeL* from *A. baumannii* 64797 strain	This study
pPLS025	Ap^r, pGEM®-T-easy *adeL* from *A. baumannii* 63487 strain	This study
pPLS035	Ap^r, Gm^r, pUC18 Δ*adeMNO::*Gm-*FRT*	This study
pPLS036	Ap^r, Gm^r, pUC18 Δ*adeL::*Gm-*FRT*	This study
pPLS041	Km^r, Gm^r, pEXKm5 Δ*adeMNO::*Gm-*FRT*	This study

Table 2.2. List of strains used in the study

***A. baumannii* strains**	**Relevant characteristics**	**Source**
ATCC19606	*A. baumannii* (Bouvet and Grimont deposited as *Bacterium anitratum* Schaub and Hauber)	ATCC
A. baumannii 63169	Clinical isolate (isolated from respiratory system of a 75 years old patient from Winnipeg)	(Zhanel *et al*., 2008)
A. baumannii 58352	Clinical isolate (isolated from respiratory system of a 1 year old patient from Winnipeg)	(Zhanel *et al*., 2008)
A. baumannii 59960	Clinical isolate (isolated from respiratory system of a 64 years old patient from Vancouver)	(Zhanel *et al*., 2008)
A. baumannii 59973	Clinical isolate (isolated from respiratory system of a 33 years old patient from Vancouver)	(Zhanel *et al*., 2008)
A. baumannii 64130	Clinical isolate (isolated from blood of a 30 years old patient from Hamilton)	(Zhanel *et al*., 2008)
A. baumannii 64153	Clinical isolate (isolated from respiratory system of a 26 years old patient from Hamilton)	(Zhanel *et al*., 2008)
A. baumannii 64397	Clinical isolate (isolated from respiratory system of a 75 years old patient from Vancouver)	(Zhanel *et al*., 2008)
A. baumannii 63487	Clinical isolate (isolated from respiratory system of a 54 years old patient from Winnipeg)	(Zhanel *et al*., 2008)
A. baumannii 65239	Clinical isolate (isolated from respiratory system of a 4 year old patient from Victoria)	(Zhanel *et al*., 2008)

A. baumannii 66310	Clinical isolate (isolated from respiratory system of a 20 years old patient from Vancouver)	(Zhanel *et al*., 2008)
A. baumannii 66985	Clinical isolate (isolated from blood of a 25 years old patient from Montreal)	(Zhanel *et al*., 2008)
A. baumannii AB016	Gm^r, *A. baumannii* ATTC 19606:mini Tn7T-Gm-LAC-*A1S2304-2306*	This study
A. baumannii AB017	AB016 without Gm marker	This study
***P. aeruginosa* strains**	**Relevant characteristics**	**Reference**
P. aeruginosa PA01	*P. aeruginosa* prototroph	(Holloway and Zhang, 1990)
P. aeruginosa PAO200	PA01:Δ(*mexAB-oprM*)	(Schweizer, 1998)
P. aeruginosa PAO397	PAO01: Δ(*mexAB-oprM*), Δ*(mexCD-oprJ),* Δ(*mexEF*-oprN), Δ(*mexJK*), Δ(*mexXY*), Δ*opmH*	(Chuanchuen *et al*., 2005)
P. aeruginosa PAO750	PAO397:Δ*pscC*	(Kumar *et al*., 2006)
P. aeruginosa PA007	Gm^r; PAO750 with chromosomally integrated pUC18-mini-Tn*7*T-Gm-LAC-*adeMNO*	This study
P. aeruginosa PA008	PA007 without Gm marker	This study
P. aeruginosa PA021	Gm^r, PAO200 with chromosomally integrated pUC18-mini-Tn*7*T-Gm-LAC	This study
P. aeruginosa PA025	PA021 without Gm marker	This study
P. aeruginosa PA029	Gm^r; PAO200 with chromosomally integrated pUC18-mini-Tn*7*T-Gm-LAC-*adeMNO*	This study

P. aeruginosa PA030	PA029 without Gm marker	This study
P. aeruginosa PA022	Gm^r, PAO397 with chromosomally integrated mini-Tn*7*T-Gm-LAC	This study
P. aeruginosa PA026	PA022 without Gm marker	This study
P. aeruginosa PA031	Gm^r; PAO397 with chromosomally integrated pUC18-mini-Tn*7*T-Gm-LAC-*adeMNO*	This study
P. aeruginosa PA032	PA031 without Gm marker	This study
P. aeruginosa PA033	Gm^r, PAO750 with chromosomally integrated pUC18-mini-Tn*7*T-Gm-LAC	This study
P. aeruginosa PA034	PA033 without Gm marker	This study
***E. coli* strains**	**Relevant characteristics**	**Reference**
E. coli JM109	*recA1, endA1, gyrA96, thi,hsd*R17(r_{K-}, m_{K+}), relA1,supE44, Δ(lac-proAB), [F',traD36, proAB, $lacI_qZ\Delta M15$]	Promega
E. coli GBE180	F- Φ80d *lacZ*ΔM15 Δ(*lacZYA-argF) U169 recA1endA1 hsdR17 (*r_k-, m_k+) *phoA supE44 λ- thi-1 gyrA96 relA1 pcnB*	(Pierais *et al*., 1999)
E. coli DH5α	F- Φ80d *lacZ*ΔM15 Δ(*lacZYA-argF) U169 deoR recA1endA1 hsdR17 (*r_k-, m_k+) *phoA supE44 λ- thi-1 gyrA96 relA1*	Laboratory collection

Table 2.3.List of oligonucleotides used in the study

Target Gene	Oligonucleotide Name	Sequence	Reference
adeB	adeB_R_RT	5'-AATACTGCCGCCAATACCAG-3'	This study
	adeB_F_RT	5'-GGATTATGGCGACTGAAGGA-3'	This study
adeN	Ab-A1S-2305-RTr	5'-ATCGCGTAGTCACCAGAACC-3'	This study
	Ab-A1S-2305-RTf	5'-CGTAACTATGCGGTGCTCAA-3'	This study
adeE	adeE_R_RT	5'-GCCTGCGTTATTTCTACCC-3	This study
	adeE_F_RT	5'-GAAACAGAGCGGGTTGGTAA-3'	This study
A1S_2818	2818_R_RT	5'-AATTGAGCCAAGCTCATGCT-3'	This study
	2817_R4	5'-TATTGATTTCCCATTCGTAG-3'	This study
adeJ	adeJ_F1_RT	5'-CATCGGCTGAAACAGTTGAA-3'	This study
	adeJ_R1_RT	5'-GCCTGACCATTACCAGCACT-3'	This study
A1S_3217	3219_R_RT	5'-GTGACTTGGGAAAGCCCATA-3'	This study
	3219_F_RT	5'-ACCGCTTTAGAGGTCGAACA-3'	This study
23S rRNA	23S_R_RT	5'-GGGAGAACCAGCTATCACCA-3'	This study
	23S_F_RT	5'-GCAGGTTGAAGGTTGGGTAA-3'	This study
adeMNO	Ab_A1S_2304_SpF	5'-AACtaGTATCCAAATAACTAG GAG-3'* (*SpeI*)	This study
	Ab_A1S_2306_KpR	5'-AAAGgtaCCGTAAGGCGCTTT-3'* (*KpnI*)	This study
adeL	Ab2303_R	5'-GCATTAATAGGAGTGTGCGTG-3'	This study
	Ab2303_F	5'-AGCAATGATCTGTATTGACGG-3'	This study
glmS from *P. aeruginosa*	PglmSDN1	5'-GCACATCGGCGACGTGCTCTC-3'	(Choi *et al*., 2005)
pUC18-miniTn7T-Gm-lac	Tn7R	5'-CACAGCATAACTGGACTGATT TC-3'	(Choi *et al*., 2005)
LacZ gene	LacZ-R	5'-CGATAATTTCACCGCCGAAAG G-3'	This study
glmS from *A. baumannii*	AB_glmSF	5'-GGCGGTCAGTTGTATGTCTT-3'	This study
aacC1 gene	GmFRT-Dn	5'-CGAATTGGGGATCTTAAGTTC CT-3'	(Choi and Schweizer, 2005)
	GmFRT-UP	5'-CGAATTAGCTTCAAAAGCGCTC TGA-3'	(Choi and Schweizer, 2005)

Target Gene	Oligonucleotide Name	Sequence	Reference
aacC1 gene	Gm_F	5'-TGGAGCAGCAACGATGTTAC-3'	(Choi and Schweizer,2005)
	Gm_R	5'-TGTTAGGTGGCGGTACTTGG-3'	(Choi and Schweizer,2005)
adeO-Gm SOEing reaction	Ab_2306_DNF-Gm	5'-AGGAACTTCAAGATCCCCAATT CGTCAAGCAAGAGCGGCTTATG AG-3'	This study
adeM-Gm SOEing reaction	Ab_2304_UPR-Gm	5'-TCAGAGCGCTTTTGAAGCTAATT CGACGAGGCTTCCATCTTTGAAAT G-3'	This study
adeL-Gm SOEing reaction	Ab2303_DNF_Gm	5'-AGGAACTTCAAGATCCCCAATT CGGGCAACATCCGCTAAAATACG GGC-3'	This study
	Ab2303_UPR_Gm	5'-TCAGAGCGCTTTTGAAGCTAA TTCGCAGGCTGTCCATTACTTGG CGGTACAG-3'	This study
adeMNO promoter	A1S2303_Lac_PsF	5'-CTTTGCTGCAGACTCTCATG-3'	This study
	A1S2304_Lac_KpR	5'-TTGGTACCGGCATTTTCATG-3'	This study

* Restriction site indicated by underlined bases and restriction enzyme indicate in parentheses, the base modifications introduced to generate the new restriction sites are annotated in lowercase.

CHAPTER 3

RESULTS

A. EXPRESSION OF RND EFFLUX PUMPS IN CLINICAL ISOLATES OF *Acinetobacter baumannii* FROM CANADIAN HOSPITALS

3. A.i. DETECTION OF RND COMPONENT-ENCODING GENES IN CLINICAL ISOLATES OF *Acinetobacter baumannii*

PCR was used to determine the presence of RND component-encoding genes in eleven *A. baumannii* isolates using primers specific to the individual RND pump genes. All of the clinical isolates contained at least three different RND component-encoding genes (Table 3.1). Two genes, *adeJ* from the *adeIJK* operon, and *adeN* from the *adeMNO* operon, were present in all isolates (Figs. 3.1.D.i and 3.1.E.i). The *adeB* gene of the *adeABC* operon was found in seven (Fig.3.1.B.i), *adeE* of the *adeDE* operon in 10 (Fig. 3.1.C.i), *A1S_2818* of the *A1S_2817-A1S_2818* operon in six of the 11 isolates tested (Fig. 3.1.F.i), while *A1S_3217* of the *A1S_3219-A1S_3218-A1S_3217-A1S_3214* operon was found in only one isolate (Fig. 3.1.G.i). The reference strain ATCC19606 was found to harbour the *adeB, adeJ, adeN, A1S_2818* and *A1S_3217* genes.

3.A.ii. EXPRESSION OF RND PUMP-ENCODING GENES

Expression of efflux pumps was determined by end-point RT-PCR using cDNA samples synthesized from RNA of different *A. baumannii* strains as templates. Results of RT-PCR reactions are summarized in Table 3.1. The genes *adeB* and *adeJ* were detected in cDNA samples after 25 PCR cycles, while all other RND pump-encoding genes, namely *adeE*, *A1S_2818*, *adeN*, and *A1S_3217*, were detected after 30 cycles. In the cases of *adeE* and *adeN*, they were the most prominently expressed RND component-encoding genes in the clinical isolates, with *adeE* being expressed in eight of the 10 isolates (80%) and *adeN* in eight of the 11 isolates (72%) that harboured the respective gene (Figs. 3.1.C.iii and 3.1.E.iii). The *adeB* gene was expressed in four out of

seven (57%) and *adeJ* was expressed in four out of 11 isolates (36%) that harboured the respective genes (Figs. 3.1.B and 3.1.D). *A1S_2818* was expressed in five out of six strains (83%) harbouring the gene (Fig. 3.1.F), while *A1S*_1 *3217* was expressed in the only strain that contained the gene (Fig. 3.1.G). Control strain *A. baumannii* ATCC 19606, which sequence has been published, showed expression of only three of the five RND genes present in its genome, namely *adeB*, *adeJ* and *A1S_3217*, *A. baumannii* 64130, isolated from a 30 year old patient in Hamilton, ON, was found to express the highest number of efflux pumps among all isolates, expressing all but the *adeE* gene, even though this gene was found in this strain. *A. baumannii* 59960 and *A. baumannii* 64397, both isolated from Vancouver, BC, were found to express the *adeE* gene only. The reference strain *A. baumannii* ATCC19606 was found to express the *adeB, adeJ,* and *A1S_3217* genes.

Table 3.1. Summary of PCR results for the detection of RND pump-encoding genes and their expression in *A. baumannii* isolates.

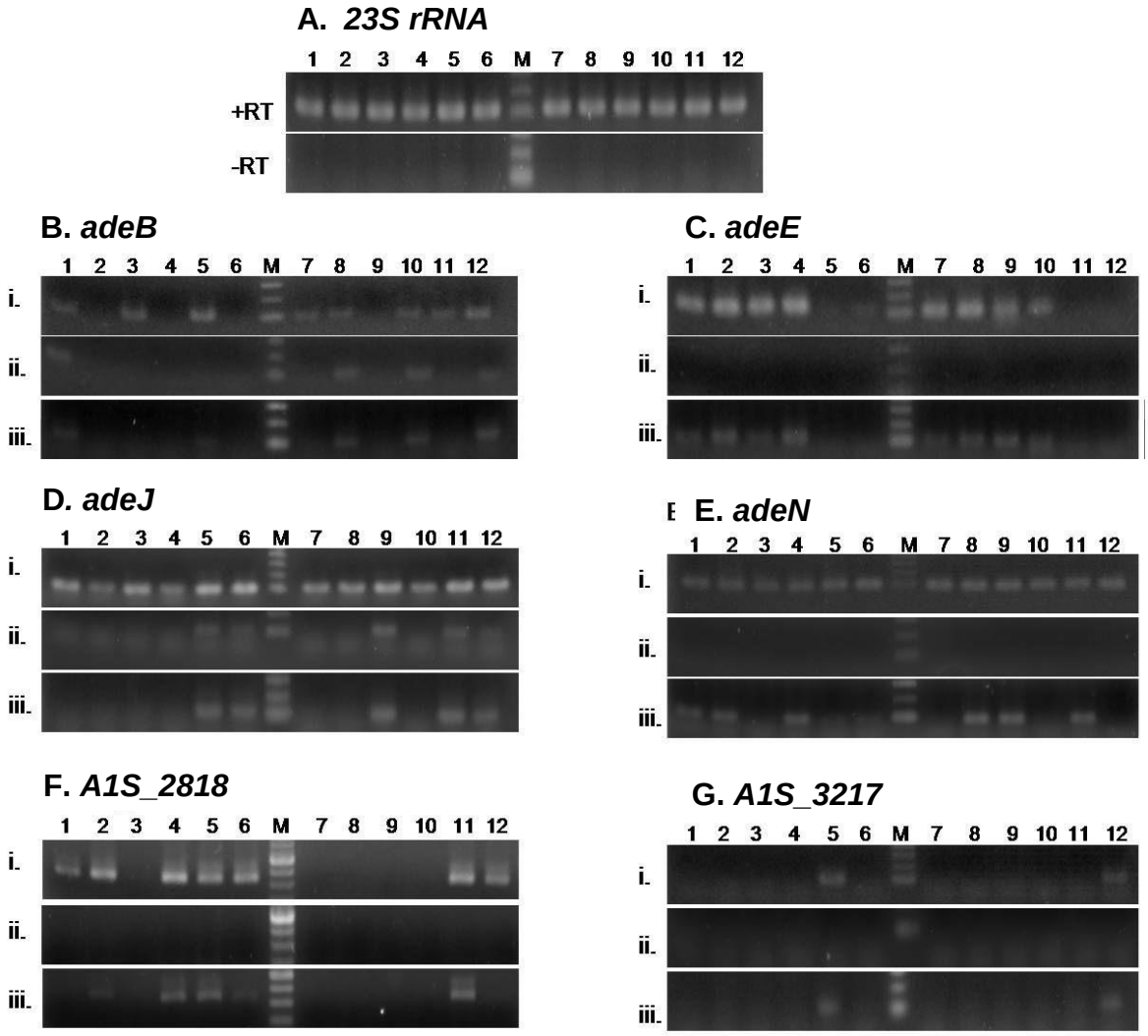
	Detection of RND-pump-encoding gene / detection of mRNA						
Strain	***adeB***	***adeE***	***adeJ***	***adeN***	***A1S_2818***	***A1S_3217***	**Total**
A. baumannii 63169	+ / +	+ / +	+/ -	+ / +	+ / -	- / -	5/3
A. baumannii 58352	- / -	+ / +	+/ -	+ / +	+ / +	- / -	4/3
A. baumannii 59960	+ / -	+ / +	+/ -	+ / -	- / -	- / -	4/1
A. baumannii 59973	- / -	+ / +	+/ -	+ / +	+ / +	- / -	4/3
A. baumannii 64130	+ / +	+ / -	+/ +	+ / +	+ / +	+ / +	6/5
A. baumannii 64153	- / -	+ / -	+/ +	+ / +	+ / +	- / -	4/3
A. baumannii 64797	+ / -	+ / +	+/ -	+ / -	- / -	- / -	4/1
A. baumannii 63487	+ / +	+ / +	+/ -	+ / +	- / -	- / -	4/3
A. baumannii 65239	- / -	+ / +	+/ +	+ / +	- / -	- / -	3/3
A. baumannii 66310	+ / +	+ / +	+/ -	+ / -	- / -	- / -	4/2
A. baumannii 66985	+ / -	- / -	+/ +	+ / +	+ / +	- / -	4/3
A. baumannii ATCC19606	+ / +	- / -	+/ +	+ / -	+ / -	+ / +	5/3
Total	8/5	10/8	12/5	12/8	7/5	2/2	

+, positive detection and/or expression of the gene

-, negative detection and/or expression of the gene

Figure 3.1. Analysis of the presence and expression of *adeB* (B), *adeE* (C), *adeJ* (D), *AdeN* (E), *A1S_2818* (F), and *A1S_3217* (G) RND-pump encoding genes in *A. baumannii* isolates. 1, *A. baumannii* 63169; 2, *A. baumannii* 58352; 3, *A. baumannii* 59960; 4, *A. baumannii* 59973; 5, *A. baumannii* 64130; 6. *A. baumannii* 64153; 7, *A. baumannii* 64797; 8, *A. baumannii* 63487; 9, *A. baumannii* 65239; 10, *A. baumannii* 66310; 11, *A. baumannii* 66985; 12, *A. baumannii* ATCC19606; M, 100 bp molecular weight marker. PCR was performed using genomic DNA (i) and cDNA as template. Amplification of cDNA was performed for 25 cycles (ii) and 30 cycles (iii). 23S rRNA (A) was used as the housekeeping control gene and amplified using the cDNA preparations with minus-RT (-RT) samples as controls to rule out the genomic DNA contamination of the samples.

