CHARACTERIZATION OF TWO COMPONENT SYSTEMS OF *Acinetobacter baumannii*

SUBMITTED BY

Yasser Alsaadi

Faculty of Science

A Thesis Submitted in Partial Fulfillment of the Requirements

For the Degree of

Masters of Science in Applied Bioscience

University of Ontario Institute of Technology

Oshawa, Ontario, Canada

ABSTRACT

Acinetobacter baumannii is an important opportunistic pathogen of hospital acquired infection, particularly in intensive care units. The emergence and rapid spread of multidrug-resistant *A. baumannii* strains has become a major health threat worldwide which severely limits the treatment options for this pathogen. This work investigated global mechanisms of antibiotic resistance and virulence of the problematic pathogen *A. baumannii*, in particular those mediated by two component regulatory systems (TCSs), that typically consist of a membrane bound sensor kinase and a cognate response regulator. Bacterial TCSs play an important role in the regulation of adaptation to different environmental conditions. Five TCSs in *A. baumannii* have been characterized; however, there are a number of putative two component systems encoded in the genome of *A. baumannii* that await detailed characterization. Differential expressions of six different TCSs was observed in two clinical isolates of *A. baumannii* AB030 and AB031, and whole-genome sequencing of both clinical isolates was performed. Data obtained from the comparative whole-genome analysis revealed the presence of an insertion element in the orphan TCSs response regulator *A1S_2006* in AB030, mutation in the promoter region and an 1189 DNA insertion element were present in AdeRS system in AB031. The whole-genome sequencing analysis of TCSs operons in AB030 and AB031 also identified sequence polymorphisms that could alter the activities of these TCSs in AB030 and AB031. Finally, we identified *A1S_3229_30* an excellent candidate that may act as global regulator of antibiotic and virulence in *A. baumannii*. The sequence of *A1S_3229_30* was highly conserved among the wild-type ATCC17978, AB030 and AB031, and showed 73% identity to the *amgRS* operon that encodes for the well characterized AmgRS system that confer resistance to aminoglycoside antibiotics and required for the virulence of the problematic pathogen *P. aeruginosa.*

Acknowledgments

I am using this opportunity to express my gratitude to my research supervisor **Dr. Ayush Kumar** who supported me throughout my master's studies. I am thankful for his aspiring guidance, invaluably constructive criticism and friendly advice during my work at his lab. I am sincerely grateful to him for giving me this amazing opportunity to do research and learn about science in a very exciting way, and also for giving me the chance to achieve my dream. This thesis would not have been possible without his support and help.

I express my warm thanks to my Co-supervisor **Dr. Andrea Kirkwood** and committee member **Dr. Sean Forrester** for their support and their advice, and their constructive criticism.

Special thanks to my lab-mate Dinesh Fernando for all of his help throughout my research project. Dinesh has been and continues to be an inspiration, source of ideas and laughs. More than anything, he is a fantastic friend. His generosity is endless. His intelligence and work ethics are overwhelming and I am honored and deeply thankful to be his friend.

It has been an honor to work with these graduate students Malaka De Silva, Andrei Bazyleu, Sarah Warren, and Veena Premjani, and I wish them all the best.

It gives me great pleasure in acknowledging all of the undergraduate students that I have met and taught during my studies.

I am deeply grateful to the all APBS graduate students in UOIT.

My thanks and appreciations also go to the department of microbiology in university of Manitoba (UofM) for their warm welcoming and co-operation which help me in completion of this project. Many thanks to all of the graduate students who have willingly helped me out to deal with Winnipeg's winter.

I would also like to convey thanks to the Ministry of Higher Education in Saudi Arabia for providing the financial means to complete my MSc.

To all my friends in Toronto (M5er's) and Oshawa (my roommates), you were and will be a great supporting network. Thank you so much for making this an amazing experience in my life.

Finally, I would like to thank the most important people in my life my mother (Fatimah) and my beloved sister (Nada) and my brother (Fiasal), who are the constant source of love and inspiration in every aspect of my life.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

APPENDICES

LIST OF ABBREVIATIONS

A600nm	Absorbance at 600nm
aacC1	Acetyltransferase 3-1 encoding gene conferring Gm-resistance
Ap	Ampicillin
bp(s)	Base pair(s)
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxyribonucleic acid
Ct	Cycle threshold
Δ	Deletion
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate(s)
dhfr	dihydrofolate reductase
FRT	Flp Recombinase Target
Gm	Gentamicin
Kb(s)	Kilobase(s)
KDa	Kilodalton
Kv	Kilovolts
LB	Lysogeny broth
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
mRNA	Messenger ribonucleic acid
µL	microliter(s)
mL	milliliter(s)
mM	Millimolar
ng	nanograms
NRT	No reverse transcriptase control
NTC	No template control

OMP	Outer membrane protein
ORF	Open reading frame
ori	origin of replication
oriT	origin of transfer
PacBio	Pacific bioscience
PCR	Polymerase chain reaction
r	resistance/resistant
RND	Resistance nodulation division
sacB	*Bacillus subtilis* levan sucrase-encoding gene
SK	Sensor kinase
SMRT	Single molecule real time sequencing
Tet	Tetracycline
TCSs	Two component systems
Tmp	Trimethoprim
TFBI	Transformation buffer I
TFBII	Transformation buffer II
v/v	Volume / volume
w/v	Weight / volume
xg	Gravitational force

1. INTRODUCTION

1.1 *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram-negative coccobacilli and that is being increasingly recognized as an important pathogen that causes severe infections in hospitalized patients (Cisneros *et al*., 1996). The genus *Acinetobacter* is diverse, and bacteria belonging to this genus can be found in soil, surface water, vegetables, animals, and humans. The presence of *Acinetobacter* species on healthy individuals' skin was reported, and majority of the skin isolates belonged to *A. lwoffii, A. johnsonii and A. junii*, whereas *A. baumannii* skin carriage is rare in healthy individuals (Dijkshoon *et al*., 2007; Zordan S *et al*., 2011). The natural reservoirs of *A. baumannii* remain unclear, and this species has been isolated mainly from the hospital environment.

1.2 *Acinetobacter baumannii* clinical relevance

A. baumannii has been implicated in a variety of nosocomial infections, including bacteremia, urinary tract infection, and secondary meningitis as well as ventilator-associated pneumonia in patients confined to hospital intensive care units (ICUs) (Peleg *et al*., 2008; Michalopoulos and Falagas, 2010). Mortality rate of 26% -70% has been reported for *A. baumannii* infection in ICUs (Alsan and Klompas, 2010). In the United States, occurrence of multi-drug resistant (MDR) *A. baumannii* has increased from 6.7% in 1993 to 29.9% in 2004, which is twice the rate of MDR emergence of any other ICU-relevant Gram-negative bacillus (Maragakis and Perl, 2008).

During the conflicts in Iraq and Afghanistan, *A. baumannii* infections were reported among severely injured returning Canadian and American soldiers (Tien *et al*., 2007). It is hypothesized that this has played a major role in triggering an increase of the occurrence of *A.*

baumannii infections among civilian hospitalized patients in North America (Hujer *et al.*, 2006). The recent Centers for Disease Control and Prevention (CDC) 2013 report has highlighted *A. baumannii* as a serious level of threat to human health. The report has also documented that *A. baumannii* causes 12,000 infections per year in the US, of which 7,300 are multidrug resistant, and it is responsible for 500 deaths (Centers for Disease Control and Prevention (CDC), 2013).

1.3 Antimicrobial resistance in *Acinetobacter baumannii*

A major factor that contributes to the pathogenesis of *A. baumannii* is its resistance to a number of antibiotics commonly used to treat infections caused by Gram-negative bacteria (Gaynes and Edwards, 2005). It displays a remarkable capacity to develop clinical resistance to broad-spectrum β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, and in recent years, to carbapenems, which have been the most powerful agents against infection caused by multidrug resistance since the late 1980s (Vaara *et al.*, 2010). Pandrug resistance of *A. baumannii* has left tigecycline (one of the most recent antibiotics approved for clinical use) and polymyxin B (an older antibiotic that is known for its nephrotoxicity) as the drugs of last resort (Afzal-Shah *et al*., 2001; Valenzuela *et al*., 2007; Mark *et al*., 2009).

However, with recent reports of *A. baumannii* resistance to polymyxins (Ko *et al*., 2007) and tigecycline (Valencia *et al.*, 2009) it is feared that this organism may become resistant to every single antimicrobial agent currently available (Maragakis and Perl, 2008).

i. Mechanisms

A. baumannii has become resistant to most of the antibiotics available. Drug resistance in *A. baumannii* can either result from intrinsic mechanisms present in Gram-negative species, or through acquisition of novel genetic information through horizontal gene transfer (Magnet *et al.*,

2001; Mammeri *et al.*, 2003). Antibiotic resistance mechanisms for *A. baumannii* can be classified into three categories (Piddock, 2006). The first category is the production of antimicrobial-inactivating enzymes (β-lactamases, cephalosporinases, and carbapenemases) that hydrolyze and confer resistance to penicillins, cephalosporins, and carbapenems (Drawz *et al.*, 2010). The most common mechanism of β-lactam resistance in *A. baumannii* is inactivation of the drug by β-lactamase enzymes, which can be either chromosomally- or plasmid-encoded (Roca *et al.*, 2012). The second category involves modification of membrane permeability or increased efflux (Limansky *et al.*, 2002); however, little is known about the permeability of the outer membrane in *A. baumannii* or its outer membrane porins (Vila *et al.*, 2007). The third category of resistance mechanisms involves alterations that change antibiotics' targets or bacterial cell functions, this type of mechanism in *A. baumannii* confer resistance to quinolone agents by mutations in the bacterial targets *gyrA* and *parC* topoisomerase enzymes (Maragakis and Perl, 2008). Overall, *A. baumannii* can express all mechanisms of resistance as described above such as enzymatic inactivation of the antibiotic, mutation(s) in the structural or regulatory genes of the target protein, decreased permeability of the outer membrane transport of the agent out of the cell by efflux (Damier-Piolle *et al.*, 2008; Roca *et al.*, 2011).