Figure 3.1.


A. 23S rRNA
1 2 3 4 5 6 M 7 8 9 10 11 12
+RT
-RT
B. adeB
C. adeE
D. adeJ
E. adeN
F. A1S_2818
G. A1S_3217
i.
ii.
iii.

B. CLONING AND CHARACTERIZATION OF AdeMNO RND EFFLUX PUMP OF *Acinetobacter baumannii*

3.B.i. IDENTIFICATION AND CLONING OF a*deMNO* OPERON

The *adeMNO* operon was identified from the genome sequence of *A. baumannii* ATCC17978. This pump was named AdeMNO, where AdeM is the membrane fusion (MF) protein; AdeN is the RND protein, while AdeO is the outer-membrane (OM) protein. Fig 3.2. shows the procedure through which the entire *adeMNO* operon, including 25 bp upstream of the *adeM* and 28 bp downstream of the *adeO* gene, was cloned into pUC18-miniTn7T-Gm-LAC vector. This plasmid allowed the insertion of a single copy of *adeMNO* operon into a surrogate *P. aeruginosa* strain using electroporation procedure as described in Fig.3.3. The resulting *P. aeruginosa* strain was named PA008 and was used to characterize AdeMNO efflux pump.

Figure 3.2. Identification and cloning of *adeMNO* operon. The *adeMNO* operon was identified from the genome sequence of *A. baumannii* ATCC17978. This operon contains the gene encoding the MFP (*adeM*, 1214 bp), RND protein (*adeN*, 3176 bp), and the OMP (*adeO*, 1406 bp). PCR was used to amplify the entire *adeMNO* operon including 25 bp upstream of the *adeM* and 28 bp downstream of the *adeO* gene. PCR product was cloned in vector pGEM-T-easy obtaining the plasmid pPLS017 and then subcloned into the pUC18T-mini-Tn*7*-Gm-LAC to yield the plasmid pPLS018. Abbreviation: *bla, beta-lactamase gene* conferred beta-lactams resistance (Ap^r)**;** *FRT*, Flp recombinase target; pTNS2, T7 transposes expression vector; pFLP2, Flp *S. cerevisiae* recombinase expression vector; *oriT*, RK2-derived origin for conjugal plasmid transfer; *lacI*^{q}, gene encodes the repressor protein; P*tac*, strong hybrid promoter composed of the -35 region of the *trp* promoter and the -10 region of the lacUV5 promoter/operator.

Figure 3.2.

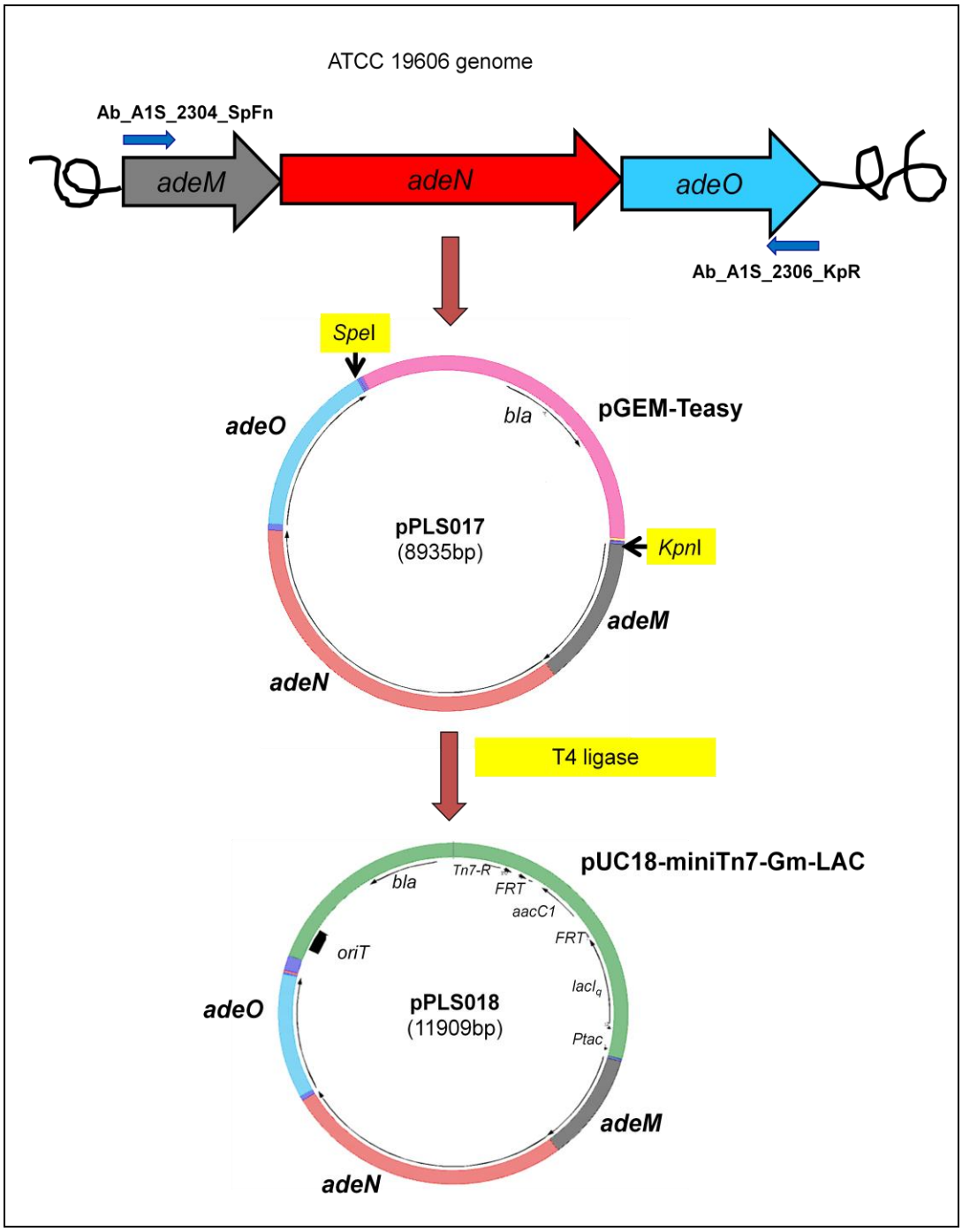
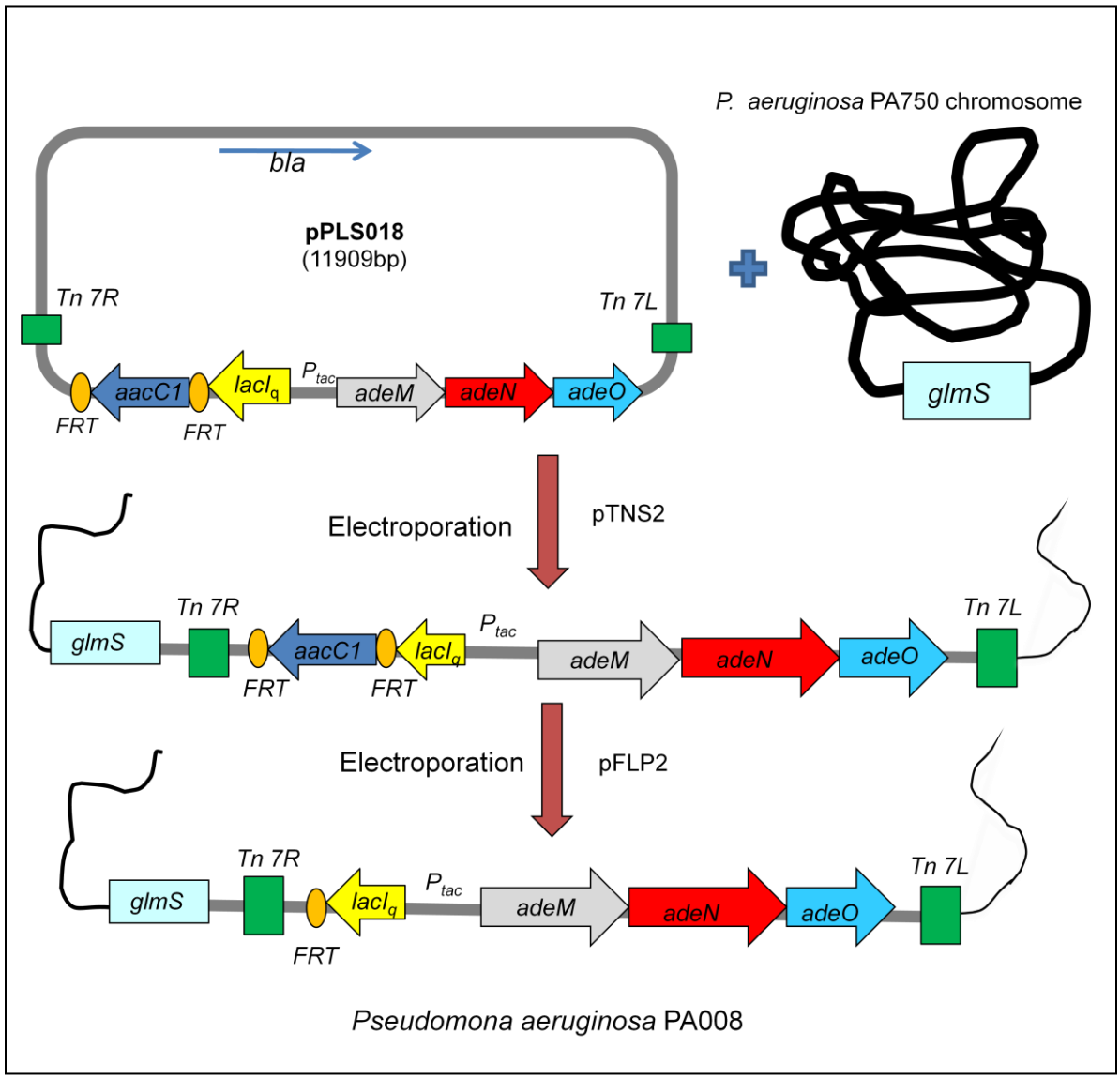

ATCC 19606 genome
Ab_A1S_2304_SpFn
adeM
adeN
adeO
Ab_A1S_2306_KpR
SpeI
adeO
bla
pGEM-Teasy
pPLS017
(8935bp)
KpnI
adeM
adeN
T4 ligase
pUC18-miniTn7-Gm-LAC
bla
Tn7-R
FRT
aacC1
FRT
oriT
lacIq
adeO
pPLS018
(11909bp)
Ptac
adeM
adeN

Figure 3.3. Insertion of *adeMNO* operon in single copy in the surrogate *P. aeruginosa* strain to obtain PA008. Insertion of the *adeMNO* operon in the surrogate *P. aeruginosa* strain PAO750 in single copy was carried out by using the method previously described (Kumar *et al*. 2006). Briefly, competent *P. aeruginosa* cells were prepared using a previously described method (Choi *et al*., 2006) and electroporated with 50 ng each of pPLS008 and the helper plasmid pTNS2 (Choi *et al*., 2005). Transformants were selected on LB agar supplemented with Gm (30 µg/mL). Abbreviation: *aacC1*, *gene* encode for gentamycin acetyltransferase-3-1 which provide gentamycin resistance; *bla, beta-lactamase gene* provide beta-lactams resistance (Ap^r)***;*** *FRT*, Flp recombinase target; *glmS*, D-fructose-6-phosphate aminotransferase; pTNS2, T7 transposes expression vector; pFLP2, Flp *S. cerevisiae* recombinase expression vector; *oriT*, RK2-derived origin for conjugal plasmid transfer; $lacI^q$, gene encode the repressor protein; P*tac*, strong hybrid promoter composed of the -35 region of the trp promoter and the -10 region of the *lac*UV5 promoter/operator; Tn7, transposable element.

Figure 3.3.


P. aeruginosa PA750 chromosome
bla
pPLS018
(11909bp)
Tn 7R
Tn 7L
aacC1
lacIq
Ptac
adeM
adeN
adeO
FRT
FRT
glmS
Electroporation
pTNS2
Tn 7R
glmS
aacC1
lacIq
Ptac
adeM
adeN
adeO
Tn 7L
FRT
FRT
Electroporation
pFLP2
Tn 7R
glmS
lacIq
Ptac
adeM
adeN
adeO
Tn 7L
FRT
Pseudomona aeruginosa PA008

3.B.ii. IDENTIFICATION OF THE SUBSTRATES OF AdeMNO PUMP

The antibiotic susceptibility testing performed using Vitek 2 system indicated an increase in resistance of PA008 to trimethoprim/sulfamethoxazole and imipenem under inducing conditions (Table 3.2.). In addition the MIC test indicated that the induction of the expression of *adeMNO* pump resulted in 16- and 4-fold increase in the resistance to chloramphenicol and trimethoprim, respectively, in PA008. In addition, the MIC for clindamycin increased by 32-fold. No significant difference was observed for the susceptibilities to ethidium bromide, gentamicin, tobramycin, imipenem, levofloxacin, ciprofloxacin, and cefotaxime (Table 3.3.). *P. aeruginosa* PA034 was obtained by using the procedure highlight in Fig. 3.3., this strain containing a single insertion of the miniTn7 vector was utilized as the control strain against PA008.

Table 3.2. Antibiotic susceptibility *P. aeruginosa* PA008 obtained in a Vitek®2 automated system version 03.01.

Antimicrobial	PA008 (µg/mL)	PA008 + IPTG (µg/mL)
Ampicillin	≥32	≥32
Piperacillin/Tazobactam	≤4	≤4
Cefazolin	≥64	≥64
Cefoxitin	≥64	≥64
Cefotaxime	32	32
Ceftazidime	≤1	≤1
Cefepime	≤1	≤1
Imipenem	**≤1**	**2**
Amikacin	≤2	≤2
Gentamicin	≤1	≤1
Tobramycin	≤1	≤1
Ciprofloxacin	≤0.25	≤0.25
Levofloxacin	≤0.12	≤0.12
Tigecycline	≤0.5	≤0.5
Nitrofurantoin	128	128
Trimethoprim/Sulfamethoxazole	**≤20**	**40**

Samples were prepared according to CLSI guidelines (NCCLS 2010), and induction testing was performed supplementing the samples with 1mM IPTG. Antibiotic in bold showed an increase in resistance when *adeMNO* operon was induced by IPTG.

Table 3.3. Antibiotic susceptibilities of *P. aeruginosa* PA008 expressing the AdeMNO pump of *A. baumannii* and PA034 *P. aeruginosa* control strain.

Antibiotic	**MIC (µg/mL) measured by broth microdilution method**			
	PA008	**PA008 + IPTG**	**PA034**	**PA034+IPTG**
Ciprofloxacin	0.125	0.125	ND	ND
Chloramphenicol	**0.5**	**8**	**1**	**1**
Trimethoprim	**16**	**64**	**1**	**2**
Gentamycin	1	1	ND	ND
Clindamycin	**16**	**512**	**8**	**8**
Ethidium Bromide	64	64	ND	ND
	Zone of inhibition (mm) measured by disk diffusion method			
Tobramycin	9.50±0.58	10.50±0.58	9.00±0	9.00±0
Levofloxacin	17.50±0.58	16.00±0	17.00±0	16.00±0
Imipenem	10.00±0	10.75±0.50	ND	ND
Nalidixic Acid	12.00±0	12.00±0	ND	ND
Cefotaxime	13.00±0	13.00±0	7.00±0	8.00±0

Susceptibilities were measured using the two-fold microdilution and the disc diffusion methods, all the tests were performed in triplicates. Antibiotics that were identified as substrates for AdeMNO efflux pump were listed in bold. The zones of inhibition measurements in this table were expressed as average± SD. Results were representative of thee independent assays.

3.B.iii. N-ACYL HOMOSERINE LACTONE (AHL) BIOASSAY

The study also investigated the efflux of non-antibiotic compounds by AdeMNO. The induction of the expression of *adeMNO* operon in *P. aeruginosa* PA008 resulted in increased fluorescence of *E. coli* MT102 harbouring the plasmid pJBA132 indicating increased secretion of AHL by *P. aeruginosa* strain upon induction of the *adeMNO* operon (Fig. 3.4.). In the case of the reference culture, wild type PA01 and parental strain PAO750, both strains showed secretion of the AHL molecule (Fig. 3.4.C. and D. respectively).

3.B.iv. CONSTRUCTION OF *adeMNO* GENE DELETION FRAGMENT

The *adeMNO* gene deletion fragment shown in Fig. 2.1., was constructed with the purpose of studying the effect of the disruption of the *adeMNO* operon in *A. baumannii* and confirm the results obtained in the *P. aeruginosa* strain PA008. The resulting fragment Δ*adeMNO*::Gm-*FRT*, which carried Gm^r selective marker, was first cloned into pUC18 plasmid pre-digested with *Sma*I as a storage plasmid. The resulting plasmid was named pPLS035, and its identity was confirmed by PCR using primers Ab_A1S_2304-SpF and Ab_A1S_2306-KpR, which amplified a 1.7 Kb fragment as seen in Fig. 3.5.B. Screening was also performed by restriction enzyme using *EcoR*V and *Xba*l, as seen in Fig. 3.5.C. and D respectively. The plasmid contains only one restriction site for *EcoR*V, and the product of this digestion was a 4395 bp fragment, as seen in Fig 3.5.C. ln the case of *Xba*l, there are five restriction sites in the resulting plasmid, the size of the fragments expected are 3070, 901, 360 and 67 bp. as seen in Fig 3.5.D. (the smallest fragments could not be seen in this gel).

The product obtained from the SOEing reaction shown in Fig. 2.1. was ligated into pEX-Km5 plasmid pre-digested with *Sma*I*,* obtaining the plasmid pPLS041. The confirmation of the identity of the suicide vector containing the Δ*adeMNO*::Gm-*FRT* was performed using PCR and restriction enzymes; the results are illustrated in Fig. 3.6.

Figure 3.4. Bioassay for AHL secretion. *E. coli* strain harbouring the reporter plasmid pJBA132 with the *gfp* gene was monitored for green fluorescence in the presence of *P. aeruginosa* PA008 in the absence (A) and presence of 1mM of IPTG (B), wild type PA01 (C) and parental strain PAO750 (D) on LB plates after 48 hr of incubation at 30 ºC.

Figure 3.4.

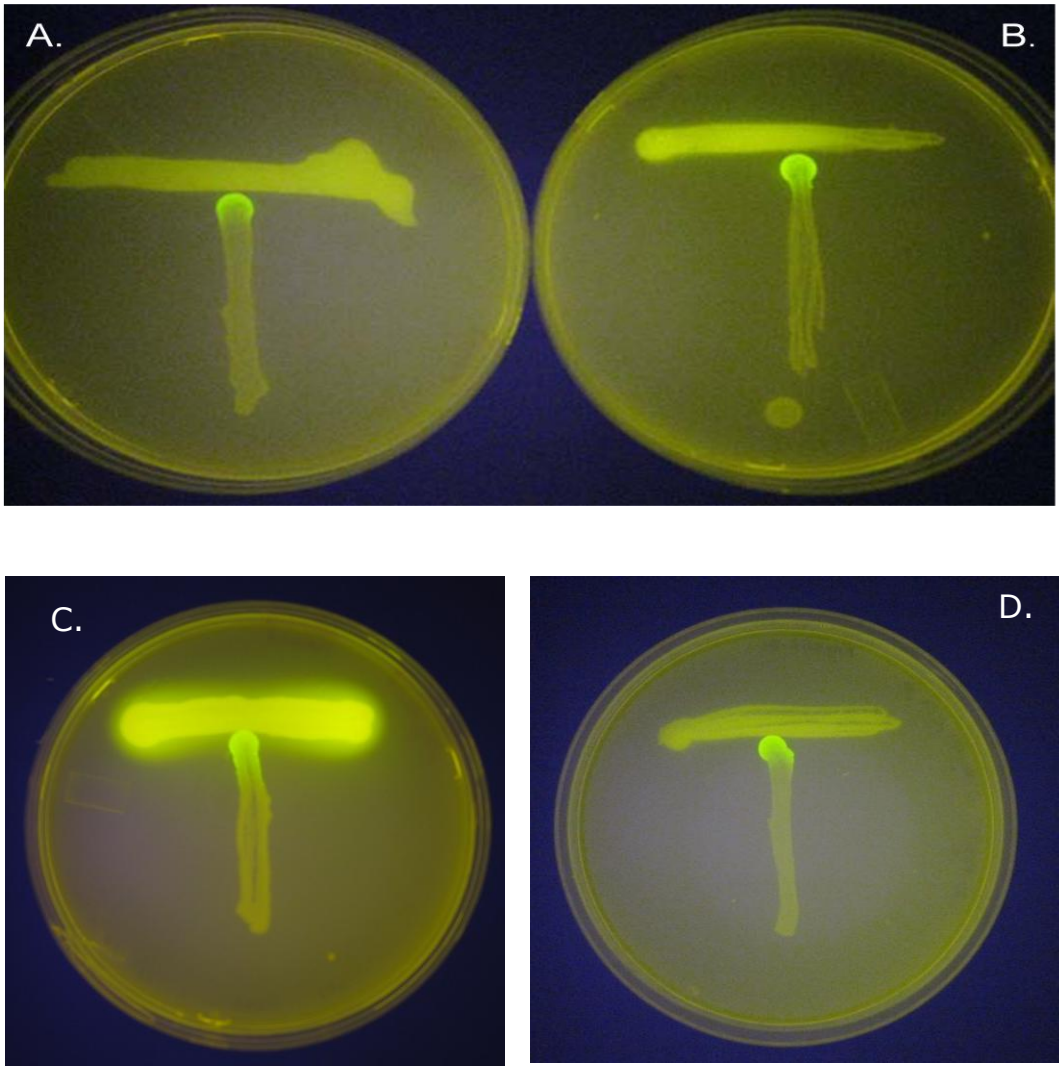


Figure 3.5. Construction of pPLS035 containing *adeMNO*::Gm-FRT deletion fragment. PCR was used to detect the presence of *aacC1* gene and Δ*adeMNO*::Gm-*FRT* fragment. Detection of *aacC1* gene took place using primers GmFRT-Dn and GmFRT-UP, and the resulting amplicon was 1053 bp. Restriction digestions with *EcoR*V and *Xba*l were performed to confirm the identity of the plasmid. Abbreviations: *bla,* beta-lactamase gene conferred beta-lactams resistance (Ap^r); *FRT,* Flp recombinase target; *aacC1*, gentamycin acetyltransferase-3-1, the gene conferring gentamycin resistance. S1, S2, S3, samples screened during the study; M, marker; C-, control negative reaction.

Figure 3.5.

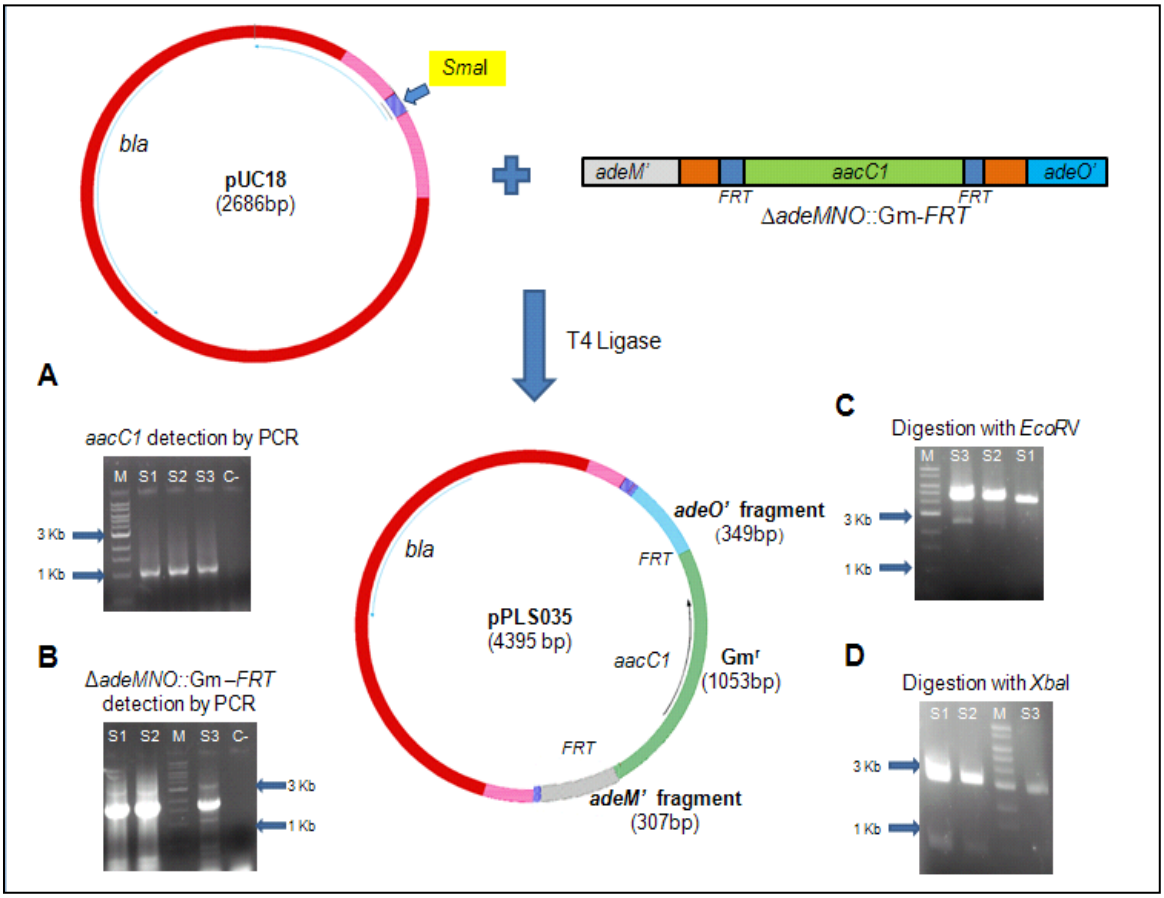
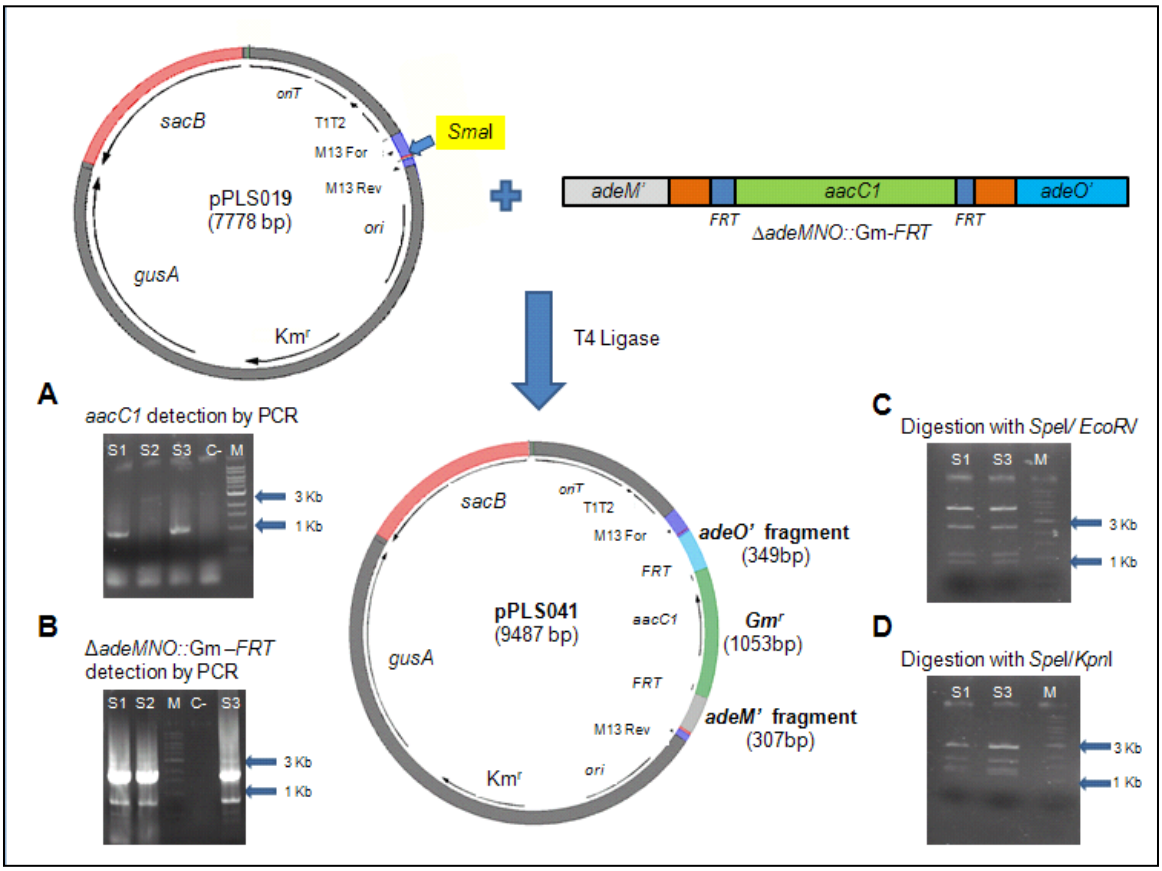

SmaI
bla
pUC18
(2686bp)
adeM'
aacC1
adeO'
FRT
FRT
ΔadeMNO::Gm-FRT
T4 Ligase
A
aacC1 detection by PCR
M S1 S2 S3 C-
3 Kb
1 Kb
B
ΔadeMNO::Gm–FRT
detection by PCR
S1 S2 M S3 C-
3 Kb
1 Kb
bla
pPLS035
(4395 bp)
adeO' fragment
(349bp)
FRT
aacC1
Gmr
(1053bp)
FRT
adeM' fragment
(307bp)
C
Digestion with EcoRV
M S3 S2 S1
3 Kb
1 Kb
D
Digestion with XbaI
S1 S2 M S3
3 Kb
1 Kb

Figure 3.6. Construction of the pPLS041 suicide plasmid containing *adeMNO* gene deletion fragment. Verification of the plasmid composition was done by restriction digestion using *Spe*I/*EcoR*V and *Spe*I/*Kpn*I. For the *Spe*I/*EcoR*V digestion four fragments were expected of 4718, 2470, 1140, 931 and 231bp sizes as seen in Fig. 3.6.C. In the case of *Spe*I/*Kpn*I digestion, the size of the fragments expected were 2664, 2658, 1698, 1297 and 1173 bp as seen in Fig. 3.6.D. Abbreviations: *sacB*, *Bacillus subtilis l*evansucrase counter selection marker; g*usA, Escherichia coli* beta-glucuronidase reporter gene; *oriT*, RK2-derived origin for conjugal plasmid transfer; T0T1, transcriptional terminators T0 and T1 from bacteriophage α and *E. coli rrnB* operon, respectively; Km^r, *nptI* gene coding for neomycin phosphotransferase, which confers kanamycin resistance; *ori*, *E. coli* pMB9 origin of replication; *FRT,* Flp recombinase target; *aacC1*, gentamycin acetyltransferase-3-1, the gene conferring gentamycin resistance.