However, recent studies have highlighted that presence of the energy-mediated efflux of antibiotics plays a crucial role in the intrinsic resistance of Gram-negative species (Li *et al*., 2002; Lin *et al*., 2009). *A. baumannii* possesses efflux pumps that are capable of actively removing a broad range of antimicrobial agents from the bacterial cell, and responsible for multidrug resistance phenotype of this organism (Magnet *et al*., 2001; Damier-Piolle *et al.*, 2008).

ii. RND efflux pumps

Multidrug efflux pumps belonging to the Resistance-Nodulation cell-Division (RND) family have been identified as the most common and important efflux system among MDR Gram-negative bacteria (Morita *et al*., 2012). RND pumps are comprised of an outer membrane protein (OMP), an inner membrane RND transporter and a membrane fusion protein (MFP) that links OMP and RND components together. RND pumps are driven by a substrate/H+ antiport mechanism and have been linked not only to efflux of antibiotics, but also broad range of structurally unrelated molecules/ions, such as: biocides, heavy metals, dyes, organic solvents, detergents, metabolic inhibitors, bile salts and homoserine lactones, involved in quorum sensing (Poole, 2004). To date, three RND-efflux pumps have been described in *A. baumannii* AdeABC Magnet S *et al.*, 2001) AdeIJK (Damier-Piolle *et al*., 2008) and AdeFGH (Coyne *et al*., 2010). These three efflux pumps have shown capability to confer resistance to a variety of antibiotic classes including β-lactams, aminoglycosides, fluoroquinolones, tigecycline, trimethoprim, and sulfamethoxazole.

iii. Porins

The first line of defense for Gram-negative bacteria against toxic compounds is the outer membrane. This barrier is resistant to large, charged molecules. Permeability of the outer membrane largely controlled by porins, which are water-filled open channels that span the outer membrane and allow the passive penetration of hydrophilic molecules (Galdiero *et al*., 2012). Outer membrane proteins (OMPs), such as porins and efflux pumps, play a fundamental role in the organism pathogenicity and its antibiotic resistance. It has been reported the role of altered porins expression in antibiotic resistance to a number of hydrophilic antibiotics like β-lactams

and carbapenems. To date, three porins have been identified in *A. baumannii* CarO (Catel-Ferreira *et al.,* 2011), OprD (Catel-Ferreira *et al.,* 2011), and 33kDa porin (Clark, 1996). These porins showed association to carbapenems resistance in *A. baumannii* (Mussi *et al.,* 2005; Del Mar Tomas *et al.,* 2005).

1.4 Virulence of *Acinetobacter baumannii*

In the past, *A. baumannii* was considered to be an organism of low virulence, but the occurrence of several infections, such as community-acquired *Acinetobacter* pneumonia indicates that this organism displays high pathogenicity and causes invasive disease. The treatment of *A. baumannii* infections is complicated by ignorance of the molecular and cellular mechanisms of pathogenesis in the host. Much still needs to be discovered about *A. baumannii* virulence factors that are involved in producing biofilms, killing the host cells, and causing infections (Gaddy and Actis, 2009).

Several bacterial virulence factors are required for *A. baumannii* to colonize and infect the host. However, very little information is known about these virulence factors in *A. baumannii*. There have been recent studies characterizing some virulence factors, such as phospholipase D and phospholipase C , biofilm formation, outer membrane protein A (OmpA), penicillin-binding protein 7/8 (PBP-7/8), and ferric iron chelators (siderophores) (Roca *et al*., 2012).

The surface protein OmpA is involved in the adherence to and invasion of epithelial cells and induces apoptosis in the early stages of *A. baumannii* infection (McConnell *et al*., 2011), while the hydrolytic enzymes PLD and PLC play a role in the organism ability to survive in the human serum, epithelial cell invasion, and enhance cytotoxicity on epithelial cells (Antunes *et*

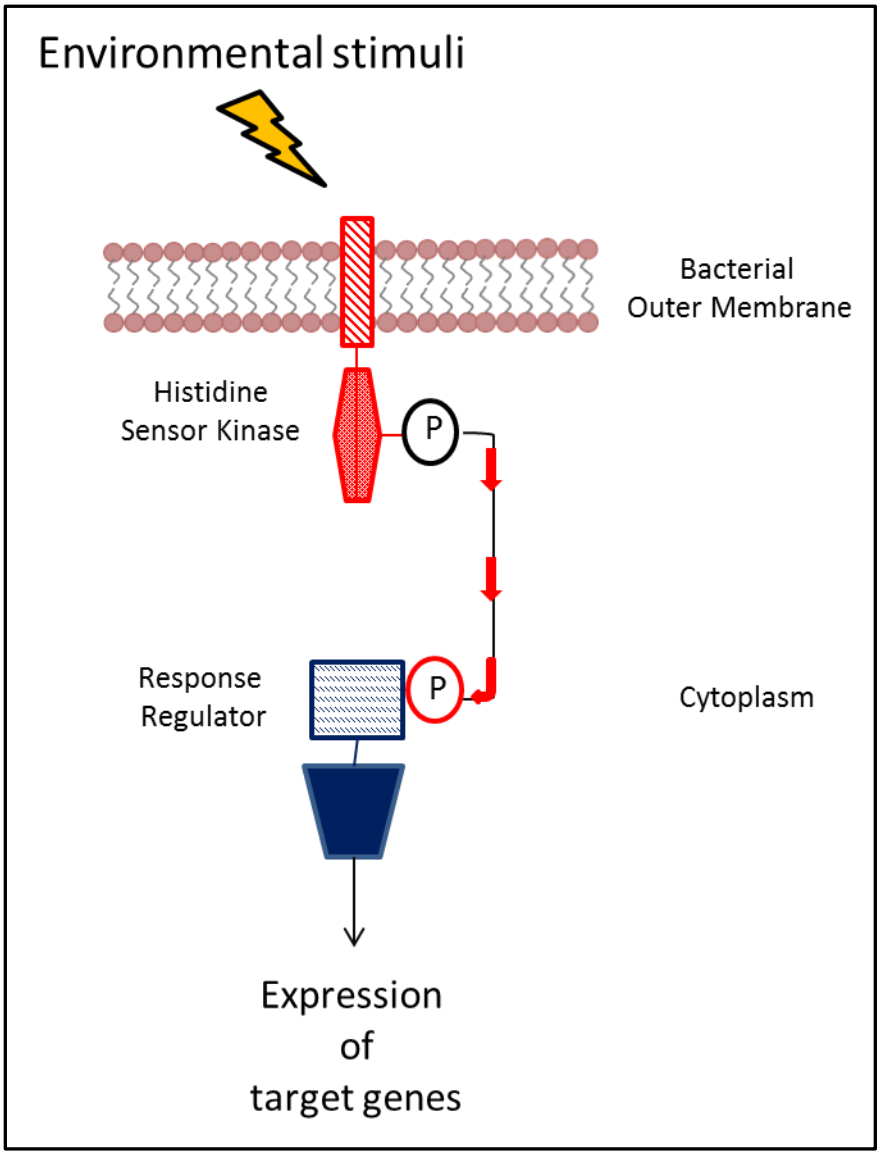
al., 2011). During colonization, *A. baumannii* forms biofilm, a highly structured microbial community, which results in adhesion to host cells, survival on other surfaces, and resistance to antimicrobial agents (Gaddy and Actis, 2009). Penicillin-binding protein 7/8 (PBP-7/8) contribute in the growth and survival of *A. baumannii* in human ascites, and showed indirect participation in human serum resistance (Cayo *et al.,* 2011). The ability of *A. baumannii* to produce siderophores helps the organism survive and multiply under iron-limiting environments in the host (Dorsey *et al.,* 2003).

1.5 Two component systems in bacteria

Rapid adaptation to environmental challenge is essential for bacterial survival. To adapt to changes in their surroundings, bacteria mainly use the two component systems (TCSs). Such systems are signal transduction pathways in bacteria (as well as certain archaea, protozoa, plants, and fungi) and respond to change in environmental conditions (Mitrophanov and Groisman, 2008). The prototypical TCSs consist of a membrane bound sensor kinase, and a response regulator that is present in the cytoplasm. The role of the sensor kinase is to direct a phosphorylation of its cognate response regulator in response to an extracellular environmental signal, this phosphorylation activates the response regulator to regulate the expression of a large number of genes (Stock *et al.,* 2000) (Figure 1.).

Figure 1. A schematic representation of common two component system in bacteria. The common TCSs are usually composed of a sensor kinase located in the outer membrane, which is able to detect one or several environmental stimuli, and a response regulator in the cytoplasm, which is phosphorylated by the sensor kinase and which, in turn, activates the expression of genes necessary for the appropriate physiological response.

Figure 1.1.



(Modified from Mitrophanov and Groisman, 2008)

Different environmental conditions detected by TCSs in bacteria are well characterized. These signals are thought to be chemical and physical parameters such as temperature, pH, oxygen pressure, osmolality, autoinducer compounds and antibiotics (Krell *et al.*, 2010). These TCSs can be characterized into several groups based on their actions: control of metabolism, respiration, influx and efflux, chemotaxis, and stress response (Mitrophanov and Groisman, 2008). Moreover, the role of the TCSs in the pathogenicity of bacteria has been reported in different microorganisms.

i. Role of TCSs in antibiotic resistance and virulence

Different TCSs have been reported to play a role in the regulation of antibiotics resistance and virulence in several organisms. For example, PhoPQ in *Salmonellae* regulates the protein and lipid content of the bacterial envelope that modifies the inner and outer membrane results in increased resistance to cationic antimicrobial peptides and production of some virulence factors associated with secretion systems (Dalebroux and Miller, 2013). In another study, Manoil *et al*., (2009) identified AmgRS system in the pathogen *Pseudomonas aeruginosa* that appears to control a conserved membrane stress response. Inactivation of *amgRS* has led to increase aminoglycoside efficacy in clinical situations in which antibiotic treatment often fails. Interestingly, the *amgRS* mutant was considerably less virulent than its parent in acute murine infections in the absence of antibiotics, which suggests that AmgRS response is required for full virulence, and its inhibition is expected to provide the dual benefit of compromising infection directly as well as enhancing antibiotic sensitivity (Lee *et al.*, 2009). These findings strongly highlight the control of TCSs in regulating virulence and drug resistance in pathogenic organisms. Thus, TCSs seem to be a good therapeutic target for the discovery of new treatment options against multidrug resistant microorganisms' infections.

ii. TCSs as new targets options for treatment

The rise in infections caused by MDR pathogens is becoming a global health problem, this creates a need for development of new antibiotics to treat these deadly infections. Over the last few decades, the introduction of new antibiotics is not keeping pace with the rapid evolution of resistance to almost all clinically available drugs, this created a need to look for alternative strategies to overcome the MDR problem. Inhibiting TCSs is a promising strategy to identify new novel antimicrobial agents that inhibit microbial virulence without inhibiting growth, which leads to less pressure for the generation of resistance (Gotoh *et al.,* 2010).

Rasko *et al.,* (2008) have reported a small molecule that inhibits the histidine sensor kinase QseC in *Salmonella* Typhimurium by inhibiting the signals to the sensor kinase, preventing its phosphorylation. This compound inhibits virulence of several pathogens in animal studies without killing the bacterial cells. WalK/WalR is another novel target for the development of antibacterial agents against multidrug resistant bacteria including methicillin-resistant *S. aureus.* Singermycin B was discovered as a new antibiotics that targets the dimerization domain of WalR and inhibits bacterial growth (Watanabe *et al.,* 2012). In another study, a small molecule has been used as an adjuvant that suppresses the colistin resistance by interfering with the expression of the TCSs *pmrAB* in *A. baumannii* and *Klebsiella pneumonia* (Harris *et al.,* 2013).

In summary, TCSs are potentially a great target for the development of new therapeutic options against MDR bacterial infections. Since TCSs are major players of the regulation of virulence and antibiotics resistance, better understanding of the mechanisms of these TCSs in multidrug resistance and virulence the emerging MDR pathogens is required.