Figure 3.6.


sacB
oriT
T1T2
M13 For
M13 Rev
pPLS019
(7778 bp)
ori
gusA
Km^r
SmaI
adeM'
aacC1
adeO'
FRT
ΔadeMNO::Gm-FRT
FRT
T4 Ligase
A
aacC1 detection by PCR
S1 S2 S3 C- M
3 Kb
1 Kb
B
ΔadeMNO::Gm–FRT
detection by PCR
S1 S2 M C- S3
3 Kb
1 Kb
pPLS041
(9487 bp)
adeO' fragment
(349bp)
Gm^r
(1053bp)
adeM' fragment
(307bp)
C
Digestion with SpeI/ EcoRV
S1 S3 M
3 Kb
1 Kb
D
Digestion with SpeI/KpnI
S1 S3 M
3 Kb
1 Kb

C. REGULATION OF THE EXPRESSION OF THE *adeMNO* OPERON

3.C.i. IDENTIFICATION OF THE PUTATIVE *lysR* FAMILY GENE *adeL*

The analysis of the region upstream of *adeMNO* operon revealed the presence of a gene *A1S_2303*, it was named *adeL*. This gene was identified as the possible local regulator for AdeMNO efflux pump expression. Gene *adeL* from eight different *A. baumannii* isolates was sequenced, and these results were input into the Basic Local Alignment Search Tool (BLAST). The results indicated that this gene belongs to the *lysR* family of regulators (Fig. 3.7.).

3.C.ii. PRESENCE OF *adeL* GENE IN THE CLINICAL ISOLATES OF *A. baumannii*

The presence of the *adeL* gene, putative regulator for *adeMNO*, was determined by PCR technique. Eight of the eleven isolates of *A. baumannii* tested showed the presence of this gene (Fig. 3.8.). From those that tested positive, six were found to express *adeN* and three showed no expression of such a gene (Table 3.4.). The control strain *A. baumannii* ATCC19606 showed presence of the putative regulator gene, without showing expression of the pump operon (Fig. 3.1.).

3.C.iii. SEQUENCE ANALYSIS OF *adeL* GENE

Gene *adeL* from eight clinical isolates of *A. baumannii* strains was amplified using PCR and the products were sequenced. The alignment analysis of these sequences revealed differences in nucleotides between strains expressing the *adeN,* gene that encodes the RND efflux pump, and those strains that showed no-expression of this gene

(Fig. 3.9.). Exceptions to these results were *A. baumannii* ATCC19606 and the clinical isolate *A. baumannii* 59973. The *adeL* sequence obtained from the control strain was similar to the sequence found in strains expressing *adeN*, but in the expression study this strain showed no-expression of such a gene. In the case of *A. baumannii* isolate 59973 the sequence for *adeL* was analogous to the group of isolates not expressing the *adeN* gene, nevertheless this strain showed expression of the *adeN* gene.

Figure 3.7. Three-dimensional structure prediction of AdeL Three-dimensional sturucture of AdeL protein was predicted using the Conserved domain database (CDD). The structure shows similarity to LysR-type transcriptional regulator (LTTR) proteins. Residues shown in red are similar/identical to the LTTR CrgA from *Neisseria meningitide*, while those shown in pink are different from the CrgA protein.

Figure 3.7.

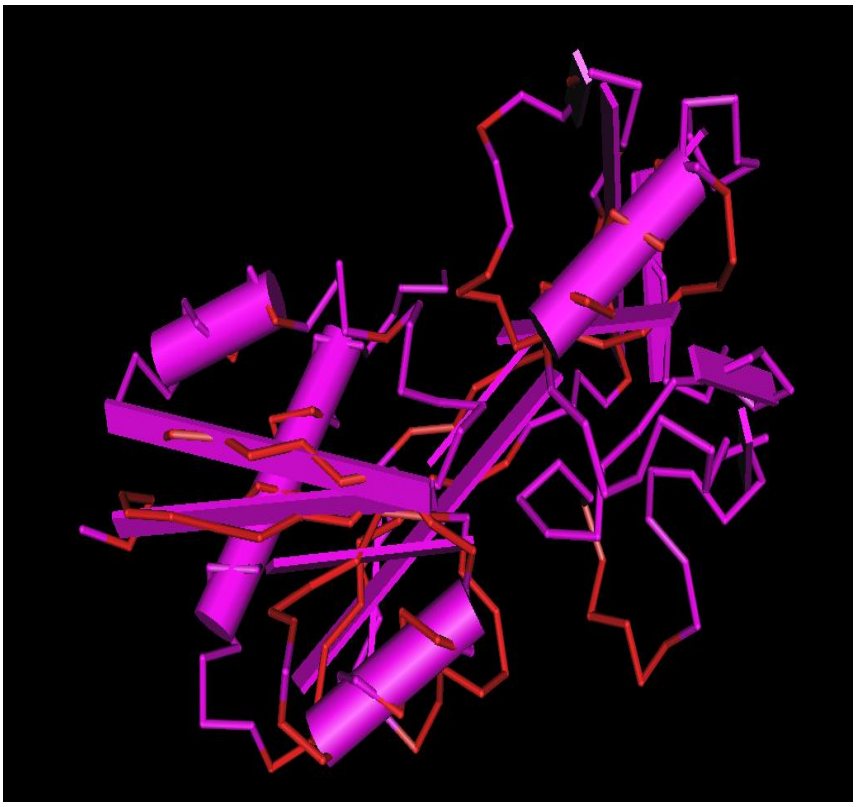


Figure 3.8. PCR-based detection of putative *lysR* family gene*, adeL* in various *A. baumannii* clinical isolates*.*** 1*. A. baumannii* 63169; 2. *A. baumannii* 58352; 3. *A. baumannii* 59960; 4. *A. baumannii* 59973; 5*. A. baumannii* 64130; 6. *A. baumannii 64153; 7. A. baumannii* 64797; 8*. A. baumannii* 63487; 9. *A. baumannii* 65239; 10. *A. baumannii* 66310; 11*. A. baumannii* 66985; 12*. A. baumannii* ATCC19606 (control).

Figure 3.8.

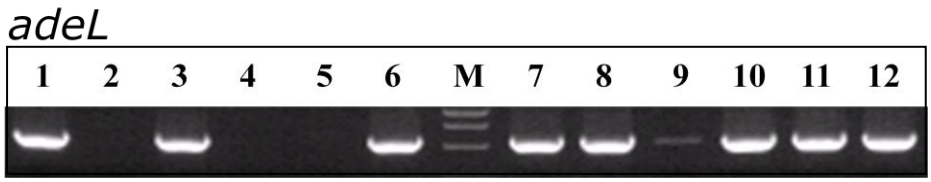


Table 3.4. Presence and expression of *adeN* gene and the presence of *adeL* putative regulator. The results summarized in this table were obtained by PCR technique

	adeN		***adeL***
Strain	**DNA**	**mRNA**	**DNA**
A. baumannii 63169	+	+	+
A. baumannii 58352	+	+	-
A. baumannii 59960	+	-	+
A. baumannii 59973	+	+	-
A. baumannii 64130	+	-	-
A. baumannii 64153	+	+	+
A. baumannii 64797	+	-	+
A. baumannii 63487	+	+	+
A. baumannii 65239	+	+	+
A. baumannii 66310	+	-	+
A. baumannii 66985	+	+	+
A. baumannii ATCC19606	+	-	+

+, indicate the positive detection of the gene and/ or its expression

-, indicate the negative detection of the gene and/or its expression in the strain.

Figure 3.9. DNA sequence analysis of putative LysR-family regulator encoding *adeL* from eight *A. baumannii* clinical isolates. Strains found to express the *adeN* gene are shown with an asterisk (*). Difference in nucleotides sequence between strains expressing and not expressing the *adeN* gene are underlined and in bold. Underlined strains showed a different tendency from the rest of the isolates studied, *A. baumannii* 59973 was found to express the *adeN* gene in spite of its homology with the sequences of those strains that showed expression of *adeN.* In the case of *A. baumannii* ATCC19606, it did not show expression of the RND pump-encoding *adeN* gene even in absence of the nucleotide difference. Completed *adeL* sequences from the strains shown in this figure and their alignment can be found in Appendix 2.

Figure 3.9.

Amino acids	D127	L	V	G	E	A	V	D	C	A	I	R	V	G	E
A. baumannii 63169*	GAC	TTG	GTT	GGA	GAA	GCA	GTT	GAT	TGT	GCA	ATT	CGG	GTG	GGA	GAA
A. baumannii 59960	GAC	TTG	GTG	GGA	GAG	GCT	GTC	GAT	TGT	GCA	ATT	CGG	GTC	GGT	GAA
A. baumannii 59973	GAC	TTG	GTG	GGA	GAG	GCT	GTC	GAT	TGT	GCA	ATT	CGG	GTC	GGT	GAA
A. baumannii 64153*	GAC	TTG	GTT	GGA	GAA	GCA	GTT	GAT	TGT	GCA	ATT	CGG	GTG	GGT	GAA
A. baumannii 64797	GAC	TTG	GTG	GGA	GAG	GCT	GTC	GAT	TGT	GCA	ATT	CGG	GTC	GGT	GAA
A. baumannii 63487*	GAC	TTG	GTT	GGA	GAA	GCA	GTT	GAT	TGT	GCA	ATT	CGG	GTG	GGA	GAA
A. baumannii 66310	GAC	TTG	GTG	GGA	GAG	GCT	GTC	GAT	TGT	GCA	ATT	CGG	GTC	GGT	GAA
A. baumannii 66985*	GAC	TTG	GTT	GGA	GAA	GCA	GTT	GAT	TGT	GCA	ATT	CGG	GTG	GGT	GAA
A. baumannii ATCC19606	GAC	TTG	GTT	GGA	GAA	GCA	GTT	GAT	TGT	GCA	ATT	CGG	GTG	GGT	GAA
	***	***	**^	***	**^	* *^	**^	***	***	***	***	***	**^	***	***

3.C.iv. CREATION OF *adeL* GENE DELETION FRAGMENT

A gene knock-out for the *adeL* regulator was created using the technique described by Choi and Schweizer (2005) with the purpose of studying *adeL* gene function in the native strains of *A. baumannii*. Annealing of the three amplicons 3' and 5' ends from *adeL* gene and *aacC1* gene is described in Fig 3.10.A. permitted the construction of the gene deletion fragment for *adeL* gene, Δ*adeL*::Gm-*FRT, as* seen in Fig. 3.10.B. The pUC18 plasmid was digested with *Sma*I, and ligated with Δ*adeL*::Gm-*FRT*, to obtain the pPLS036 plasmid and transformed into DH5α competent cells. PCR was used to screen the colonies for the presence of *aacC1* gene and Δ*adeL*::Gm-*FRT* fragment, the results are shown in Fig. 3.11.A. and B respectively. A second screening was performed by restriction enzymes using *EcoR*I in a single digestion and a *Pst*I/ *Kpn*I in a double digestion, results are illustrated in Fig. 3.11.C. and D.

Figure 3.10. Schematic illustration *adeL* knock out fragment generation by overlap extension PCR. (A) During the first round PCR (PCR1), a 200 bp fragment from the 5' end and 250 bp fragment from the 3' ends of *adeL* were annealed together with *aacC1* using the splicing overlap extension reaction as described in section 2.9. (B) The 1.5 Kb assembled fragment was then amplified using common primers Ab_2303F and Ab_2303R.

Figure 3.10.

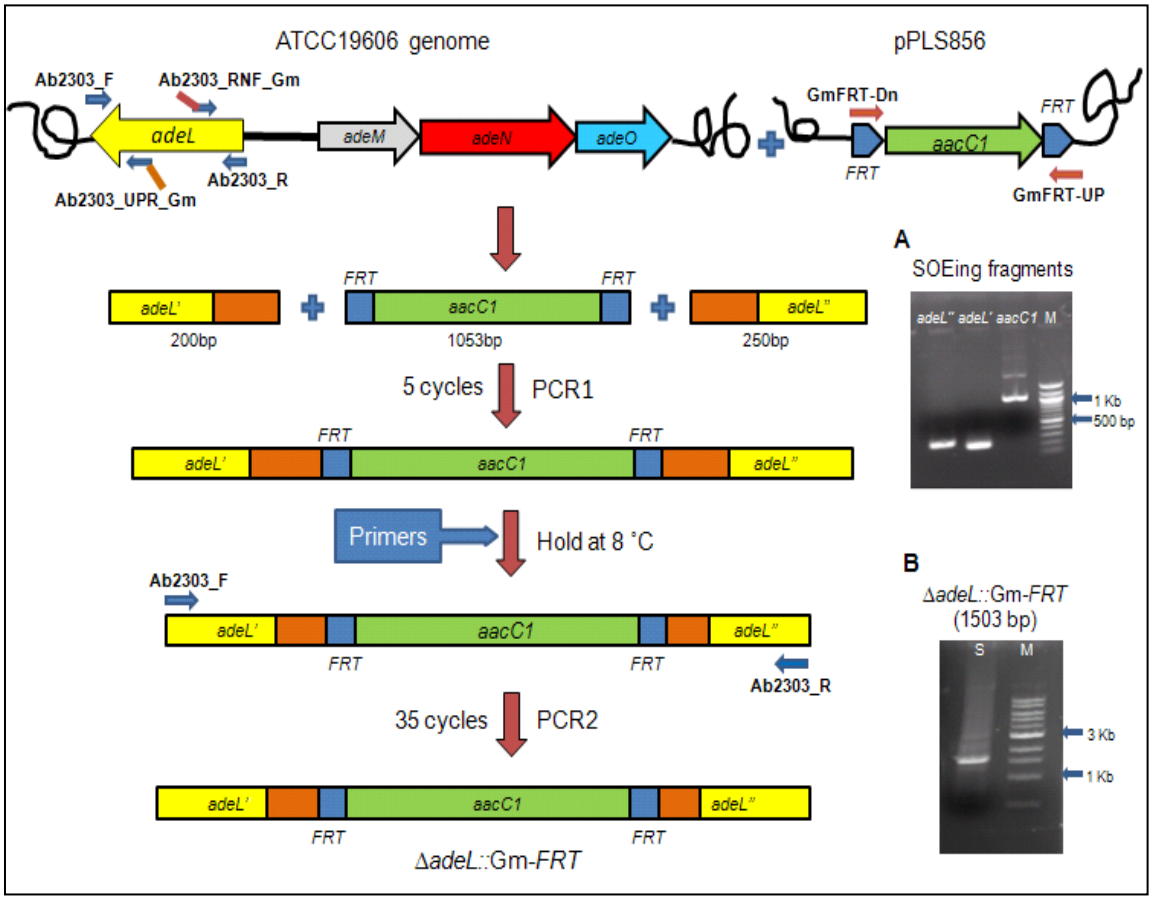
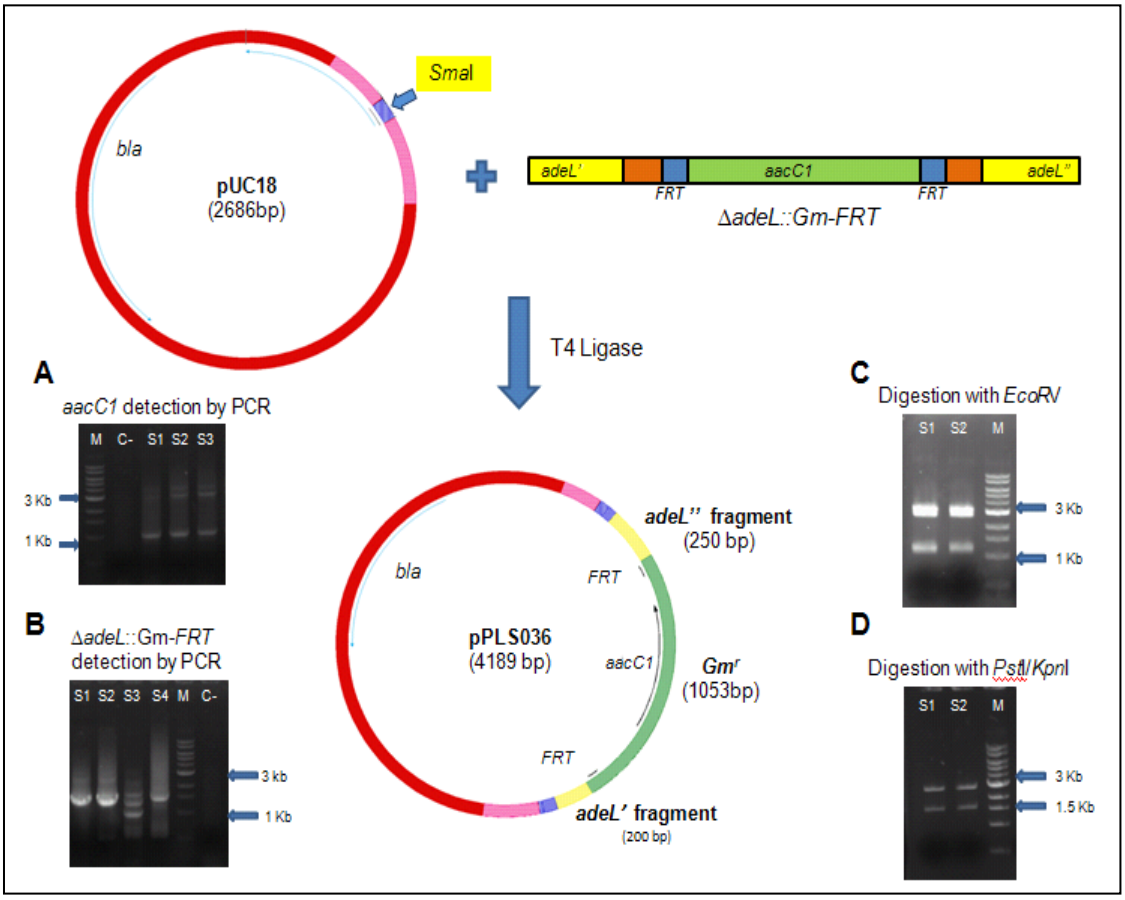

ATCC19606 genome
pPLS856
Ab2303_F
Ab2303_RNF_Gm
adeL
adeM
adeN
adeO
Ab2303_R
Ab2303_UPR_Gm
GmFRT-Dn
FRT
aacC1
FRT
GmFRT-UP
A
SOEing fragments
adeL'
FRT
aacC1
FRT
adeL"
200bp
1053bp
250bp
adeL" adeL' aacC1 M
1 Kb
500 bp
5 cycles
PCR1
Primers
Hold at 8 °C
B
ΔadeL::Gm-FRT
(1503 bp)
Ab2303_F
Ab2303_R
35 cycles
PCR2
S
M
3 Kb
1 Kb
ΔadeL::Gm-FRT

Figure 3.11. Cloning of Δ*adeL::*Gm-*FRT* fragment into pUC18 plasmid. The *adeL* gene deletion fragment obtained from the SOEing PCR reaction shown in Fig. 3.7. was ligated into pUC18 plasmid pre-digested with *Sma*I restriction enzyme. Screening of the resulting plasmids was performed by PCR and restriction digestion. Pictures A and B show the results of the screening PCR performed to detect the *aacC1* gene and the Δ*adeL*::Gm-*FRT* fragment respectively. Gm^r was detected using primers GmFRT_Dn and GmFRT_UP resulting in the amplification of a 1053 bp fragment. The presence of Δ*adeL*::Gm-*FRT* fragment was detected using primers Ab2303_F and Ab2303_R amplifying an amplicon of around 1.5 Kb. Picture C shows the digestion of the pPLS036 plasmid with *EcoR*V which generated two fragments a 3029 bp and a 1163 bp. In the case of the double digestion with *Pst*I*/ Kpn*I shown in picture D, the results fragments were 1533 bp and 2659 bp.

Figure 3.11.


SmaI
bla
pUC18
(2686bp)
adeL'
aacC1
adeL''
FRT
FRT
ΔadeL::Gm-FRT
T4 Ligase
A
aacC1 detection by PCR
M C- S1 S2 S3
3 Kb
1 Kb
B
ΔadeL::Gm-FRT
detection by PCR
S1 S2 S3 S4 M C-
3 kb
1 Kb
bla
pPLS036
(4189 bp)
FRT
aacC1
FRT
adeL'' fragment
(250 bp)
Gmr
(1053bp)
adeL' fragment
(200 bp)
C
Digestion with EcoRV
S1 S2 M
3 Kb
1 Kb
D
Digestion with PstI/KpnI
S1 S2 M
3 Kb
1.5 Kb

3.C.v. SEQUENCING AND ANALYSIS OF THE *adeMNO* PROMOTER REGION FROM *A. baumannii* ISOLATES

The promoter regions from six clinical isolates were sequenced with the purpose of establishing possible differences between strains expressing and those not expressing the *adeN* gene. For this purpose, strains *A. baumanni* 63169, 64153 and 63487 were selected as examples of strains expressing the *adeN* gene. In the case of the *A. baumannii* isolates which showed no expression of the gene, the isolates 59960, 64797 and 66310 were selected for sequencing. The alignment of the six *adeMNO* promoter sequences indicated that there is a one nucleotide difference between these two groups of isolates as seen in Fig. 3.12.

Figure 3.12. Sequence alignment of *adeMNO* promoter region between nucleotides from six *A. baumannii* isolates. Strains marked with an asterisk correspond to strains that express *adeN*, and their sequences showed the presence of a Adenine (A) nucleotide in position 128 from the start codon of *adeM,* the nucleotide in this position is showed bold and underlined. The group of isolates that showed expression of the RND efflux pump gene presented a Guanidine (G) in this position instead. The signature sequence for the LysR-binding site is shown in the bottom of the alignment (Maddocks and Oyston, 2008).

Figure 3.12.


Strain
78
A. baumannii 63169*
AAAAATACGAATAGTGTTATCAAATTTAATATATTTATTCGT
A. baumannii 59960
AAAAATACGAATAGTGTTATCGAATTTAATATATTTATTCGT
A. baumanni 64153*
AAAAATACGAATAGTGTTATCAAATTTAATATGTTTATTCGT
A. baumannii 64797
AAAAATACGAATAGTGTTATCGAATTTAATATATTTATTCGT
A. baumannii 63487*
AAAAATACGAATAGTGTTATCAAATTTAATATATTTATTCGT
A. baumannii 66310
AAAAATACGAATAGTGTTATCGAATTTAATATATTTATTCGT
LysR conserved binding sequence
TTA -N7- TTAA

CHAPTER 4

DISCUSSION AND CONCLUSIONS

A. EXPRESSION OF RND EFFLUX PUMPS IN CLINICAL ISOLATES OF *Acinetobacter baumannii* FROM CANADIAN HOSPITALS

It is now a widely accepted that RND efflux pumps play a major role in the antibiotic resistance of clinically relevant Gram-negative bacteria. Therefore, characterization of these pumps is essential in order to design effective drug therapy. *A. baumannii* is no exception and with increasing reports of multidrug resistance in this organism (Falagas *et al*., 2007; Navon-Venezia *et al*., 2007), this study intended to elucidate the role of RND pumps in its resistance. For this purpose, this research studied the expression of six RND efflux pump-encoding genes (characterized as well as yet uncharacterized) in clinical isolates of *A. baumannii* from Canadian hospitals. Since the expression of RND efflux pumps is usually under very tight regulatory control, this study identifies only the genes that are expressed either constitutively or under the experimental conditions. While the absence of the PCR product could indicate absence of the gene or its transcription, this could also result from inability of the experimental primers to anneal to their target gene as result of possible variations in the sequence. In spite of these limitations and the small sample size, this study provided convincing evidences that the expression of RND pumps is widespread in clinical isolates of *A. baumannii,* as well as confirmed the presence RND efflux pumps that are yet to be characterized in this organism.

AdeABC and AdeIJK are the two major pumps described in *A. baumannii* (Damier-Piolle *et al*., 2007; Magnet *et al*., 2001). AdeABC has been shown to efflux amikacin, chloramphenicol, cefotaxime, erythromycin, gentamicin, kanamycin, norfloxacin, netilmicin, ofloxacin, perfloxacin, sparfloxacin, tetracycline, tobramycin, and trimethoprim (Magnet *et al*., 2001), while AdeIJK effluxes β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, pyronine, safranin, and sodium dodecyl sulfate (SDS) (Damier-Piolle *et al*., 2007). Interestingly, the *adeJ* gene was in all of the 11 clinical

isolates from the study in contrast to the *adeB* gene that was found only in seven isolates, suggesting that AdeIJK is likely to be the major pump in these isolates. Also, five out of six clinical isolates that harbored both the *adeB* and *adeJ* genes, namely *A. baumannii* 63169, *A. baumannii* 59960, *A. baumannii* 64130, *A. baumannii* 64397, *A. baumannii* 63487, and *A. baumannii* 66985, expressed only one of the two genes. One exception was *A. baumannii* 64130 that was found to express both *adeB* and *adeJ* genes together (Table 3.1.). The AdeDE pump is the third pump previously described in *A. baumannii* that effluxes amikacin, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, ethidium bromide, meropenem, rifampin, and tetracycline (Chau *et al.*, 2004). From the 11 isolates tested, 10 were found to harbor the gene and 80% of those isolates were found to express the *adeE* gene (Table 3.1). All the strains used in this study were tested for their susceptibility to ceftazidime, ciprofloxacin, tetracycline, and chloramphenicol which are known substrates of the AdeDE pump (Chau *et al.*, 2004), and the results are shown in Appendix 1. According to these results, strains that expressed the *adeE* gene were not found to be any more resistant to these antibiotics than *A. baumannii* 66985 that did not contain the *adeE* gene. These observations could be the result of overlapping substrate which makes difficult to identify the subtrates efflux by this pump. Also there is a possibility that this RND pump was not functional in these strains, this could be caused by the lack of its outer membrane protein or by presence of a defective sequence of the operon. In this case more research is necessary in order to validate any of these explanations.

A result that is important to mension is the detection of *adeB* and *adeE* genes together in *A. baumannii* isolates analysed during this study. From the six strains harbouring both genes, three of them showed concurrent expression, this observation is unique and has not been reported in literature. Therefore, more research is needed to

understand the implication of these results in the resistance mechanism present in this pathogen. Further analyses including Southern blot analysis are required to confirm the concurrent presence of the entire *adeABC* and *adeDE* operons in *A. baumannii* strains.

The clinical isolates of *A. baumannii* were also tested for the presence and expression of three uncharacterized RND pump encoding genes, namely *A1S_2818*, *A1S_3217*, and *adeN* that are part of the *A1S_2817-2818*, *A1S_3219-3218-3217-3214,* and *adeMNO* operons, respectively. The *A1S_2817-2818* operon consists of genes encoding the MF (*A1S_2817*) and the RND (*A1S_2818*) proteins, but not for the OMF. The *A1S_2818* gene was found to be present in six out of 11 isolates tested (55%), while five out those six isolates were found to be expressing the gene. Two possible homologs of the OMF-encoding genes were found in the genome of *A. baumannii* ATCC17989, *A1S_0255* and *A1S_1241*. It is possible that *A1S_2817-2818* utilizes one of the gene products as its outer membrane component. It is also possible that *A1S_2817-2818* functions with one of the outer membrane proteins that is encoded as a part of RND complex operons in a fashion similar to the MexXY pump of *P*. *aeruginosa*, which functions with the OprM protein that is encoded as part of the *mexAB-oprM* operon (Mine *et al.*, 1999).

The *A1S_3219-3218-3217-3214* operon is different from other RND complex encoding operons because it contains two genes encoding the OMF (*A1S_3219* and *A1S_3214*), as shown in Fig. 1.2. If *A1S_3218-3217* is a functional pump, having two outer membrane proteins can possibly result in a wider substrate range for this pump. An example of this type alternative structure is MexJK pump which is able to function with OpmH or OprM outer membrane proteins, this flexible tripartite assembly allows the pump to efflux different substrates depending on the outer membrane forming the complex (Chuanchuen *et al*., 2005). However, the *A1S_3217* gene was found to be

present and expressed in only *A. baumannii* 64130, suggesting that it may not be of significant clinical relevance.

The purpose of this part of the study was to examine the expression of different RND pump encoding genes in clinical isolates of *A. baumannii*; the results indicated that the clinical isolates of *A. baumannii* extensively express RND efflux pumps. The lack of conclusive correlations between efflux pump expression and the antibiotic susceptibilities of clinical isolates of *A. baumannii* is most likely due to concurrent expression of multiple pumps and/or due to the presence of other resistance mechanisms. As for the gene products for the yet uncharacterized genes, the lack of correlation could be connected to the possibility that the drugs tested during this research were not substrates of these pumps or maybe to the lack of a functional pump structure. This study also indicated that as yet uncharacterized pumps may play an important role as part of the antibiotic resistance mechanism of *A. baumannii*, since at least two of the three uncharacterized pumps were found to be expressed in a large proportion of clinical isolates.

The results obtained durig this study support the idea that RND pumps played an essential role in the antibiotic resistance mechanism present in Gram-negative bacteria, suggesting that energy-dependent efflux system should be considered while designing therapeutic options for organisms like *A. baumannii.*

B. CLONING AND CHARACTERIZATION OF AdeMNO RND EFFLUX PUMP OF *Acinetobacter baumannii*

Expression analysis of the RND pumps from clinical isolates of *A. baumannii* showed that the operon *A1S_2304-A1S_2305-A1S_2306*, designated as A*deMNO* in this study, was present in all of the isolates tested showing expression in 8 out of the 11 isolates tested.These results point to the necessity of characterizing this RND efflux pump*,* in order to further investigate its role in the antibiotic resistance mechanism of emergent MDR *A. baumannii*. This was accomplished using a surrogate *P. aeruginosa* strain PAO750 which presents the advantage of lacking its native RND efflux systems; this allowed the functional characterization of the AdeMNO efflux pump without interferences. The AdeMNO pump was found to efflux clinically-relevant antibiotics, chloramphenicol, trimethoprim, and clindamycin. Preliminary screening using Vitek 2 system also showed imipenem as substrate for this pump, but posterior analysis by disc diffusion technique discarded this possibility.

As mentioned above, the *adeN* gene was found in all of the clinical isolates studied, as well as the control strain, *A. baumannii* ATCC19606. None of the previously characterized pumps (AdeABC, AdeDE, AdeIJK) were found to be as prevalent in the clinical isolates, and even though the expression levels of this gene were lower than the *adeB* and *adeJ* genes, the presence of the *adeN* gene in 100% of the isolates tested suggests that this pump may be of clinical and/or physiological significance in *A. baumannii*. Indeed, a BLAST search for the *adeMNO* operon reveals that it is present in all strains of *A. baumannii* that have been sequenced (either partially or completely) to date, including the pathogenic strains *A. baumannii* AYE (Vallenet *et al*., 2008), *A. baumannii* ACICU (Iacono *et al*., 2008), and *A. baumannii* AB900, and also the human louse symbiont *A. baumannii* SDF (Vallenet *et al*., 2008), in addition to *A. baumannii* ATCC17989 (Smith *et al.*, 2007) and *A. baumannii* ATCC19606 (PATRIC, Pathosystems Resource Integration Center, Virginia Bioinformatics Institute).

Among RND pumps characterized from other bacterial species, the closest homolog of AdeN is the BpeF protein from BpeEF-OprC efflux system of *Burkholderia pseudomallei* (Kumar *et al.*, 2006) and the CeoB protein from CeoAB-OpcM efflux system of *Burkholderia cenocepacia* (Nair *et al*., 2004). The BpeEF-OprC and CeoAB-OpcM systems have both been shown to efflux trimethoprim and chloramphenicol, but not clindamycin. A number of clinical isolates used in this study were found to be resistant to various antibiotics (Appendix 1); including substrates of the AdeMNO pump (chloramphenicol, trimethoprim, and clindamycin). However, it was impossible to ascertain the role of AdeMNO in the antibiotic resistance of the clinical isolates due to the simultaneous expression of multiple RND efflux pump genes.

The AHL assay performed in this study established that the AdeMNO pump introduced in the surrogate strain of *P. aeruginosa* not only effluxes the antibiotics mentioned above, but also its presence results in increased efflux of AHL. N-Acyl homoserine lactone is a group of compounds utilized by bacteria as quorum sensing molecule; this biological signal trigger a synchronized respond of the microorganism based in population density (Chan *et al*., 2007). This is an interesting finding since it opens the possibility that the AdeMNO pump plays a physiological role in biofilm formation by *A. baumannii*. Although previous reports have shown that RND pumps are involved in the efflux of cell signalling molecules in bacteria (Kohler *et al.*, 2001; Tian *et al*, 2009a), this has not been described for any of the characterized efflux pumps of *A. baumannii*. Further studies are necessary to determine the role of AdeMNO in cell-to-cell communication, biofilm formation and secretion in *A. baumannii*.

To summarize, this study identified and characterized AdeMNO, a novel RND efflux pump of *A. baumannii* that is able to efflux clinical relevant antibiotics as well as AHL, a quorum sensing signalling molecule in *P. aeruginosa* PA008. Since this pump

was found to be present in all of the clinical isolates tested in this study, with its expression observed in a very high proportion of the isolates, it is likely to be of clinical and/or physiological relevance warranting further characterization.

C. REGULATION OF THE EXPRESSION OF THE *adeMNO* OPERON

The last objective of this thesis was to study the regulatory mechanisms involved in the expression of the AdeMNO pump. It is known that efflux pumps decrease the antibacterial efficiency of structurally unrelated substances, by limiting the accumulation of these compounds in the bacteria cytoplasm (Borst *et al.*, 1999; Koronakis *et al.,* 2000; Borges-Walmsley *et al*., 2003). The overexpression of these proteins can be responsible for an extended cross-resistance that can confer low sensitivity to a variety of drug types (Coyne *et al*., 2010; Lin *et al*., 2009). As a consequence, the identification and understanding of the transcription regulation system associated with RND efflux pumps could result in the creation or discovery of molecules to repress their expression allowing the inactivation of the efflux system, decreasing the resistance of this pathogen.

In this study, examination of the upstream region of the *adeMNO* operon of *A. baumannii* revealed the presence of a *lysR*-type open reading frame. In Gram-negative bacteria, a publication described this type of regulator associated with the expression of the MexEF-OprN operon in *P. aeruginosa* (Kohler *et al*., 1999) and BpeEF-OprC in *Burkholderia pseudomallei* (Kumar *et al.*, 2006). LysR-family transcription regulator is widely spread among prokaryotes associated to a diversity of genes and complex regulons, but this is the first time described in connection to RND efflux pumps operons in *A. baumannii*.