1.6 Two component systems in *Acinetobacter baumannii*

Around nineteen different TCSs have been identified in *A. baumannii*, most of these were present in sequenced clinical strains (*A. baumannii* AB0057, AYE, ACICU, AB307-0294, AB900, SDF, and ADP1). Fifteen of these are conserved in all sequenced strains including the type strain *A. baumannii* ATCC17978 (Adams *et al.,* 2008). Of these, five TCSs have been characterized so far, PmrAB, BfmRS, AdeRS, BaeSR and GacSA.

i. PmrAB

Colistin and polymyxins are antibiotics that were commonly used in the 1960s-1970s but were abandoned because of reports of toxicity. Outbreaks of polymyxin-resistant *A. baumannii* have been reported (Fernandez-Reyes *et al.,* 2009), and resistance to these drugs is mediated by replacement of Lipid A with aminoarabinose which is controlled by *pmrC*. The TCSs PmrAB plays a role in the expression of genes implicated in lipid A modification and thus influence sensitivity to colistin. Knockout of the *pmrB* gene in two clinical isolates has led to a decrease in the polymyxin B and colistin susceptibility, these findings suggested that alterations in the sequence of the PmrCAB are responsible for resistance to polymyxins and colistin in *A. baumannii* (Arroyo *et al*., 2011; Beceiro *et al.*, 2011).

ii. BfmRS

It has been demonstrated that *A. baumannii* has the ability to form pili to adhere and form biofilm which depends on the expression of the *csuE* gene. Inactivation of the *csuE* disrupted pili production and biofilm formation suggesting that CsuA/BABCDE-mediated pili play an important role in the biofilm formation. The expression of the *csuE* gene is controlled by a two component system consisting of a sensor kinase encoded by *bfmS* and a response regulator

encoded by *bfmR*. Inactivation of the sensor kinase does not abolish the biofilm formation; however, inactivation of the response regulator *bfmR* results in a loss of the expression of the *csu* operon and, therefore, abolition of the pili production and biofilm formation. These findings suggest that the expression of a responsible gene for biofilm formation in *A. baumannii* is controlled by the two component system BfmRS (Tomaras *et al*., 2008).

iii. AdeRS

The expression of AdeABC efflux pump is tightly regulated by the two-component system that contains a sensor kinase (SK) *adeS* and a response regulator (RR) *adeR*, encoded by the *adeRS* operon. The *adeRS* operon is located at the upstream of *adeABC* operon and is transcribed in the opposite direction contributing to resistance to aminoglycosides, tetracycline, erythromycin, chloramphenicol, trimethoprim, and fluoroquinolones (Sun *et al.,* 2012). Disruption of *adeR* and *adeS* genes by insertional mutagenesis resulted in increased susceptibility of the mutant strains of *A. baumannii* to aminoglycosides and other substrates of the pump (Marchand *et al.,* 2004). Two component systems have been reported to control the expression of drug efflux transporters in other bacterial species, but are considered to be an uncommon mode of regulation of efflux pumps.

iv. BaeSR

In *Escherichia coli,* BaeSR system is known to upregulate the expression of efflux pumps in response to specific envelope damaging agents (Leblanc *et al.*, 2011). A recent study done by Lin *et al.,* (2013) have identified BaeSR system in *A. baumannii*, and showed that a *baeSR* deletion mutant was susceptible to tigecycline. The study also hypothesized that this system could play a role in the regulation of the RND efflux pump *adeABC*, which suggests that there is

other TCSs involved in the regulation of the RND efflux pump AdeABC other than AdeRS system.

v. GacSA

The sensor kinase *gacS* deletion mutant was analyzed using transcriptional profiling and functional assays. GacS was shown to regulate key virulence factors including pili production, biofilms and motility. Transcriptomic profiling showed that 674 genes were affected by the deletion of *gacS,* and based on in slico analysis an orphan response regulator was identified and predicted as GacS response regulator GacA. The TCSs GacSA is thought to be the first characterized global virulence regulator in *A. baumannii* (Cerqueira *et al.*, 2013).

1.7 Knowledge gaps

TCSs are absent in mammalian cells which emphasizes the need for inhibitors of such systems that are considered a great strategy to overcome MDR pathogens infections. Relatively, few virulence factor have been characterized in *A. baumannii,* and much needs to be explored about the global mechanisms that control antibiotic resistance regulation and production of virulence factors of *A. baumannii*. The knowledge obtained during this study will contribute to the understanding of the action of TCSs in *A. baumannii,* as well as their participation in the global mechanisms used by this microorganism to overcome adverse conditions in clinical settings. Finally, this research will supply additional information to create a better understanding to the pathogenicity and resistance of *A. baumannii*.

1.8 Hypothesis

Two component systems act as global regulators of antibiotic resistance and virulence and their better characterization will lead to novel therapeutic options for treating infections caused by MDR *A. baumannii*.

Therefore, the objectives of this thesis are:

A. Cloning of two component systems-encoding operons of *A. baumannii*

B. Creation of gene knockouts for two component systems

C. Analysis of TCSs in clinical isolates of *A. baumannii*

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and oligonucleotides

Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Tables 2.1, 2.2, and 2.3, respectively. The clinical isolates of *Acinetobacter baumannii* were collected from Canadian Hospitals provided by Dr. George Zhanel, University of Manitoba, Canada (Fernando *et al*., 2013). *A. baumannii* ATCC17978 was used as the wild-type strain.

2.2 Growth and culture conditions

Bacteria were routinely cultured in lysogeny broth (LB) at 37 °C (Biobasic Inc., Markham, ON, Canada) and supplemented with the appropriate antibiotic where necessary to maintain plasmids. The following concentrations: 100 µg/mL ampicillin (Bioshop Canada Inc., Burlington, ON., Canada) and 30 µg/mL gentamicin (Bioshop Canada Inc., Burlington, ON., Canada), 50 µg/mL trimethoprim (Bioshop Canada Inc., Burlington, ON., Canada), 15 µg/mL streptomycin (Sigma Aldrich Co., St Louis, MO, USA), and 10 µg/mL tetracycline (Bioshop Canada Inc., Burlington, ON., Canada) for selection of *E. coli* strains. Concentrations of antibiotics used for *A. baumannii* are 200 µg/mL carbenicillin (Bioshop Canada Inc., Burlington, ON, Canada), 50 µg/mL gentamicin (Bioshop Canada Inc., Burlington, ON, Canada).

2.3 DNA manipulation

i. DNA extraction

Genomic DNA was extracted using the DNeasy Kit from Qiagen® (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions. Briefly, cells from 1.5 mL of overnight culture were harvested and directly lysed by incubating with proteinase K at 56˚C for 10 min, 96-100% ethanol was added and the mix was transferred to a silica membrane column. The

column was washed twice with ethanol solution and the DNA was extracted from the column using 100 to 200 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 the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific In, Mississauga, ON, Canada) and all samples were stored either at 4°C or -20°C.

ii. Extraction of DNA fragments from agarose gels

GeneJET Gel Extraction Kit from Thermo Scientific (Thermo Fisher Scientific In, Mississauga, ON, Canada) was used to purify DNA fragments from 0.8% agarose gels (Biobasic Inc., Markham, ON, Canada) following manufacturer's instructions. Briefly, a gel containing DNA was excised and dissolved in Binding buffer by incubating at 56°C for 10 minutes. The mixture was transferred to a silica membrane column and washed once with ethanol solution and the DNA was extracted from column with adding 20 to 30 µl of elution buffer. DNA concentration was measured using the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific In, Mississauga, ON, Canada) and samples were stored at 4°C.

iii. DNA restriction digestion, ligation, and polishing

Restriction enzymes were used to confirm the identity of plasmids, as well as for cloning DNA. Enzymes were used following the manufacturer's instructions (New England Biolabs Ltd., Pickering, ON, Canada). Ligations were performed using T4 DNA ligase (Invitrogen Inc., Burlington, ON, Canada) or from (Promega, Madison, WI, USA) using varying molar ratios of the insert and vector. The ligation mixtures were incubated at 14°C overnight. DNA fragments were polished with a T4 DNA polymerase (NEB England Biolabs Ltd., Pickering, ON, Canada)

at 12°C for 20 minutes and the enzyme was heat inactivated at 75°C for 15 minutes to create a blunt-ended product for ligation.

iv. Construction of the suicide vector pPLS103

The suicide plasmid pKNG101 (Kaniga *et al.,* 1991) was digested with the restriction enzymes *Sac*I and *Not*I (NEB England Biolabs Ltd., Pickering, ON, Canada) to remove the 1460 bp selection marker *strAB* encoding for streptomycin phosphotransferase and was replaced with the 739 bp *dhfr* selection marker encoding for dihydrofolate reductase. The Tmp^r marker was obtained from the plasmid pPLS076 by restriction digestion using *Pst*I and *Kpn*I (NEB England Biolabs Ltd., Pickering, ON, Canada) and was then polished using T4 DNA polymerase (NEB England Biolabs Ltd., Pickering, ON, Canada). The polished *dhfr* fragment was sub-cloned into a polished 5526 bp DNA fragment of pKNG101.

v. Construction of the suicide vector pPLS109

The suicide plasmid pKNG101 (Kaniga *et al.,* 1991) was digested with the restriction enzymes *Sac*I and *Not*I (NEB England Biolabs Ltd., Pickering, ON, Canada) to remove the 1460 bp selection marker *strAB* encoding for streptomycin phosphotransferase and was replaced with the 1359 bp *tet* selection marker encoding tetracycline-resistance gene. The tet^r marker was obtained from the plasmid pPLS077 by restriction digestion using *Pst*I and *Kpn*I (NEB England Biolabs Ltd., Pickering, ON, Canada) and was then polished using T4 DNA polymerase (NEB England Biolabs Ltd., Pickering, ON, Canada). The polished *tet* fragment was sub-cloned into a polished 5526 bp DNA fragment of pKNG101.

2.4 Preparation *of E. coli* chemically competent cells and transformation

E. coli competent cells were prepared according to Inoue and colleagues (Inoue *et al*., 1990). Briefly, *E. coli* were subcultured with a 1% v/v inoculum from an overnight culture and was grown to an absorbance (600nm) of 0.5. The culture was then chilled on ice for 15 minutes. Cells were harvested in a pre-chilled rotor at 5000 x g and resuspended in 0.4% of original volume of ice cold transformation buffer I (TFBI– 100 mM rubidium chloride (Fisher Scientific, Markham, ON, Canada), 50 mM manganese chloride (Bioshop Canada Inc., Burlington, ON., Canada), 30 mM potassium acetate (Bioshop Canada Inc., Burlington, ON., Canada), 10 mM calcium chloride (Bioshop Canada Inc., Burlington, ON, Canada), 15% w/v glycerol (Bioshop Canada Inc., Burlington, ON, Canada), pH 5.8). Cells were incubated on ice for exactly 5 minutes, harvested as above and resuspended in 0.04% of original volume of ice cold transformation buffer II (TFBII– 10 mM 3-(N-morpholino) propanesulfonic acid, 10 mM rubidium chloride, 75 mM calcium chloride, 15% w/v glycerol, pH 6.5). Cells were incubated on ice for 60 minutes and 100 µl aliquots were made and immediately frozen on dry ice to be stored long term at -80°C. DNA Transformations were performed using the heat shock method (Sambrook and Russell, 2001). Briefly, 100-500 ng of DNA was added to 100 µl competent cells and incubated on ice for 10 minutes, then incubated at 42 °C for 45 seconds followed by incubation on ice for another 2 minutes. LB media (890 µL) was added to cells at room temperature and cells were recovered at 37 °C for 1 hour with shaking and plated on LB agar media containing the appropriate antibiotic for selection.