The analysis of the *adeL* sequences obtained from multiple *A. baumannii* clinical isolates showed differences in sequence between strains expressing and those not expressing *adeN* gene. These nucleotide replacements were all identified in the wobble base, so none of them translated into amino acids substituted in the resulting protein. This observation suggests that the differences found in the *adeN* gene expression is not related with the amino acids constituent of the *adeL*, but may be connected with the nucleotide sequence that encodes the protein. According to the available literature, a

single nucleotide variation in the third base of a codon that encodes the same amino acid could result in fluctuation of the gene expression (Kudla e*t al.*, 2009). This control could be explained by codon bias, in which the efficiency of the gene translation and accuracy correlates with the abundance of the isoaccepting tRNA (Zuckerkandl and Pauling, 1965; Ikamura, 1985). In addition, recent research has stablished that mRNA folding near the ribosomal binding site affects the levels of a protein expression (Kudla e*t al.*, 2009). In the case of *adeL*, if the nucleotide substitutions found in its sequence alters the proper mRNA folding affecting its interaction with the ribosomal structure, this could result in fluctuations of the transcription leves of the regulator, causing variations in the levels of *adeMNO* transcription.

In addition to the sequence differences found in the regulator *adeL* region, the analysis of the promoter region of six isolates of *A. baumannii* revealed the existence of a single nucleotide variation between strains expressing and those not expressing the *adeN* gene. As seen Fig. 3.12., the recognized as the promoter region for *adeM* gene showed a one base substitution of a G for an A in the nucleotide 128 up-stream from *adeMNO* start codon. It is possible that the *adeL* regulator binds tighter to the DNA sequence of the promoter region in presence of a Guanine (G) nucleotide resulting in the repression of the transcription of *adeN*.

Another characteristic of LysR-family regulator that must be considered when attenting to explain the difference in the *adeN* transcription, is the possibility of multiple binding sites within the promoter region associated to the *adeMNO* operon. Several reports have shown that the LysR-type of transcription regulator could bind simultaneously to multiples sites within the intergenic region between the regulator sequence and the associated gene or operon (Belitsky *et al.*, 1995; Lochoswska *et al.*, 2001; Porrua *et al*., 2007). This family of regulators has been reported as functionally

active as tetramer, and DNase-I protection assay studies support the multibinding capacity of the LysR polymer proteins (Muraoka *et al.*, 2003). This function could result in the bending of the DNA strand interfering with the DNA-RNA-polymerase interaction modulating the gene transcription (Lochowska *et al*., 2004).

In summary, many questions remain unanswered in relation to the transcription regulation mechanism(s) associated not only with the adeMNO operon, but also with RND efflux pumps from *A. baumannii*. This study has made the novel discovery of AdeL, a LysR-transcription regulator associted to *adeMNO*. The study of the *adeL* sequence, as well as the promoter region from different *A. baumannii* isolates revealed variations in nucleotide sequence that could explain the differences in the *adeN* expression observed in this study. Nevertheless, it is essential to continue this line of investigation to confirm this hypothesis.

CHAPTER 5

FUTURE DIRECTIONS

The creation of a deletion mutant of *adeMNO* is essential in order to confirm the substrate profile of this pump. A gene deletion construct for *adeMNO* was prepared utilizing a SOEing reaction as seen in Fig. 2.1. This product was ligated into a mobilizable pEXKm5 vector (Fig. 3.6) to be used as delivery plasmid. There are various advantages associated with the use of this plasmid; it contains multiples cloning sites inserted into the *LacZ*α gene that facilitate the recombinant recognition, it possesses a constitutively expressed *gusA* gene which allows the visual recognition of the merodiploids, and lastly, the presence of a resolution element *sacB* gene that works as a counterselection marker faciliates the curing of the plasmid (Lopez *et al.,* 2009). In addition, this plasmid possesses the *nptII* gene encoding for neomycin phosphotransferase, which confers kanamycin resistance making easy the manipulation and identification of mutants containing pEX-based plasmid (Choi e*t al*., 2008). *A. baumannii* will be used to conjugate a mutant strain containing the pEXKm5-Δ*adeMNO*::Gm-*FRT* plasmid followed by the flipping of Gm marker. This *A. baumannii* mutant will lack a functional AdeMNO efflux pump facilitating the verification of the substrate profile obtained in the *P. aeruginosa* PA008.

Gene *adeL,* located downstream from *adeMNO,* was identified as the possible local regulator for this pump operon, nevertheless this association could be confimed by the use of the homologous recombination technique highlighted above. The innactivation of AdeL in *A. baumannii* will verify the role of this gene in the regulation of *adeMNO* expression, as well as to gather details about the nature of this control.

In summary, more research is required in order to understand the relation between *adeL* and promoter sequence variations and the pump expression. All this information is indispensable to understand and resolve the increasing resistance observed among clinical isolates. This research has provided exciting results associated

with RND efflux pump in *A. baumannii*, nevertheless more research is required before this information can be translated into a treatment against MDR *A. baumannii*.

REFERENCES

1. **Abrutyn E, GL Goodhart, K Roos, R Anderson, A Buxton.** 1978. *Acinetobacter calcoaceticus* outbreak associated with peritoneal dialysis. American Journal of Epidemiology **107**:328-35
2. **Afzal-Shah M, N Woodford, DM Livermore.** 2001. Characterization of OXA-25, OXA-26, and OXA-27, molecular class D beta-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii.* Antimicrobial Agents and Chemotherapy **45**:583-588
3. **Andersen JB, A Heydorn, M Hentzer, L Eberl, O Geisenberger, BB Christensen, S Molin, M Givskov**. 2001. Gfp-based N-acyl homoserine-lactone sensor systems for detection of bacterial communication. Applied and Environmental Microbiology. **67**:575-585
4. **Belitsky BR, PJ Janssen, AL Sonenshein**. 1995. Sites requires for GltC-dependent regulation of *Bacillus subtilis* glutamate synthase expression. Journal of Bacteriology **177**:5686-5695
5. **Bergogne-Berezin E, KJ Towner.** 1996. *Acinetobacter spp.* as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clinical Microbiology Reviews **9**:148-165
6. **Berlau J, HM Aucken, H Malnick, T Pitt.** 1999a. Distribution of *Acinetobacter* species on skin of healthy humans. European Journal of Clinical Microbiology and Infectious Diseases **18**:179-183
7. **Berlau J, HM Aucken, E Houang, TL Pitt.** 1999b. Isolation of *Acinetobacter spp*. including *A. baumannii* from vegetables: implications for hospital-acquired infections. The Journal of Hospital Infection **42**:201-204
8. **Borges-Walmsley MI, KS McKeegan, AR Walmsley.** 2003. Structure and function of efflux pumps that confer resistance to drugs. The Biochemical Journal **376**:313-38
9. **Borst P, Evers R, Kool M, Wijnholds J.** 1999. The multidrug resistance protein family. Biochimica et Biophysica Acta **1461**:347-357
10. **Brown, D. G., J. K. Swanson, and C. Allen**. 2007. Two host-induced *Ralstonia solanacearum* genes, *acrA* and *dinF*, encode multidrug efflux pumps and contribute to bacterial wilt virulence. Applied and Environmental Microbiology **73**:2777–2786

11. **Centers for Disease Control and Prevention (CDC)**. 2004. *Acinetobacter baumannii* infections among patients at military medical facilities treating injured U.S. service members, 2002-2004. MMWR Morbidity and Mortality Weekly Report **53**:1063-1066
12. **Centers for Disease Control and Prevention** www.cdc.gov/ncidod/dhqp/ar_*Acinetobacter*.html, downloaded on Aug 20, 2010
13. **Chan, Y. Y., H. S. Bian, T. M. Tan, M. E. Mattmann, G. D. Geske, J. Igarashi, T. Hatano, H. Suga, H. E. Blackwell, and K. L. Chua**. 2007. Control of quorum sensing by a *Burkholderia pseudomallei* multidrug efflux pump. Journal of Bacteriology. **189**:4320–4324
14. **Chau SL, YW Chu, ET Houang.** 2004. Novel resistance-nodulation-cell division efflux system AdeDE in *Acinetobacter* genomic DNA group 3. Antimicrobial Agents and Chemotherapy **48:** 4054-4055
15. **Choi KH, A Kumar, HP Schweizer.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. Journal of Microbiological Methods **64**: 391- 397
16. **Choi KH, HP Schweizer**. 2006. mini-Tn7 insertion in bacteria with single *att*Tn7 sites: example *Pseudomonas aeruginosa*. Nature Protocols **1**:153-161
17. **Choi KH, JB Gaynor, KG White, C Lopez, CM Bosio, RR Karkhoff-Schweizer, HP Schweizer.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nature Methods **2**:443-448
18. **Choi KH, P Schweizer**. 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. BMC Microbiology **5**:30-41
19. **Choi KH, T Mima, Y Casart, D Rholl, A Kumar, IR Beacham, HP Schweizer**. 2008. Genetic Tools for Select-Agent-Compliant Manipulation of *Burkholderia pseudomallei.* Applied and Environmental Microbiology **74**:1064-1075
20. **Chu YW, L Chau, ET Houang**. 2006. Presence of active efflux systems AdeABC, AdeDE and AdeXYZ in different *Acinetobacter* genomic DNA groups. Journal of Medical Microbiology **55:**477-478
21. **Chuanchuen R, T Murata, N Gotoh, HP Schweizer.** 2005. Substrate dependent utilization of OprM or OpmH by the *Pseudomonas aeruginosa* MexJK efflux pump. Antimicrobial Agents and Chemotherapy **49:**2133-2136

22. **Cisneros JM, MJ Reyes, J Pachón, B Becerril, FJ Caballero, JL García-Garmendía, C Ortiz, AR Cobacho.** 1996. Bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical findings, and prognostic features. Clinical infectious diseases **22**:1026-1032
23. **Coyne S, G Guigon, P Courvalin, B Perichon**. 2010. Screening and quantification of the expression of antibiotic resistance genes in *Acinetobacter baumannii* with a microarray. Antimicrobial Agents and Chemotherapy **54**:333-340
24. **Damier-Piolle L, S Magnet, S Brémont, T Lambert, P Courvalin.** 2008. AdeIJK, a Resistance-Nodulation-Cell Division Pump Effluxing Multiple Antibiotics in *Acinetobacter baumannii.* Antimicrobial Agents and Chemotherapy **52:**557-562
25. **Dent LL, DR Marshall, S Pratap, RB Hulette**. 2010. Multidrug resistant *Acinetobacter baumannii*: a descriptive study in a city hospital. BMN Infectious Diseases **10**:196-204
26. **Dijkshoom L, A Nemec, HA Seifert**. 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nature Reviews Microbiology **5:**939-951
27. **Dijkshoorn L, H Aucken, P Gerner-Smidt, P Janssen, ME Kauffmann, J Garaizar, G Ursing, TL Pitt.** 1996. Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotype and phenotype methods. Journal of Clinical Microbiology **34**:1519-1525
28. **Eswaran J, E Koronakis, M K Higgins, C Hughes, V Koronakis.** 2004. Three's company: component structures bring a closer view if tripartite drug efflux pumps. Current Opinion in Structural Biology **14**:741-747
29. **Falagas, ME, EG Mourtzoukou, M Polemis, AC Vatopoulos.** 2007. Trends in antimicrobial resistance of *Acinetobacter baumannii* clinical isolates from hospitalised patients in Greece and treatment implications. Clinical Microbiology and Infection **13:**816-819
30. **Fournie PE, H Richet.** 2006. The epidemiology and control of *Acinetobacter baumannii* in Health care facilities. Clinical Infectious Diseases **42**:692-699
31. **Fournier PE, D Vallenet, V Barbe, S Audic, H Ogata, L Poirel, H Richet, C Robert, S Mangenot, C Abergel, P Nordmann, J Weissenbach, D Raoult, JM Claverie**. 2006. Comparative Genomics of Multidrug Resistance in *Acinetobacter baumannii*. PLoS Genetics **2**:e7

32. **French, GL, MW Casewell, AJ Roncoroni, S Knight, I Phillips.** 1980. A hospital outbreak of antibiotic-resistant *Acinetobacter anitratus*: epidemiology and control. The Journal of Hospital Infection **1:**125–131

33. **Getchell-White SI, LG Donowitz, DH Gröschel**. 1989. The inanimate environment of an intensive care unit as a potential source of nosocomial bacteria: evidence for long survival of *Acinetobacter calcoaceticus.* Infection Control and Hospital Epidemiology **10**:402-407

34. **Goldberg JB, DE Ohman.** 1984. Cloning and expression in *Pseudomonas aeruginosa* of a gene involved in the production of alginate. Journal of Bacteriology **158**:1115-1121

35. **Govindan R, V Bharathi Srinivasan, WA Gebreye**s. 2010. Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. The Journal of Antimicrobial Chemotherapy **65**:228-232

36. **Heinemann B, M Wisplinghoff, M Edmond, H Seifert.** 2000. Comparative activities of ciprofloxacin, clinafloxacin, gatifloxacin, gemifloxacin, levofloxacin, moxifloxacin, moxifloxacin, and trovafloxacin against epidemiologically defined *Acinetobacter baumannii* strains. Antimicrobial Agents and Chemotherapy **44**:2211-2213

37. **Heritier C, L Poirel, T Lambert, P Nordmann.** 2005. Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in *Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy **49**:3198–202

38. **Hirakata, Y., R. Srikumar, K. Poole, N. Gotoh, T. Suematsu, S. Kohno, S. Kamihira, R. E. Hancock, and D. P. Speert**. 2002. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. Journal of Experimental Medicine. **196**:109–118

39. **Hoang, T. T., R. R. Karkhoff-Schweizer, A.J. Kutchma, H. P Shweizer**. 1998. A broad-host-range Flp-*FRT* recombination system for site specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomona aeruginosa* mutants. Gene **212**:77-86

40. **Holloway PW, C Zhang.** 1990. *Pseudomonas aeruginosa* PA0,p. 271-278. In S. J. O'Brien (ed.) Genetic maps. Locus map of comples genomes. 5th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

41. **Houang ET, YW Chu, CM Leung, KY Chu, J Berlau, AF Cheng**. 2001.

Epidemiology and infection control implications of *Acinetobacter spp.* in Hong Kong. Journal of Clinical Microbiology **39**:228-234

42. **Houang TSH, RT Sormunen, L Lai, CY Chan, ASY Leong.** 1998**.** Effect of desiccation on the structural appearance of *Acinetobacter baumannii* and *Acinetobacter lwoffii*. Journal of Clinical Pathology **51**:786-788
43. **Hujer KM, AM Hujer , EA Hulten , S Bajaksouzian , JM Adams , CJ Donskey, DJ Ecker, C Massire, MW Eshoo, R Sampath, JM Thomson, PN Rather, DW Craft , JT Fishbain, AJ Ewell, MR Jacobs , DL Paterson , RA Bonomo**. 2006. Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter spp.* isolates from military and civilian patients treated at the Walter Reed Army Medical Center. Antimicrobial Agents and Chemotherapy **50**:4114-4123
44. **Iacono M, L Villa, D Fortini, R Bordoni, F Imperi, RJ Bonnal, T Sicheritz-Ponten, G De Bellis, P Visca, A Cassone, A Carattoli.** 2008. Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group. Antimicrobial Agents and Chemotherapy **52:**2616-2625
45. **Ikamura T**. 1985. Codon usage and tRNA content in unicellular and multicellular organisms. Molecular Biology and Evolution **2**:13-34
46. **Jain R**. 2004. Multidrug-Resistant *Acinetobacter* Infections: An Emerging Challenge to Clinicians. The Annals of Pharmacotherapy **38:**1449-1459
47. **Juni E.** 1972. Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. Journal of Bacteriology **112**:917-931
48. **Juni E.** 1978. Genetics and physiology of *Acinetobacter*. Annual Review of Microbiology. **32**:349-371
49. **Kohler T, SF Epp, LK Curty, JC Pechere**. 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. Journal of Bacteriology **181**: 6300-6305
50. **Kohler T, C van Delden, LK Curty, MM Hamzehpour, JC Pechere.** 2001. Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. Journal of Bacteriology **183:**5213-5222
51. **Koronakis V, A Sharff, E Koronakis, B Luisi, C Hughes.** 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature **405**:914-919

52. **Kudla G, AW Murray, D Tollervey, JB Plotkin**. 2009. Coding–sequence determinants of gene expression in *Escherichia coli.* Science **324**:255-258

53. **Kumar A**, **C Kim-Lee, HP Schweizer.** 2006. Method for regulated expression of Single –Copy efflux pump genes in a surrogative *Pseudomonas aeruginosa* strain: Identification of the BpeEF-OprC Chloramphenicol and Trimethoprim efflux pump of *Burkholderia pseudomallei* 1026b. Antimicrobial Agents and Chemotherapy **50**: 3460-3463

54. **Kumar A, C Dalton , J Cortez-Cordova , HP Schweizer.** 2010. Mini-Tn7 vectors as genetic tools for single copy gene cloning in Acinetobacter baumannii. Journal of Microbiology Methods **82**:296-300

55. **La Scola B, D Raoult.** 2004. *Acinetobacter baumannii* in human body louse. Emerging Infectious Diseases **10**:1671-1673

56. **Li XZ, L Zhang, K Poole.** 2002. SmeC, an outer membrane multidrug efflux protein of *Stenotrophomonas maltophilia*. Antimicrobial Agents and Chemotherapy **46**:333-43

57. **Lin, J., C. Cagliero, B. Guo, Y. W. Barton, M. C. Maurel, S. Payot, and Q. Zhang**. 2005. Bile salts modulate expression of the CmeABC multidrug efflux pump in *Campylobacter jejuni*. Journal of Bacteriology **187**:7417–7424

58. **Lin L, BD Ling, XZ Li.** 2009. Distributions of the multidrug efflux pump genes, *adeABC, adeDE and adeIJK*, and *class 1 integron* genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex. International Journal of Antimicrobial Agents. **33**: 27-32

59. **Lochoswska A, R Iwanicka-Nowicka, D Plochocka, MM Hryniewicz**. 2001. Functional dissection of the LysR-type CysB transcriptional regulator. Regions important for DNA binding inducer response, oligomerization and positive control. The Journal of Biological Chemistry 276:2098-2107

60. **Lochowska A, R Iwanicka-Nowicka, J Zaim, M Witkowska-Zimny, MM Hryniewicz.** 2004. Identification of activating region (AR) of *Echerichia coli* LysR-type transcription factor CysB and CysB contact site on RNA polymerase alpha subunit at the *cysP* promoter. Molecular Microbiology **53:** 791-806

61. **Lopez CM, DA Rholl, LA Trunck, HP Schweizer.** 2009. Versatile dual-technology system for markerless allele replacement in *Burkholderia pseudomallei.* Applied and Environmental Microbiology **75**: 6496-6503

62. **Lopez-Hernandez S, T Alarcon, M Lopez-Brea**. 2001. Biochemical characterization of chromosomal cephalosporinases from isolates belonging to the *Acinetobacter baumannii* complex. Clinical Microbiology and Infection **7**:218-226
63. **Ma D, DN Cook, M Alberti, NG Pon, H Nikaido, JE Hearst.**1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli.* Journal of Bacteriology **175**:6299-6313
64. **Maddocks SE, PC Oyston.** 2008. Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. Microbiology **154**:3609-3623
65. **Magnet S, P Courvalin, T Lambert**. 2001. Resistance-Nodulation-Cell-division-Type Efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. 2001. Antimicrobial Agents and Chemotherapy **45**:3375-3380
66. **Mammeri H, Poirel L, Mangeney N, Nordmann P.** 2003. Chromosomal integration of a cephalosporinase gene from *Acinetobacter baumannii* into *Oligella urethralis* as a source of acquired resistance to β-lactams. Antimicrobial Agents and Chemotherapy **47**:1536-1542
67. **Marchand I, L Damier-Piolle, P Courvalin, Lambert T.** 2004. Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. Antimicrobial Agents and Chemotherapy. **48**:3298-3304
68. **Mark JK, MJ Kim, J Pham, J Tapsall, PA White.** 2009 Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*. The Journal of Antimicrobial Chemotherapy **63:**47-54
69. **McMurry LM, R Petrucci, SB Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *E. coli*. Proceedings of the National Academy of Sciences of the United States of America **77**:3974-3977
70. **Mine T, Y Morita, A Kataoka, T Mizushima, T Tsuchiya.** 1999. Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy **43:**415-417
71. **Murakami S, R Nakashima, E Yamashita, A Yamaguchi. 2002.** Crystal structure of bacterial multidrug efflux transporter AcrB. Nature **419**:587-593
72. **Muraoka S, R Okumura, N Ogawa, T Nonaka, K Miyashita, T Senda.** 2003.

Crystal structure of a full-length LysR-type transcriptional regulator, CbnR: unusual combination of two subunit forms and molecular bases for causing and changing DNA bend. Journal of Molecular Biology **328**:555-566

73. **Muraoka S, R Okumura, Y Uragami, T Nonaka, N Ogawa, K Miyashita, T Senda.** 2003. Purification and crystallization of s LysR-type transcriptional regulator CbnR from *Ralstonia eutropha* NH9. Protein and Peptide Letters **10**:325-329
74. **Mussi MA, AS Limansky, AM Viale.** 2005. Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumanii*: natural insertional inactivation of a gene encoding a member of a novel family of beta-barrel outer membrane proteins. Antimicrobial Agents and Chemotherapy **49**:1432-1440
75. **Nair BM, KJ Cheung, A Griffith, JL Burns.** 2004. Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genomovar III (*B. cenocepacia*). The Journal of Clinical Investigation **113:**464-473
76. **Navon-Venezia S, A Leavitt, Y Carmeli.** 2007. High tigecycline resistance in multidrug resistant *Acinetobacter baumannii*. The Journal of Antimicrobial Chemotherapy **59:**772-774
77. **NCCLS.** 2010. Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement. NCCLS document M02-A10 and M7-A8
78. **Nordmann P, L Poirel.** 2005. Emergence of plasmid-mediated resistance to quinolones in *Enterobacteriaceae*. Journal of Antimicrobial Chemotherapy **56**:463-469
79. **Okuso H, Ma D, Nikaido H.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. Journal of Bacteriology **178**:306-308
80. **Perez F, KM Hujer, BK Decker, PN Rather, RA Bonomo.** 2007. Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy **51**:3471-3484
81. **Piddock LJ.** 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical Microbiology Reviews **319**:382-402
82. **Pierais V, GJ Barcak.** 1999. Development of *E. coli* host strains tolerating unstable DNA sequences on ColE1 vectors. Focus **21**:18-19
83. **Poirel L, T Naas, P Nordmann.** 2010. Diversity, epidemiology, and genetics of

class D-beta-lactamases. Antimicrobial Agents and Chemotherapy **54**:24-38

84. **Poole K, K Krebes, C McNally, S Neshat**. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. Journal of Bacteriology **75**:7363-7372
85. **Porrua O, M Garcia-Jaramillo, E Santero, F Govantes**. 2007. The LysR-type regulator AtzR binding site: DNA sequences involved in activation, repression and cyanuric acid-dependent repositioning. Molecular Microbiology **66**:410-427
86. **Ribera A, J Ruiz, V Vila.** 2003. Presence of the TetM determinant in a clinical isolate of *Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy **47**: 2310-2312
87. **Ruzin A, D Keeney, PA Bradford**. 2007. AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. Journal of Antimicrobial Chemotherapy **59**:1001-1004
88. **Sambrook J, D Russell.** 2001. Molecular Cloning: A Laboratory Manual, Third Edition Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
89. **Schweizer HP.** 1998. Intrinsic resistance to inhibitors of fatty acid biosynthesis in *Pseudomonas aeruginosa* is due to efflux: application of a novel technique for generation of unmarked chromosomal mutations for the study of efflux systems. Antimicrobial Agents and Chemothery **42**: 394-398
90. **Schreckenberger PC, MI Daneshvar, DG Hollis**. 2007. *Acinetobacter, Achromobacter, Chryseobacterium, Moraxella*, and other nonfermentative Gram-negative rods. In: Murray PR, ed. Manual of clinical microbiology. Washington, Conn: ASM Press. 770-802
91. **Seeger MA, A Schiefner, T Eicher, F Verrey, K Diederichs, KM Pos.** 2006. Asymmetric structure of trimeric AcrB suggests a peristaltic drug pumping mechanism. Science **313:**1295-1298
92. **Shannon KP, I Phillips, BA King.** 1978. Aminoglycoside resistance among Enterobacteriaceae and *Acinetobacter* spp. Journal of Antimicrobial Chemotherapy **4:**131-142
93. **Shaw KJ, PN Rather, RS Hare, GH Miller.** 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiology Review **57:**138-163

94. Smith, M. G., T. A. Gianoulis, S. Pukatzki, J. J. Mekalanos, L. N. Ornston, M. Gerstein, and M. Snyder. 2007. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes & Development **21:**601-614

95. Spence RP, KJ Towner. 2003. Frequencies and mechanisms of resistance to moxifloxacin in nosocomial isolates of *Acinetobacter baumannii*. Journal of Antimicrobial Chemotherapy **52**: 687-690

96. Sulavik MC, C Houseweart, C Cramer, N Jiwani, N Murgolo, J Greene, B DiDomenico, KJ Shaw, GH Miller, R Hare, G Shimer. 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrobial Agents and Chemotherapy **45**:1126-1136

97. Tartaglia LA, C Gimcnos, G Storz, BN Ames. 1991. Multi-degenerate DNA recognition by the OxyR transcriptional regulator. Journal of Biological Chemistry **267**:2038**-**2045

98. Tian ZX, M Mac Aogáin, HF O'Connor, E Fargier, MJ Mooij, C Adams, YP Wang, F O'Gara. 2009a. MexT modulates virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump. Microbial Pathogenesis **47:**237-241

99. Tian ZX, E Fargier, M Mac Aogain, C Adams, YP Wang, F O'Gara. 2009b. transcriptome profiling defines a novel regulon modulated by the LysR-type transcriptional regulator MexT in *Pseudomonas aeruginosa*. Nucleic Acids Research **37**:7546-7559

100. Tien HC, A Battad, EA Bryce, J Fuller, M Mulvey, K Bernard, R Brisebois, JJ Doucet, SB Rizoli, R Fowler, A Simor. 2007. Multi-drug resistant *Acinetobacter* infections in critically injured Canadian forces soldiers.BMC Infectious Diseases **7**:95-101

101. Tikhonova EB, HI Zgurskaya. 2004. AcrA, AcrB, and TolC of *Escherichia coli* form a stable intermembrane Multidrug Efflux Complex. Journal of Biological Chemistry **279**:32116-32124

102. Touze T, J Eswaran, E Bokma, E Koronakis, C Hughes, V Koronakis. 2004. Interactions underlying assembly of the *Escherichia coli* AcrAB-TolC multidrug efflux system. Molecular Microbiology **53**:697-706

103. Towner KJ, E Bergogne-Berezin, CA Fewson. 1991. *Acinetobacter*: portrait of a

genus. *In* K. J. Towner, E. Bergogne-Berezin, and C. A. Fewson (ed.), The biology of *Acinetobacter*. Plenum Publishing Corp., N. Y. 1-24

104. Turton JF, ME Kaufmann, M Warner, J Coelho, L Dijkshoorn, T van der Reiden, TL Pitt. 2004. A prevalent, multiresistant clone of *Acinetobacter baumannii* in southeast England. The Journal of Hospital Infection **58**:170-179

105. Valenzuela JK, L Thomas, SR Partridge, T van der Reijedn, L Dijkshoorn, J Iredell. 2007. Horizontal gene transfer within a polyclonal outbreak of carbapenem-resistant *Acinetobacter baumannii*. Journal of Clinical Microbiology **45**:453-60

106. Vallenet D, P Nordmann, V Barbe, L Poirel, S Mangenot, E Bataille, C Dossat, S Gas, A Kreimeyer, P Lenoble, S Oztas, J Poulain, BA Segurens, C Robert, C Abergel, JM Claverie, D Raoult, C Médigue, J Weissenbach, S Cruveiller. 2008. Comparative analysis of *Acinetobacters*: three genomes for three lifestyles. PLoS One **3:**e1805

107. Wendt C, B Dietze, E Dietz, H Ruden. 1997. Survival of *Acinetobacter baumannii* on dry surfaces. Journal of Clinical Microbiology **35**:1394-1397

108. Zhanel GG, M Decorby, KA Nichol, A Wierzbowski, PJ Baudry, JA Karlowsky, P Lagace-Wiens, A Walkty, MR Mulvey, DJ Hoban. 2008. Antimicrobial susceptibility of 3931 organisms isolated from intensive care units in Canada: Canadian National Intensive Care Unit Study, 2005/2006. Diagnostic Microbiology and Infectious Disease **62:**67-80

109. Zhanel GG, M Decorby, N Laing, B Weshnoweski, R Vashisht, F Tailor, KA Nichol, A Wierzbowski, PJ Baudry, JA Karlowsky, P Lagace-Wiens, A Walkty, M McCracken, MR Mulvey, J Johnson, DJ Hoban. 2008. Antimicrobial resistant pathogens in intensive care units across Canada: Results of the Canadian national intensive care unit 1 (CAN-ICU) study 2005/2006. Antimicrobial Agents and Chemotherapy **52:**1430-1437

110. Zuckerkandl E, L Pauling. 1965. Molecules as documents of evolutionary history. Journal of Theoretical Biology **8**:357-366

APPENDICES

Appendix 1. Antibiotic Susceptibilities of *A. baumannii* Clinical Isolates Used in this Study

Strain / Antibiotic	City	Ap	A/C	PIP	CXM	CXM-AX	CTX	CAZ	AMK	Gm	TOB	CIP	TCN	NF	SXT
A. baumannii 63169	Winnipeg	≥32	4	8	32	32	8	8	16	8	2	1	4	≥512	≤20
A. baumannii 58352	Winnipeg	16	4	8	16	16	8	4	≤2	≤1	≤1	≤0.25	2	256	≤20
A. baumannii 59960	Vancouver	≥32	4	8	≥64	≥64	16	16	≤2	≤1	≤1	1	4	≥512	≤20
A. baumannii 59973	Vancouver	≥32	8	16	≥64	≥64	16	16	≤2	≤1	≤1	≤0.25	≤1	≥512	≤20
A. baumannii 64130	Hamilton	16	4	8	16	16	8	4	≤2	≤1	≤1	≤0.25	≤1	≥512	≤20
A. baumannii 64153	Hamilton	16	4	8	16	16	8	≤1	8	≤1	≤1	≤0.25	2	≥512	≤20
A. baumannii 64397	Vancouver	16	4	8	≥64	≥64	16	4	≤2	≤1	≤1	0.5	2	≥512	≥320
A. baumannii 63487	Winnipeg	≥32	4	8	32	32	8	8	8	8	≤1	1	4	≥512	≤20
A. baumannii 65239	Victoria	≥32	4	16	≥64	≥64	16	8	≤2	≤1	≤1	≤0.25	≤1	≥512	≤20
A. baumannii 66310	Vancouver	16	4	8	32	32	8	16	4	8	≤1	1	4	256	≤20
A. baumannii 66985	Montreal	16	4	16	≥64	≥64	16	4	≤2	≤1	≤1	0.5	2	≥512	≤20
A. baumannii ATCC19606	NA	≥32	16	32	≥64	≥64	16	8	≤2	4	≤1	1	≤1	128	80

Ap, Ampicillin; A/C, Amoxicillin/Clavulanic Acid; PIP, Piperacillin; CXM,Cefuroxime; CXM-AX, Cefuroxime Axetil; CTX, Cefotaxime; CAZ, Ceftazidime; AMK, Amikacin; Gm, Gentamicin; TOB, Tobramycin; CIP, Ciprofloxacin; TCN, Tetraciclyne; NF, Nitrofurantoin; SXT, Trimethoprim-sulfamethoxazole

Appendix 2. Sequence alignment of *adeL* from *A. baumannii* isolates. Differences in sequence between strains expressing and those not expressing *adeN* gene are shown underlined.