2.5 Preparation *of A. baumannii* electrocompetent cells and transformation by electroporation

A. baumannii electrocompetent cells were prepared using the electroporation method previously described by Aranada *et al.,* (2010). Briefly, *A. baumannii* were subcultured with a 1% v/v inoculum from an overnight culture and grown to an absorbance$_{600nm}$ of 0.8. Cells were harvested in a pre-chilled rotor at 8000 x g by centrifugation and then washed 3 times with 10% (v/v) glycerol and finally resuspended in 500 µL of 10% (v/v) glycerol. An aliquot of 100 µL of the cell suspension was mixed with the 50-500 ng of DNA. The mixture was placed in a pre-chilled sterile 2 mm electroporation cuvette (Thermo Fisher Scientific In, Mississauga, ON, Canada) and pulsed at 2.5 KV with default settings of 10 µF for 5 sec using Eppendorf electroporator Model 2510 (Eppendorf North America, Inc., Westbury, N.Y., USA). Immediately following the pulse, LB media (1 mL) was added to the cuvette at room temperature and cells were transferred to a microcentrifuge tube. Cells were recovered at 37 °C for 1 hour with shaking and plated on LB agar media containing the appropriate antibiotic for selection.

2.6 Polymerase chain reaction (PCR)

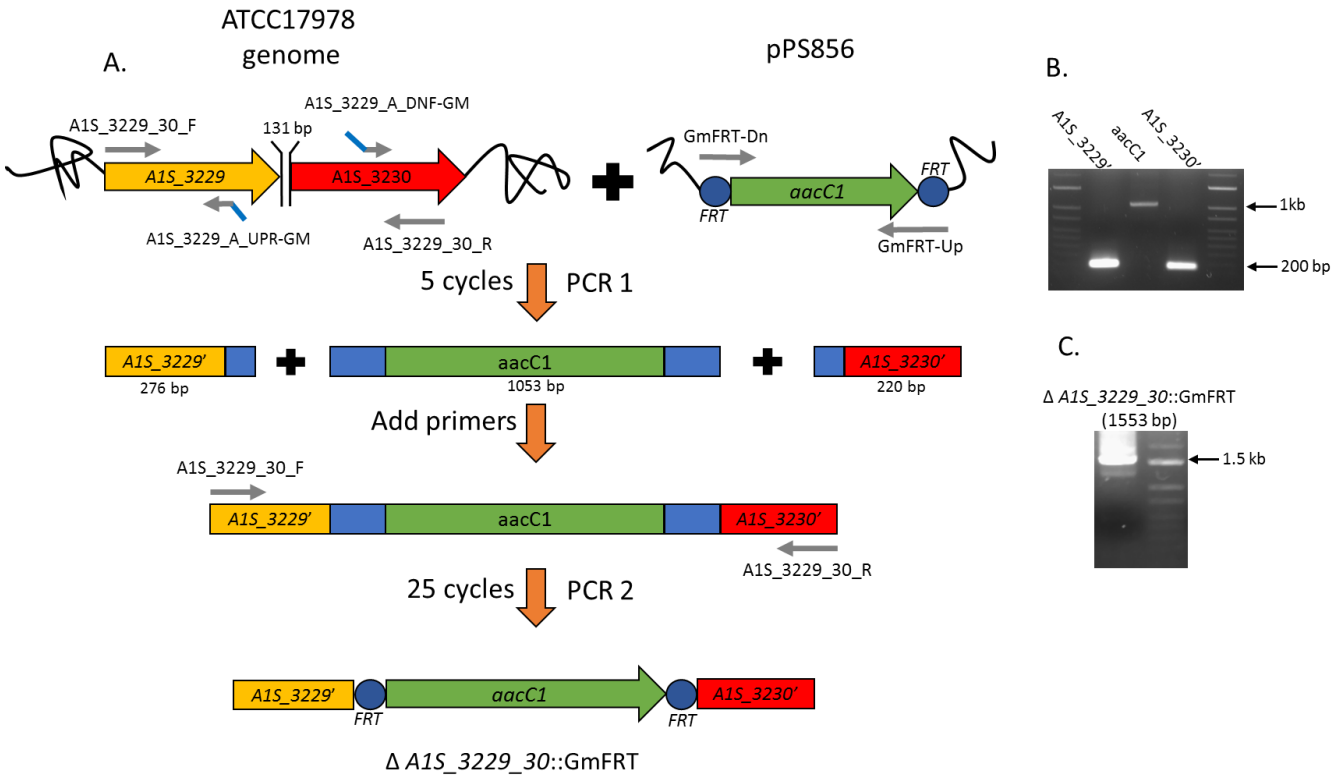
Extracted plasmids and genomic DNA were used as template for PCR amplification. PCR amplifications reactions were performed using variety of polymerases. Taq DNA polymerase (New England Biolabs, Pickering, ON, Canada) was used routinely for TA cloning and screening purposes, and Phusion HF (New England Biolabs, Pickering, ON, Canada) was used in the final step of Splicing by Overlap Extension (SOEing) reaction described below. All PCR polymerases and conditions were performed according to the manufacturer's instructions. The annealing temperature (Ta) for each PCR primer was calculated based on the equation: 4 (G+C) + 2(A +T) -5 (°C).

2.7 Splicing by overlap extension (SOEing) PCR

The splicing Overlap Extension (SOEing) reaction was used to generate disrupted fragments for *A1S_3229_30* with the purpose of creating gene knock-out using a previously described method (Choi and Schweizer, 2005) (Figure 2.1). This method consists of two steps of a PCR reaction. The first step involved amplifying the gentamycin resistant *aacC1* and the flanking regions of the target gene/operon using Taq DNA polymerase (New England Biolabs, Pickering, ON, Canada). Gm^r cassette was obtained by PCR amplification of *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* ATCC17978 and adding engineered homologous 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 an agarose gel using the method described above and their concentrations determined spectrophotometrically (260nm) using the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific In, Mississauga, ON, Canada). Fifty nanograms of each of the three fragments was used as template for the second step of the PCR reaction. The reaction proceeded without any primers for 5 cycles (annealing temperature, 60˚C) after which the reaction was paused and 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 25 additional cycles. Phusion HF and Taq DNA polymerase (New England Biolabs, Pickering, ON, Canada) were used for SOEing reactions following the manufacturer's instructions. The resulting PCR product was gel purified and cloned into the pUC18 cloning vector, followed by sub-cloning into a pPLS103 suicide plasmid for further applications.

Figure 2.1. Schematic illustration of mutant fragment generation by SOEing reaction for *A1S_3229_30* operon. A PCR reaction was performed to amplify 276 bp of the 5'-end of the *A1S_3229* gene, designed in the figure as *A1S_3229*', and 220bp of the 3'-end of the *A1S_3230* gene, designed as A1S_3230'. These fragments were mixed with Gmʳ cassette (*aacC1*) to obtain the *A1S_3229_30* deletion fragment. PCR products were visualized on a 0.8% agarose gel (A). These purified products which contained *FRT* overlapping sequences (blue boxes) were then assembled by SOEing reaction using PCR1 and PCR2 cycles. PCR1 reactions were prepared using equal amounts (50 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 25 cycles to allow the amplification of the SOEing product. The presence of Δ*A1S_3229_30*::GmFRT 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.


A.
ATCC17978
genome
pPS856
A1S_3229_30_F
131 bp
A1S_3229_A_DNF-GM
A1S_3229
A1S_3230
A1S_3229_A_UPR-GM
A1S_3229_30_R
GmFRT-Dn
aacC1
FRT
GmFRT-Up
5 cycles
PCR 1
A1S_3229'
276 bp
aacC1
1053 bp
A1S_3230'
220 bp
Add primers
A1S_3229_30_F
A1S_3229'
aacC1
A1S_3230'
A1S_3229_30_R
25 cycles
PCR 2
A1S_3229'
FRT
aacC1
FRT
A1S_3230'
Δ A1S_3229_30::GmFRT
B.
A1S_3229'
aacC1
A1S_3230'
1kb
200 bp
C.
Δ A1S_3229_30::GmFRT
(1553 bp)
1.5 kb

2.8 DNA sequencing and analysis

Sanger DNA sequencing was performed at the Genome Quebec facility at McGill University, Montreal, QC. The sequence analysis was carried out using Basic Local Alignment Search Tool (BLAST) and Gene Construction Kit Software (Textco Biosoftware Inc, West Lebanon, NH, USA).

2.9 Whole-genome sequencing for the clinical isolates *A. baumannii* AB030 and *A. baumannii* AB031

i. Genomic DNA extractions

Genomic DNA were extracted using the DNeasy Kit from Qiagen® (Qiagen, Mississauga, ON, Canada) as described above, and the MoBio® UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, US) following the detailed protocol provided by the manufacturer. The presence of DNA in each extraction was confirmed by agarose gel electrophoresis, and the final concentration and purity of DNA were determined using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific In, Mississauga, ON, Canada).

ii. Sequencing and genome assembly

The whole genome sequencing of *A. baumannii* AB030 and *A. baumannii* AB031 was carried out at the Genome Quebec facility at McGill University, Montreal, QC using the PacBio (Pacific Bioscience) sequencing platform using three SMRTcells. Assembly was carried out using the PacBio SMRT Analysis pipeline version 2.0.1, with 92X coverage for AB030 and 89X coverage for AB031 to give a single contiguous genome sequence for each strain.

iii. Annotation and comparative genomics analysis

Protein-coding genes were identified and annotated using RAST server (Aziz *et al.,* 2008), and National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Annotation Pipeline. Multiple sequence alignments were performed with Basic Local Alignment Search Tool (BLAST), and ClustalW (Larkin *et al.,* 2007). Putative promoter regions were analyzed using BPROM (Softberry Inc) (Solovyev and Salamov, 2011). The prediction of phosphorylation site was done using NetPhosBac 1.0 Server (Miller *et al.,* 2009). Circular

alignment maps of AB030 and AB031 genomes obtained using the CGView (Stothard and Wishart, 2005) application.

iv. Nucleotide sequence accession numbers

The genome sequences of AB030 and AB031 have been deposited in the NCBI/GenBank databases under the accession numbers CP009257 and CP009256, respectively.