A.baumannii strain	sequence
63169	GGGTTTACCGCGTGCTTCTGTGACTACAACCATTCAGGCTTTAGAGAAGCATTTACAAGT
59960	GGGTTTACCGCGTGCTTCTGTGACCACAACTATTCAGGCTTTAGAGAAGCATTTACAAGT
59973	GGGTTTACCGCGTGCTTCTGTGACCACAACTATTCAGGCTTTAGAGAAGCATTTACAAGT
64153	GGGTTTACCGCGTGCTTCTGTGACCACAACCATTCAGGCTTTAGAGAAGCATTTGCAAGT
64797	GGGTTTACCGCGTGCTTCTGTGACCACAACTATTCAGGCTTTAGAGAAGCATTTACAAGT
63487	GGGTTTACCGCGTGCTTCTGTGACTACAACCATTCAGGCTTTAGAGAAGCATTTACAAGT
66310	GGGTTTACCGCGTGCTTCTGTGACCACAACTATTCAGGCTTTAGAGAAGCATTTACAAGT
66985	GGGTTTACCGCGTGCTTCTGTGACTACAACCATTCAGGCTTTAGAGAAGCATTTACAAGT
ATCC19606	GGGTTTACCGCGTGCTTCTGTGACTACAACCATTCAGGCTTTAGAGAAGCATTTACAAGT
	********** ***** ********** *****
63169	TCGTTTGCTGAATCGAACAACACGAAAAATTAGTCTCACACCGGATGGCGCGGTATATTA
59960	TCGTTTGCTGAATCGGACAACACGAAAAATTAGTCTCACACCGGATGGCGCCGTATATTA
59973	TCGTTTGCTGAATCGGACAACACGAAAAATTAGTCTCACACCGGATGGCGCCGTATATTA
64153	TCGATTGCTTAATCGGACAACACGAAAAATTAGTCTCACACCGGATGGCGCCGTATATTA
64797	TCGTTTGCTGAATCGGACAACACGAAAAATTAGTCTCACACCGGATGGCGCCGTATATTA
63487	TCGTTTGCTGAATCGAACAACACGAAAAATTAGTCTCACACCGGATGGCGCGGTATATTA
66310	TCGTTTGCTGAATCGGACAACACGAAAAATTAGTCTCACACCGGATGGCGCCGTATATTA
66985	TCGATTGCTTAATCGGACAACACGAAAAATTAGTCTCACACCGGATGGCGCCGTATATTA
ATCC19606	TCGATTGCTTAATCGGACAACACGAAAAATTAGTCTCACACCGGATGGCGCCGTATATTA
	*** ***** ***** ********** ********
63169	TGATCGGACAGCCCGTATTTTAGCGGATGTTTCCGATATTGAATCTTCTTTTCATGATGC
59960	TGATCGGACAGCCCGTATTTTAGCGGATGTTTCCGATATTGAATCTTCCTTTCATGATGC
59973	TGATCGGACAGCCCGTATTTTAGCGGATGTTTCCGATATTGAATCTTCCTTTCATGATGC
64153	TGATCGGACAGCCCGTATTTTAGCGGATGTTGCCGATATTGAATCTTCTTTTCATGATGC
64797	TGATCGGACAGCCCGTATTTTAGCGGATGTTTCCGATATTGAATCTTCCTTTCATGATGC
63487	TGATCGGACAGCCCGTATTTTAGCGGATGTTTCCGATATTGAATCTTCTTTTCATGATGC
66310	TGATCGGACAGCCCGTATTTTAGCGGATGTTTCCGATATTGAATCTTCCTTTCATGATGC
66985	TGATCGGACAGCCCGTATTTTAGCCGATGTTGCCGATATTGAATCTTCTTTTCATGATGC
ATCC19606	TGATCGGACAGCCCGTATTTTAGCGGATGTTGCCGATATTGAATCTTCTTTTCATGATGC
	********** ****** ********** **********
63169	AGAGCGAGGGCCGAGAGGTCAGCTTCGTATTGATGTGCCTGTATCGATTGGCCGTTTAAT
59960	AGAGCGGGGGCCAAGGGGTCAGCTTCGTATTGATGTGCCTGTATCGATTGGCCGTTTAAT
59973	AGAGCGGGGGCCAAGGGGTCAGCTTCGTATTGATGTGCCTGTATCGATTGGCCGTTTAAT
64153	AGAGCGGGGGCCAAGAGGTCAGCTTCGTATTGATGTGCCTGTATCGATTGGACGTTTAAT
64797	AGAGCGGGGGCCAAGAGGTCAGCTTCGTATTGATGTGCCTGTATCCATTGGCCGTTTAAT
63487	AGAGCGAGGGCCGAGAGGTCAGCTTCGTATTGATGTGCCTGTATCGATTGGCCGTTTAAT
66310	AGAGCGGGGGCCAAGAGGTCAGCTTCGTATTGATGTGCCTGTATCCATTGGCCGTTTAAT
66985	AGAGCGGGGGCCAAGAGGTCAGCTTCGTATTGATGTGCCTGTGTCCATTGGCCGTCTAAT
ATCC19606	AGAGCGGGGGCCAAGAGGTCAGCTTCGTATTGATGTGCCTGTATCGATTGGACGTTTAAT
	****** ***** ** ********** ** ***** *** ****

A.baumannii strain sequence

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63169       TTTAATTCCAAGACTACGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTGATTGG
59960       TTTAATTCCAAGACTACGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTGATTGG
59973       TTTAATTCCAAGACTACGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTGATTGG
64153       TTTAATTCCAAGGCTCCGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTAATTGG
64797       TTTAATTCCAAGACTACGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTGATTGG
63487       TTTAATTCCAAGACTACGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTGATTGG
66310       TTTAATTCCAAGACTACGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTGATTGG
66985       TTTAATTCCAAGGCTCCGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTAATTGG
ATCC19606   TTTAATTCCAAGGCTCCGCGATTTTCATGCACGCTATCCTGATATTGATTTAGTGATTGG
            ************ ** ************************************ *****

63169       TCTAAATGACCGACCTGTAGACTTGGTTGGAGAAGCAGTTGATTGTGCAATTCGGGTGGG
59960       TCTAAATGACCGACCTGTAGACTTGGTGGGAGAGGCTGTCGATTGTGCAATTCGGGTCGG
59973       TCTAAATGACCGACCTGTAGACTTGGTGGGAGAGGCTGTCGATTGTGCAATTCGGGTCGG
64153       TCTGAACGACCGACCTGTAGACTTGGTTGGAGAAGCGGTTGATTGTGCAATTCGGGTGGG
64797       TCTAAATGACCGACCTGTAGACTTGGTGGGAGAGGCTGTCGATTGTGCAATTCGGGTCGG
63487       TCTAAATGACCGACCTGTAGACTTGGTTGGAGAAGCAGTTGATTGTGCAATTCGGGTGGG
66310       TCTAAATGACCGACCTGTAGACTTGGTGGGAGAGGCTGTCGATTGTGCAATTCGGGTCGG
66985       TCTGAATGACCGACCTGTAGACTTGGTTGGAGAAGCAGTTGATTGTGCAATTCGGGTGGG
ATCC19606   TCTGAACGACCGACCTGTAGACTTGGTTGGAGAAGCGGTTGATTGTGCAATTCGGGTGGG
           *** ** ********************^******^**^**^****************^**

63169       AGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGCGCAACCGC
59960       TGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGTGCAACTGC
59973       TGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGTGCAACTGC
64153       TGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGTGCAACTGC
64797       TGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGTGCAACTGC
63487       AGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGCGCAACCGC
66310       TGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGTGCAACTGC
66985       TGAATTAAAAGATTCCAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGTGCAACTGC
ATCC19606   TGAATTAAAAGATTCAAGCTTAATTGCGCGTCGTATCGGAACTTTCCAGTGTGCAACAGC
            ************** ************************************** **

63169       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
59960       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
59973       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
64153       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
64797       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
63487       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
66310       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
66985       TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
ATCC19606   TGCTTCACCGATTTATTTAGAAAAATATGGCGAACCTACCTCAATTGAAGATTTGCAAAA
            ************************************************************
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A.baumannii strain sequence

```
63169       AAACCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
59960       AAACCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
59973       AAACCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
64153       AAATCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
64797       AAACCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
63487       AAACCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
66310       AAACCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
66985       AAATCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
ATCC19606   AAATCATAAAGCGATTCACTTCTTTTCAAGCCGTACCGGACGCAACTTCGATTGGGACTT
            *** ************************************************************

63169       TGTGGTTGATGATTTAATTAAAAGTGTGTCAGTACGTGGACGTGTTTCTGTAAATGACGG
59960       TGTGGTTGATGACTTAATTAAAAGTGTATCAGTACGTGGACGTGTTTCGGTAAATGACGG
59973       TGTGGTTGATGACTTAATTAAAAGTGTATCAGTACGTGGACGTGTTTCGGTAAATGACGG
64153       TGTGGTTGATGATTTAATTAAAAGTGTGTCAGTACGTGGACGTGTTTCGGTAAATGACGG
64797       TGTCGTTGATGATTTAATTAAAAGTGTGTCAGTACGTGGACGTGTTTCGGTAAATGACGG
63487       TGTGGTTGATGATTTAATTAAAAGTGTGTCAGTACGTGGACGTGTTTCTGTAAATGACGG
66310       TGTCGTTGATGATTTAATTAAAAGTGTGTCAGTACGTGGACGTGTTTCGGTAAATGACGG
66985       TGTGGTTGATGATTTAATTAAAAGTGTGTCAGTACGTGGACGTGTTTCGGTAAATGACGG
ATCC19606   TGTGGTTGATGATTTAATTAAAAGTGTGTCAGTACGTGGACGTGTTTCGGTAAATGACGG
            *** ******** ************** ********************************

63169       GATGCTTATATCGACTTGGCTTTACAAGGTTTCGGTATAATTCAAGGTCCACGTTATAT
59960       TGATGCTTATATCGACTTGGCTTTGCAAGGTTTCGGTATAATTCAAGGCCCACGTTATAT
59973       TGATGCTTATATCGACTTGGCTTTGCAAGGTTTCGGTATAATTCAAGGCCCACGTTATAT
64153       TGATGCTTATATCGACTTGGCTTTGCAAGGTTTTGGTATAATTCAAGGCCCACGTTATAT
64797       TGATGCTTACATCGACTTGGCTTTGCAGGGTTTCGGTATAATTCAAGGCCCACGTTATAT
63487       TGATGCTTATATCGACTTGGCTTTACAAGGTTTCGGTATAATTCAAGGTCCACGTTATAT
66310       TGATGCTTACATCGACTTGGCTTTGCAGGGTTTCGGTATAATTCAAGGCCCACGTTATAT
66985       TGATGCTTATATCGACTTGGCTTTGCAAGGTTTTGGTATAATTCAAGGCCCACGTTATAT
ATCC19606   TGATGCTTATATCGACTTGGCTTTGCAAGGTTTTGGTATAATTCAAGGCCCACGTTATAT
            ********* ************** ** ***** ************** ***********

63169       GCTCACCAACCATTTAGAATCGGGTTTGTTAAAAGAAGTATTACCTCAGTGGACGCCAGC
59960       GCTCACCAACCATTTAGAATCGGGTTTGTTAAAAGAAGTATTGCCTCAGTGGACGCCAGC
59973       GCTCACCAACCATTTAGAATCGGGTTTGTTAAAAGAAGTATTGCCTCAGTGGACGCCAGC
64153       GCTCACCAACCATTTAGAATCAGGTTTATTAAAAGAGGTATTGCCTCAGTGGACGCCAGC
64797       GCTCACCAACCATTTAGAATCGGGTTTGTTAAAAGAAGTATTGCCTCAGTGGACGCCAGC
63487       GCTCACCAACCATTTAGAATCGGGTTTGTTAAAAGAAGTATTACCTCAGTGGACGCCAGC
66310       GCTCACCAACCATTTAGAATCGGGTTTGTTAAAAGAAGTATTGCCTCAGTGGACGCCAGC
66985       GCTCACCAACCATTTAGAATCAGGTTTGTTAAAAGAGGTATTGCCTCAGTGGACGCCAGC
ATCC19606   GCTCACCAATCATTTAGAATCAGGTTTGTTAAAAGAGGTATTGCCTCAGTGGACGCCAGC
            ********* *********** ***** ******** ***** ****************
```

A.baumannii sequence

strain	
63169	GCCGATGCCAATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCGCTCAAAGTAAAAGT
59960	ACCGATGCCAATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCGCTCAAAGTAAAAGT
59973	ACCGATGCCAATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCGCTCAAAGTAAAAGT
64153	GCCGATGCCGATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCGCTTAAAGTAAAAGT
64797	GCCGATGCCGATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCACTCAAAGTAAAAGT
63487	GCCGATGCCAATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCGCTCAAAGTAAAAGT
66310	GCCGATGCCGATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCACTCAAAGTAAAAGT
66985	ACCGATGCCGATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCGCTTAAAGTAAAAGT
ATCC19606	ACCGATGCCGATTTCAGCAGTTTATCTTCAAAATCGTCATTTATCGCTTAAAGTAAAAGT
	********* ********************************** ** **********
63169	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGTTGTCCATTACTTGGCGGTACGGCTTT
59960	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGTTGTCCATTACTTGGCGGTACGGCTTT
59973	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGTTGTCCATTACTTGGCGGTACGGCTTT
64153	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGCTGTCCATTACTTGGCGGTACAGCTTT
64797	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGTTGTCCATTACTTGGCGGTACGGCTTT
63487	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGTTGTCCATTACTTGGCGGTACGGCTTT
66310	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGTTGTCCATTACTTGGCGGTACGGCTTT
66985	GTTTGTTGATTGGGTCGCTGAACTTTTTGCAGGCTGTCCATTACTTGGCGGTACAGCTTT
ATCC19606	GTTTGTAGATTGGGTCGCTGAACTTTTTGCAGGCTGTCCATTACTTGGCGGTACAGCTTT
	****** ************************* ******************** *****
63169	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
59960	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
59973	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
64153	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
64797	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
63487	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
66310	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
66985	ACCTTTCGACCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
ATCC19606	ACCTTTCGATCAGAAATGTGAATTTGCCTGTGATAAAGAAACTGGTCATGAATATACAAT
	********* **
63169	CGTACTTTGGTCGAGCAGCACAATAT----------
59960	TCGTACTTTGGTCGAGCAGCACAATAT----------
59973	TCGTACTTTGGTCGAGCAGCACAATAT----------
64153	TCGTACTTTGGTCGAGCAGCATAATAT----------
64797	TCGTACTTTGGTCGAGCAGCACAATAT----------
63487	TCGTACTTTGGTCGAGCAGCACAATAT----------
66310	TCGTACTTTGGTCGAGCAGCACAATAT----------
66985	TCGTACTTTGGTCGAGCAGCATAATAT----------
ATCC19606	TCGTACTT----------
	********

Appendix 3. Sequence alignment from promoter region of six *A. baumannii* solates. Strains marked with asterisk showed expression of the *adeN*, those strains without it correspond to isolates that showed lack of expression of this gene. The one nucleotide underlined indicates the difference between these two groups of isolates.

```
A.baumannii strain                    sequence
                                                                        60
Ab59960     CATGGCATGAAATAGATCCACACGCACACTCCTATTAATGCATTTCTTTATTCAAATACA
Ab66310     CATGGCATGAAATAGATCCACACGCACACTCCTATTAATGCATTTCTTTATTCAAATACA
Ab64797     CATGGCATGAAATAGATCCACACGCACACTCCTATTAATGCATTTCTTTATTCAAATACA
Ab63487*    CATGGCATGAAATAGATCCACACGCACACTCCTATTAATGCATTTCTTTATTCAAATACA
Ab63169     CATGGCATGAAATAGATCCACACGCACACTCCTATTAATGCATTTCTTTATTCAAATACA
Ab64153*    CATGGCATGAAATAGATCCACACGCACACTCCTATTAATGCATTTTCTAGTGCAAACACA
            ********************************************  *  * **** ***

                                                                       120
Ab59960     TCAATTGTTATAAAAATACGAATAGTGTTATCGAATTTAATATATTTATTCGTGATTGAC
Ab66310     TCAATTGTTATAAAAATACGAATAGTGTTATCGAATTTAATATATTTATTCGTGATTGAC
Ab64797     TCAATTGTTATAAAAATACGAATAGTGTTATCGAATTTAATATATTTATTCGTGATTGAC
Ab63487*    TCAATTGTTATAAAAATACGAATAGTGTTATCAAATTTAATATATTTATTCGTGATTGAC
Ab63169*    TCAATTGTTATAAAAATACGAATAGTGTTATCAAATTTAATATATTTATTCGTGATTGAC
Ab64153*    TCAATTGTTATAAAAATACGAATAGTGTTATCAAATTTAATATGTTTATTCGTGATTGAC
            **********************************^********** ****************

                                                                       180
Ab59960     AAACTTTTATGCTCCGTCCATTCCATAAACCCTGTTTCTCCCATACGGACCATAACTAAA
Ab66310     AAACTTTTATGCTCCGTCCATTCCATAAACCCTGTTTCTCCCATACGGACCATAACTAAA
Ab64797     AAACTTTTATGCTCCGTCCATTCCATAAACCCTGTTTCTCCCATACGGACCATAACTAAA
Ab63487*    AAACTTTTATGCTCCGTCCATTCCATAAACCCTGTTTCTCCCATACGGACCATAACTAAA
Ab63169*    AAACTTTTATGCTCCGTCCATTCCATAAACCCTGTTTCTCCCATACGGACCATAACTAAA
Ab64153*    AAACTTTTATGCTCCGTCCATTCCATAAACCCTGTTTCTCCCATACGGACCATAACTAAA
            ************************************************************

                                                              225
Ab59960     AGTCTGAAAACAGGTATCCAAATAACTAGGAGCACCTCATGTCAT
Ab66310     AGTCTGAAAACAGGTATCCAAATAACTAGGAGCACCTCATGTCAT
Ab64797     AGTCTGAAAACAGGTATCCAAATAACTAGGAGCACCTCATGTCAT
Ab63487*    AGTCTGAAAACAGGTATCCAAATAACTAGGAGCACCTCATGTCAT
Ab63169*    AGTCTGAAAACAGGTATCCAAATAACTAGGAGCACCTCATGTCAT
Ab64153*    AGTCTGAAAACAGGTCAATAAATAACTAGGAGCACCTCATGTCAT
            *********************************************
```

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