2.10 Quantitative-Reverse transcriptase polymerase chain reaction (qRT-PCR)

i. RNA extraction

Total RNA was extracted from cells using the RNeasy RNA isolation kit (Qiagen, Mississauga, ON, Canada). Briefly, cells were subcultured with a 1% v/v inoculum from an overnight culture and was grown to an absorbance (600nm) of 0.6. Cell pellets were frozen at -80 °C for an overnight to facilitate cell lysis. Cells were then thawed at room temperature and resuspended in a containing 400 µg/mL lysozyme (Bioshop Canada Inc., Burlington, ON, Canada) and then transferred to a buffer containing guanidine-isothiocyanite, which inactivates RNases. Total RNA was precipitated with 95-100% ethanol and the solution was applied to an RNeasy silica membrane column. The membrane was washed with an ethanol containing wash buffer. RNA was eluted off of the column using RNase-free water. RNA concentrations were determined by the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific In, Mississauga, ON, Canada) RNA samples were stored at -80°C.

ii. cDNA synthesis

Contaminating genomic DNA was removed using the RNase-free DNase kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Briefly, 1 µg of total

RNA was incubated with 1.0 unit of DNase for 40 minutes at 37 °C, DNase was heat inactivated at 70 °C for 5 minutes and 800 ng of RNA was reverse transcribed with the iScript Reverse Transcriptase with random hexamer primers (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. No reverse transcriptase (NRT) controls were included with every cDNA preparation to rule out genomic DNA contamination.

iii. Real-Time PCR

Real-Time PCR was performed in the CFX-96 Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using Evagreen Sso-fast PCR supermix (BioRad Laboratories, Hercules, CA, USA). PCR conditions were as follows: 95 °C 3 minutes; (95 °C 10 seconds; 60 °C 30 seconds) for 39 cycles. High-resolution melt conditions used were: 95 °C 10 seconds, 65 °C – 95 °C in 0.5 °C increments, 5 seconds/ °C. The housekeeping gene 16S rRNA was used as a control. Reactions (15 μL) were set up using 300 nM primers and 5 μL of the cDNA template (diluted 1:10). The efficiency of each primer set was determined by creating a standard curve using 10-fold dilutions of pooled cDNA samples. Primer sets with efficiencies below 95% or above 105% were omitted. No Reverse Transcriptase (NRT) controls for the housekeeping genes were included to rule out contamination by genomic DNA. A No Template Control (NTC) for each gene was also included. Expression of target genes were normalized to the reference gene using the CFX Manager Software, Gene Expression Analysis tool (BioRad Laboratories, Hercules, CA, USA). The quality of the mRNA was assessed by ensuring the Ct-value remained constant for the housekeeping gene between runs. For gene expression analysis the ΔΔCt method was employed (Livak and Schmittgen, 2001). Data analysis was carried out using Bio-Rad CFX Manager 2.0 Gene Study software.

Table 2.1. List of strains used in the study

Strains	Relevant Characteristics	Source
	E. coli	
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Laboratory collection
MaH1	DH5α::mini-Tn*7*-*pir*-*116*	(Kvitko *et al*., 2012)
SM10	*thi-1 thr-1 leuB26 tonA21 lacY1 supE44* *recA* integrated RP4-2 Tc^r::Mu $aphA^+$ (Km^r) (RP4-2 is RP4 ΔTn*1*)	Laboratory collection
	A. baumannii	
ATCC17978	*A. baumannii* wild-type	ATCC
AB030	Clinical isolate (isolated from blood of a patient from Winnipeg, MB)	(Fernando *et al.*, 2013)
AB031	Clinical isolate (isolated from blood of a patient from Toronto, ON)	(Fernando *et al.*, 2013)

Table 2.2. List of plasmids used in the study

Plasmids	Relevant Characteristics	Source
pUC18	Ap^r, pUC18	Laboratory collection
pGEMT-Easy	Ap^r, TA PCR cloning vector	Promega
pKNG101	Str^r, suicide vector contains the *sacB* gene that can be used as counter selectable marker.	(Kaniga *et al.*, 1991)
pPLS076	Ap^r, Tmp^r, pUC18-*dhfr*	Laboratory collection
pPLS077	Ap^r, Tet^r, pUC18-*tet*	Laboratory collection
pPLS103	Tmp^r, pKNG101-dhfr, Str^r marker replaced with the *dhfr* gene.	This Study
pPLS104	Ap^r, pGEMT-Easy-*A1S_2883_84*	This study
pPLS105	Ap^r, pGEMT-Easy-*A1S_2006*	This Study
pPLS106	Ap^r, pGEMT-Easy-*A1S_3375_76*	This Study
pPLS107	Ap^r, pGEMT-Easy-*A1S_1232*	This Study
pPLS108	Ap^r, pGEMT-Easy-*A1S_2906*	This Study
pPLS109	Tet^r, pKNG101-tet, Str^r marker replaced with the *tet* gene.	This Study
pPLS115	Ap^r, pGEMTEasy-*A1S_1753_54*	This Study
pPLS116	Ap^r, pGEMTEasy-*A1S_3229_30*	This Study
pPLS117	Ap^r, pGEMTEasy-A1S_*0260_61*	This Study
pPLS125	Ap^r, Gm^r, pUC18-*3229::*GmFRT	This Study
pPLS126	Tmp^r, Gm^r, pPLS103-*Δ3229*::GmFRT	This Study
pPLS143	Ap^r, pGEMTEasy-*A1S_0478_79*	This Study
pPLS144	Ap^r, pGEMTEasy-*A1S_2137_38*	This Study
pPLS146	Ap^r, pGEMTEasy-A1S_*2750_51*	This Study

Table 2.3. List of oligonucleotides used in the study

Target Gene	Oligonucleotide Name	Sequence	Reference
A1S_2883_84	A1S_2883_2884_R	CATTGTGGTTATCCACAGC	Kumar Lab Database
	A1S_2883_2884_F	GCTAGATCGGCTCATTACTA	
A1S_2750_51	A1S_2750_2751_R	TTATGGACAGGCTGGGTCGT	Kumar Lab Database
	A1S_2750_2751_F	CTGGACATGTTGCACTCTTG	
A1S_2006	A1S_2006_R	GCCGAGTTGCAATTGAGTC	Kumar Lab Database
	A1S_2006_F	CGCGTCGAATTGGCGCATTT	
A1S_1977_78	A1S_1977_1978_R	CGGCTAAATTAAATTAGCCGC	Kumar Lab Database
	A1S_1977_1978_F	CAATGAAGATGCCATTGCG	
A1S_1753_54	A1S_1753_1754_R	ATGGCGAGAAGAGATTCGTAG	Kumar Lab Database
	A1S_1753_1754_F	GCGTATGATGAGTTGAAGCAC	
A1S_1393_94	A1S_1393_1394_R	TAGCAGTGAGTCTGTATGCT	Kumar Lab Database
	A1S_1393_1394_F	GGATTGCTGGGTTTAATGCAGC	
A1S_0260_61	A1S_0260_0261_R	TTGCAAGTAAAATCTCACAAGC	Kumar Lab Database
	A1S_0260_0261_F	CATACCTGAGGTTTGGTTTGG	
A1S_1232	A1S_1232_R	GACGCGCAAGGTTAGCAATTA	Kumar Lab Database
	A1S_1232_F	CATAGGAGATCGCTGGTCAC	
A1S_3375_76	A1S_3375_3376_R	GCCGACTTTTCATGAGTCGG	Kumar Lab Database
	A1S_3375_3376_F	GTCGCCGTTTAAAACGTGATG	
A1S_2906	A1S_2906_R	GTTATAGCAGTTCAAGTAGTAG	Kumar Lab Database
	A1S_2906_F	AGCGGTTCCGACCAACAATG	
A1S_0748_49	A1S_0748_0749_R	TGGAACCTGATGCAACTCAG	Kumar Lab Database
	A1S_0748_0749_F	GATACATGTAATGAGATTTATAGG	
A1S_3229_30	A1S_3229_3230_F	CCTAACCGATCCAATAATATATA	Kumar Lab Database
	A1S_3229_3230_R	GCGCTTTAAATGTTCAATTGCTC	
A1S_2137_38	A1S_2137_2138_R	CTTTG AAACCCTTAGGGAG	Kumar Lab Database
	A1S_2137_2138_F	GCCATTAAAGCAATTGATGTAG	
A1S_0236	A1S_0236_RT_R	AACGACGGGTTGTTTCTACG	Kumar Lab Database
	A1S_0236_RT_F	TATCGTTCGCCAACAACATC	
A1S_3375	A1S_3375_RT_R	TGCAAGCAGACGGTATTCTG	Kumar Lab Database
	A1S_3375_RT_F	CAATTGATGCCAATGGTTTG	

A1S_2883	A1S_2883_RT_F	GATGTTGCCGACCGAGTTAT	Kumar Lab Database
	A1S_2883_RT_R	TCCACTCATGACGGTTACCA	
A1S_2751	A1S_2751_RT_R	ATACGGGCAAGCAACTCATC	Kumar Lab Database
	A1S_2751_RT_F	TTACAAAACCGTGTCGATGG	
A1S_3229	A1S_3229_RT_F	CTCGTGAGGGTCAAATCGTT	Kumar Lab Database
	A1S_3229_RT_R	TGTCGCGTGTTAAAGGTTCA	
A1S_0261	A1S_0261_RT_F	CCAGCTAACACAAGCACAGC	Kumar Lab Database
	A1S_0261_RT_R	CCAGCTAACACAAGCACAGC	
A1S_2137	A1S_2137_RT_R	GTGCTTCTTGCTGGATTGGT	Kumar Lab Database
	A1S_2137_RT_F	TGCTTTGCTAGATGCTGGTG	
A1S_2006	A1S_2006_RT_F	GTTGAAAAGGCCAAAGTGCT	Kumar Lab Database
	A1S_2006_RT_R	GTGCCATTTCTCCAATCGTT	
A1S_1394	A1S_1394_RT_R	TTCACTGTCCGTGGACTCAG	Kumar Lab Database
	A1S_1394_RT_F	AAATATTGCCCGCAGTTGAC	
bfmR	bfm_RT_R	CATGAGATACCGCCCTCATT	Kumar Lab Database
	bfm_RT_F	TCCTATTGAGGGAAGCGATG	
16S	16S_F_RT	GGAGAAAGCAGGGGATCTTC	(Fernando *et al.*, 2013)
	16S_R_RT	ATCCTCTCAGACCCGCTACA	
GmFRT	GmFRT-UP	CGAATTAGCTTCAAAAGCGCTCTGA	(Choi and Schweizer, 2005)
	GmFRT-Dn	CGAATTGGGGATCTTGAAGTTCCT	
A1S_3229_30-GmFRT	A1S_3229_A_DNF-GM	AGGAACTTCAAGATCCCCAATTCGGGGAAGCGGTTTGGGTCTTGC	This study
	A1S_3229_A_UPR-GM	TCAGAGCGCTTTTGAAGCTAATTCGACGGGCAACATAAAGTCGAGC	

3. RESULTS

3.1 Cloning of two component systems operons of *Acinetobacter baumannii*

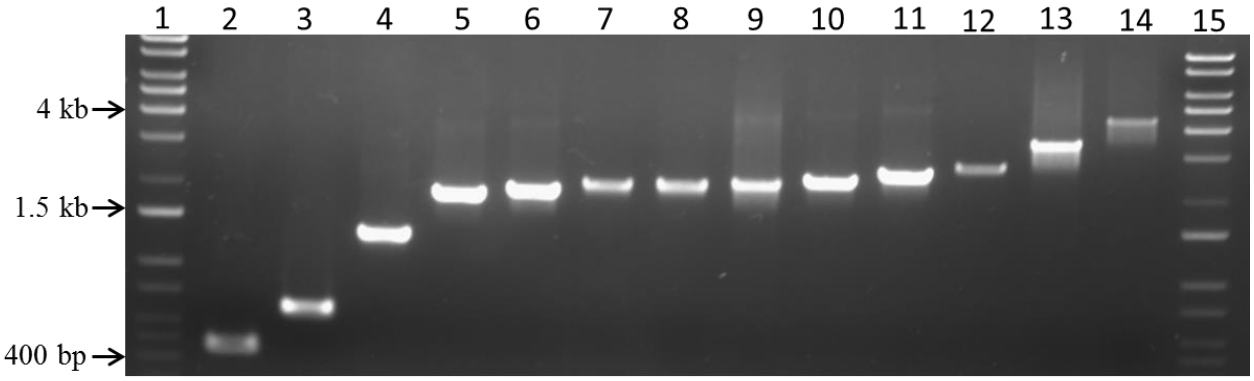
i. Amplification and cloning of two component systems operons.

The PCR amplifications of *A1S_1232*, *A1S_2006*, *A1S_2906*, *A1S_3375_76*, *A1S_0260_61*, *A1S_2750_51*, *A1S_1753_54*, *A1S_2883_84*, *A1S_0748_49*, *1977_78*, *A1S_3229_30*, *A1S_2137_38*, and *A1S_1394_95* have been standardized using the *A. baumannii* ATCC17978 genomic DNA as template, as seen in Figure 3.1.A. The PCR product of each TCSs operon was purified and then cloned into pGEMT-Easy vector. Successful clones were confirmed by restriction digestion, and DNA sequencing (Figure 3.1.B).

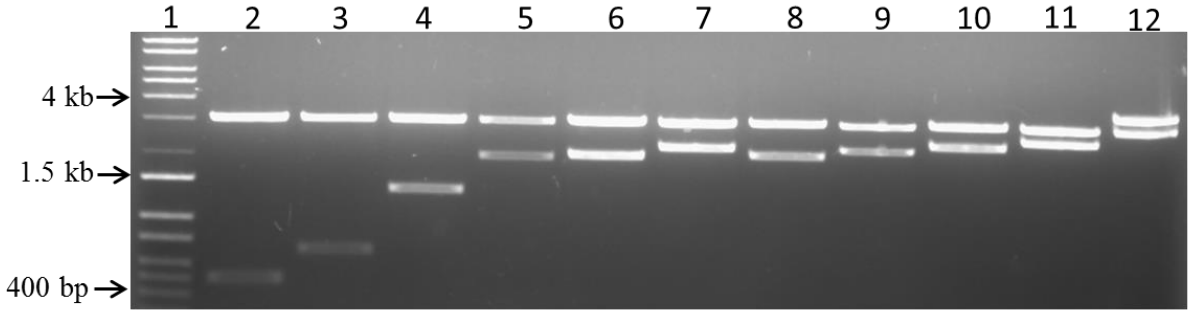
Figure 3.1. Amplification and cloning of TCSs operons of *A. baumannii*. A) The TCSs operon was amplified using genomic DNA of *A. baumannii* as a template using PCR. Lane 1 was the 10kb DNA marker; 2. *A1S_1232*; 3. *A1S_2006*; 4. *A1S_2906*; 5. *A1S_3375_76*; 6. *A1S_0260_61*; 7. *A1S_2750_51*; 8. *A1S_1753_54*; 9. *A1S_2883_84*; *10. A1S_0748_49*; 11. *A1S_3229_30*; 12. *A1S_1977_78*; 13. *A1S_2137_38*; 14. *A1S_1394_95*; 15. 10kb DNA marker. B) The TCSs operons were cloned into pGEMT-Easy vector, and clones were confirmed using restriction digestion. The size of the fragments expected are 3kb represents the size of the vector. Lane 1 was the 10kb DNA marker; 2. pPLS107; 3. pPLS105; 4. pPLS108; 5. pPLS106; 6. pPLS117; 7. pPLS146; 8. pPLS115; 9. pPLS104; 10. pPLS143; 11. pPLS116, and 12. pPLS144.

Figure 3.1.

A.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
4 kb
1.5 kb
400 bp

B.


1 2 3 4 5 6 7 8 9 10 11 12
4 kb
1.5 kb
400 bp

3.2 Creation of genetic tools for *Acinetobacter baumannii*

i. Construction of the suicide plasmid pPLS103 to create gene knockouts in A. baumannii.

The trimethoprim-resistant suicide plasmid pPLS103 was constructed with the purpose of creating mark-less gene knockouts in MDR *A. baumannii*. The resulting plasmid was named pPLS103, and its identity was confirmed by restriction digestion, as seen in Figure 3.2.A.The sizes of the fragments are as expected: 3097 bp, 1570 bp, and 1565 bp (Figure 3.2).

ii. Construction of the suicide plasmid pPLS109 to create gene knockouts in A. baumannii.

The tetracycline-resistant suicide plasmid pPLS109 was constructed with the purpose of creating mark-less gene knockouts in intrinsically streptomycin resistant *A. baumannii*. The resulting plasmid was named pPLS103, and its identity was confirmed by restriction digestion, as seen in Figure 3.3.A.The sizes of the fragments are as expected: 3097 bp, 2162 bp, and 1570 bp (Figure 3.3).

Figure 3.2. Construction of the suicide vector pPLS103. The suicide vector pKNG101 (6986 bp) contains the *pir* minus origin of replication of R6K (*oriR6K*), multiple cloning sites (MCS), the *strAB* genes encoding for streptomycin phosphotransferase, origin of transfer (*oriT*), and *sacB* encodes of levansucrase as a positive selection marker for the excision of the plasmid. The trimethoprim-resistant suicide vector was named pPLS103, and restriction digestion was performed to confirm the plasmid identity. Lane 1, 10 kb DNA marker. Lane 2, pKNG101 digested with *Eco*RI, and the expected fragments sizes are 3097 bp, 2319 bp, and 1570. Lane 3, pPLS103 digested with *Eco*RI, and the expected fragments sizes are 3097 bp, 1570 bp, and 1565 bp.

Figure 3.2.

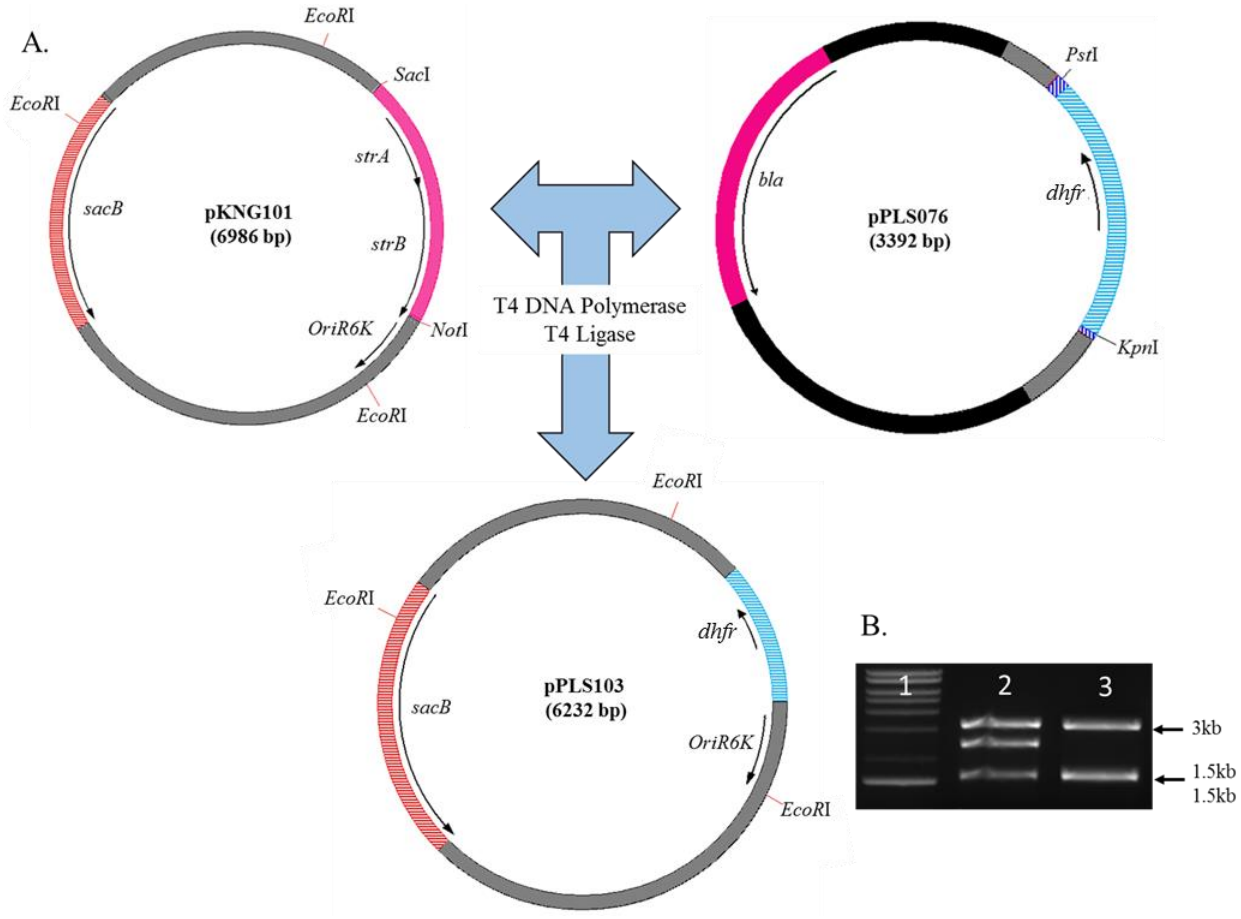
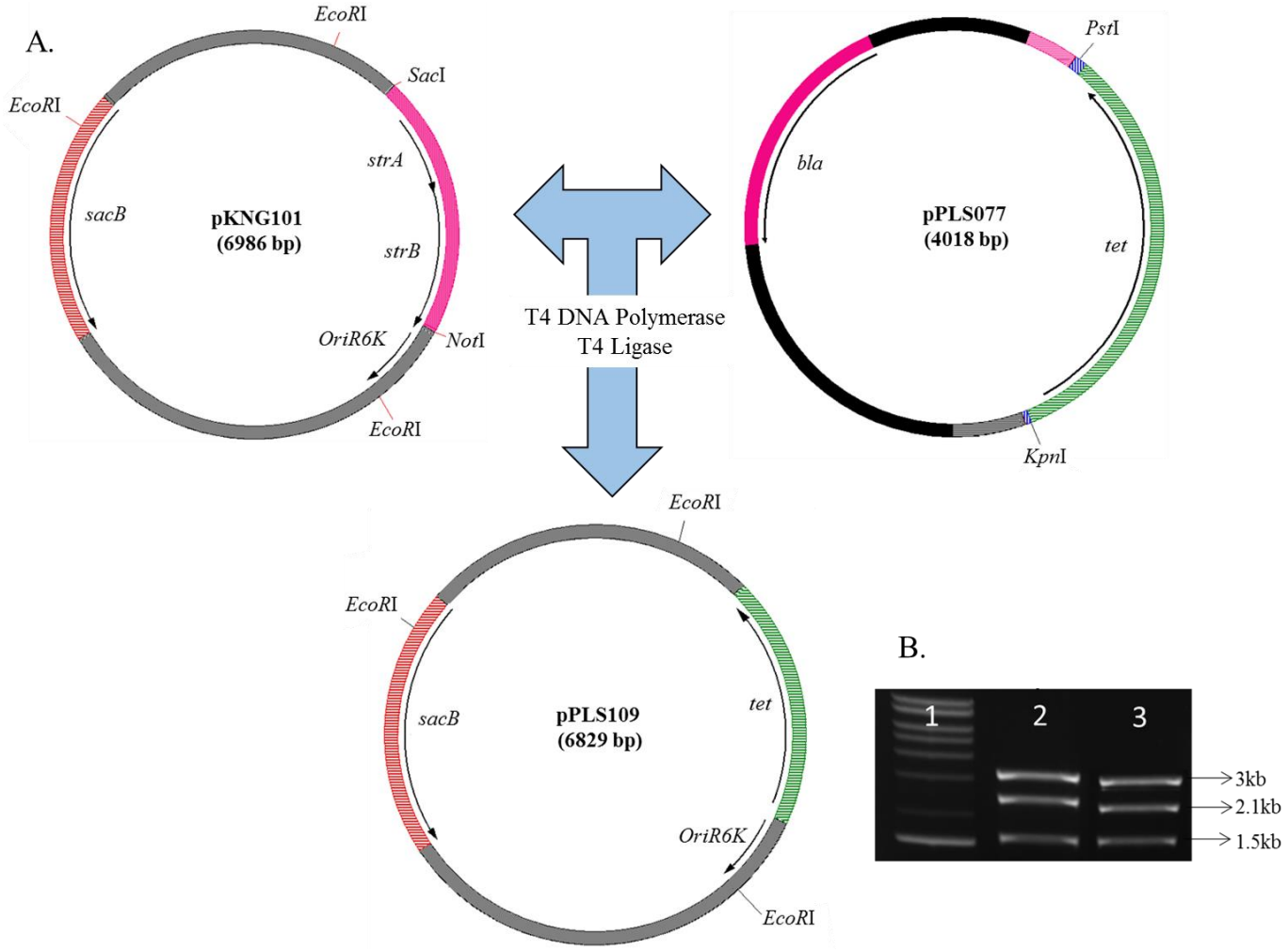

A.
EcoRI
SacI
EcoRI
strA
sacB
pKNG101
(6986 bp)
strB
OriR6K
NotI
EcoRI
T4 DNA Polymerase
T4 Ligase
PstI
bla
dhfr
pPLS076
(3392 bp)
KpnI
EcoRI
EcoRI
dhfr
sacB
pPLS103
(6232 bp)
OriR6K
EcoRI
B.
1
2
3
3kb
1.5kb
1.5kb

Figure 3.3. Construction of the suicide vector pPLS109. The suicide vector pKNG101 (6986 bp) contains the *pir* minus origin of replication of R6K (*oriR6K*), multiple cloning sites (MCS), the *strAB* genes encoding for streptomycin phosphotransferase, origin of transfer (*oriT*), and *sacB* encodes of levansucrase as a positive selection marker for the excision of the plasmid. The tetracycline-resistant suicide vector was named pPLS109, and restriction digestion was performed to confirm the plasmid identity. Lane 1, 10 kb marker. Lane 2, pKNG101 digested with *Eco*RI, and the expected fragments sizes are 3097 bp, 2319 bp, and 1570. Lane 3, pPLS109 digested with *Eco*RI, and the expected fragments sizes are 3097 bp, 2162 bp, and 1570 bp.

Figure 3.3.


A.
EcoRI
SacI
EcoRI
strA
sacB
pKNG101
(6986 bp)
strB
OriR6K
NotI
EcoRI
T4 DNA Polymerase
T4 Ligase
PstI
bla
pPLS077
(4018 bp)
tet
KpnI
EcoRI
EcoRI
sacB
pPLS109
(6829 bp)
tet
OriR6K
EcoRI
B.
1
2
3
3kb
2.1kb
1.5kb

3.3 Expression of two component systems in clinical isolates of *Acinetobacter baumannii*

i. Comparative quantitative real-time (qRT-PCR) analysis of mRNA expression of twelve TCSs.

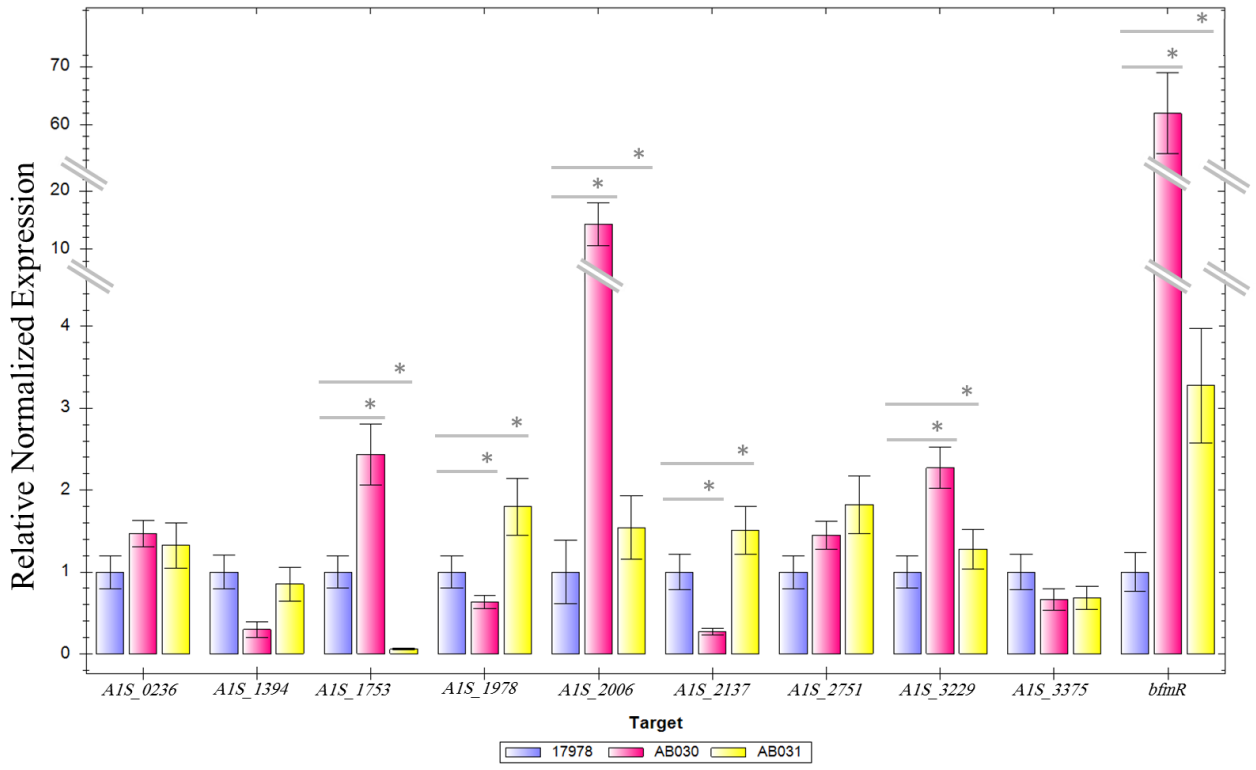
Analysis of the expression of TCSs was carried out on two blood isolates *A. baumannii* AB030 and *A. baumannii* AB031, which display very different antibiotic sensitivity profiles (Fernando *et al.,* 2013). Comparison of global regulatory mechanisms that control multidrug resistance between these two strains can be an effective way to understand the mechanisms of multidrug resistance in *A. baumannii*. Since TCSs are known to play a role in regulating multidrug resistance and virulence in various pathogens, characterization of genes that encode TCSs in AB030 and AB031 is the first logical step in deciphering these mechanisms.

Ten putative response regulators genes were selected for this analysis based on the fact that these are conserved among the all *A. baumannii* sequenced strains (Adams *et al.,* 2008). These ten putative and characterized response regulators are *A1S_0236, A1S_2006, A1S_1978, A1S_3375, A1S_1753, A1S_1394, A1S_2137, A1S_2751, A1S_3229,* and *bfmR.*

Differential expressions were observed in the well characterized TCSs (*bfmR* and *adeR*) between *A. baumannii* AB030 and *A. baumannii* AB031 compared to the control strain *A. baumannii* ATCC17978. *bfmR* showed 70-fold overexpression in *A. baumannii* AB030 and 5-fold overexpression in *A. baumannii* AB031. On the other hand, *A1S_0236, A1S_3375, A1S_2751,* and *A1S_1394* were all expressed in *A. baumannii* AB030 and *A. baumannii* AB031 but did not show any significant differential expressions. (Figure 3.4)

Figure 3.4. Expression of ten putative response regulators genes in *A. baumannii* clinical isolates. Expression of *A1S_0236*, *A1S_2006*, *A1S_1978*, *A1S_3375*, *A1S_1753*, *A1S_1394*, *A1S_2137*, *A1S_2751*, *A1S_3229*, and *bfmR* was measured using qRT-PCR in *A. baumannii* AB030 and *A. baumannii* AB031. Differential expression of the six response regulators genes *A1S_1753*, *A1S_1978*, *A1S_2006*, *A1S_2137*, *A1S_3229*, and *bfmR* in *A. baumannii* AB030 and *A. baumannii* AB031 compared to the control strain *A. baumannii* ATCC17978. *16S* rRNA was used as the housekeeping gene, and *A. baumannii* ATCC17978 was used as the control strain. Each reaction contained the no-template and no-RT controls. Asterisk indicates differences in expression relative to parent strain ATCC17978, $P<0.05$. Data shown is representative of at least 2 biological replicates.

Figure 3.4.


Relative Normalized Expression
70
60
20
10
4
3
2
1
0
A1S_0236
A1S_1394
A1S_1753
A1S_1978
A1S_2006
A1S_2137
A1S_2751
A1S_3229
A1S_3375
bfmR
Target
17978
AB030
AB031

ii. Differential expression of six different response regulators genes

Six response regulators were differentially expressed between *A. baumannii* AB030 and *A. baumannii* AB031 compared to the control strain *A. baumannii* ATCC17978. These genes are *A1S_1753*, *A1S_1978*, *A1S_2006*, *A1S_2137*, *A1S_3229*, and *bfmR*. (Figure 3.4)

These results taken together, demonstrate an ideal start to identify suitable candidates to understand the global mechanisms in the regulations of antibiotic resistance and virulence of *A. baumannii*.

3.4 Whole-genome sequencing of *Acinetobacter baumannii* AB030 and AB031.

i. Whole-genome sequencing of A. baumannii AB030 and A. baumannii AB031

Genomes of AB030 *and A. baumannii* AB031 was sequenced using Pacific bioscience (PacBio) sequencing platform, assembled, and annotated for this analysis. The genome size of AB030 was 4,335,793 bp with a G+C content of 39% and the number of ORFs was 4132 (Figure 3.5).

Interestingly, the AB031 genome appears to be relatively small in comparison to AB030, and the genome size of AB031 was 3,803,317 bp with a G+C content of 38% and the number the number of ORFs was 3456 (Figure 3.6).

Circular alignment maps of AB030 and AB031 was obtained from using the CGView (Stothard and Wishart, 2005) application, a comparative genomics tool was used to visualize the sequence feature information. For each of these maps a BLAST comparison was done using AB030 and AB031 against each other; the clinical isolate AB307-0294 (GenBank accession no. CP001172), which was shown as the closest neighbor to both AB030 and AB031, and ATCC17978 (GenBank accession no. CP000521). The gaps in the BLAST comparison results indicate the unique regions in the AB030 and AB031 genomes. For the BLAST results rings, the overlapping hits appear as darker regions. The innermost two rings show GC content and GC skew respectively, which are plotted as a deviation from the average of the entire sequence.

Figure 3.5. Circular representation of genome of *A. baumannii* AB030. Circles display (from outside in order of) (i) open reading frames (>100 codons) in the clockwise and counterclockwise direction respectively; (ii) comparison with three selected genomes by BLAST (AB031, AB307-0294 and ATCC17978); (iii) GC content; and (iv) G-C skew.

Figure 3.5.

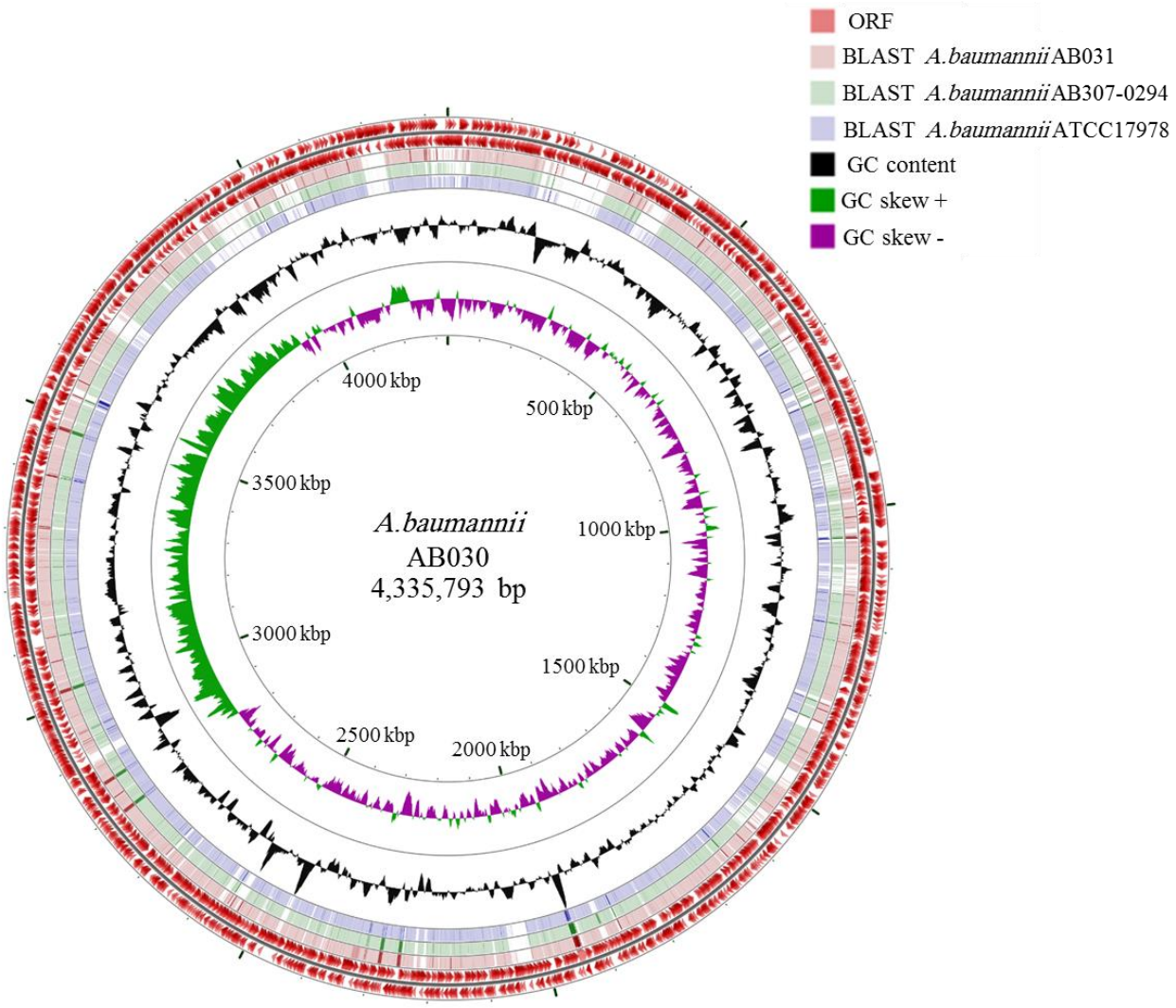
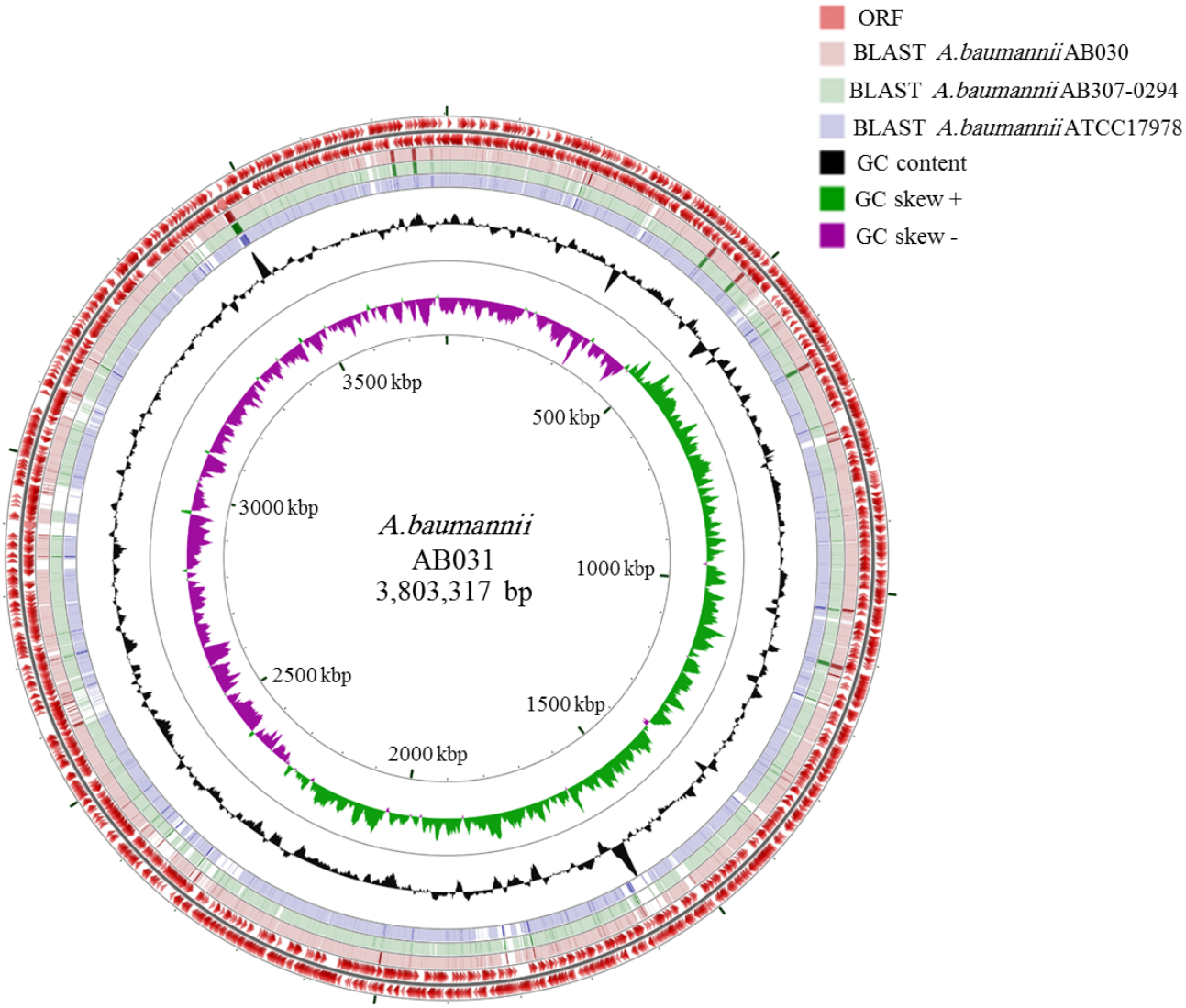

ORF
BLAST A.baumannii AB031
BLAST A.baumannii AB307-0294
BLAST A.baumannii ATCC17978
GC content
GC skew +
GC skew -
4000 kbp
500 kbp
3500 kbp
A.baumannii
AB030
4,335,793 bp
1000 kbp
3000 kbp
1500 kbp
2500 kbp
2000 kbp

Figure 3.6. Circular representation of genome of *A. baumannii* AB031. Circles display (from outside in order of) (i) open reading frames (>100 codons) in the clockwise and counterclockwise direction respectively; (ii) comparison with three selected genomes by BLAST (AB030, AB307-0294 and ATCC17978); (iii) GC content; and (iv) G-C skew.

Figure 3.6.


ORF
BLAST A.baumannii AB030
BLAST A.baumannii AB307-0294
BLAST A.baumannii ATCC17978
GC content
GC skew +
GC skew -
3500 kbp
500 kbp
3000 kbp
A.baumannii
AB031
3,803,317 bp
1000 kbp
2500 kbp
1500 kbp
2000 kbp

ii. Identification and comparison of TCSs in A. baumannii AB030 and A. baumannii AB031 genomes

The first Sensor kinase (SK) and response regulator (RR) genes in *A. baumannii* were identified in the two strains ATCC17978 and AB0057 (Adams *et al.,* 2008). A total of nineteen SK genes and seventeen RR genes were identified in the genome of the wild-type strain ATCC17978. Like in other bacterial species SKs and RRs were encoded by clusters of adjacent genes, sixteen putative clusters of complete TCSs genes were identified in ATCC17978. For the remaining SK or RR genes, their partner genes could not be predicted from genetic organization and, therefore, they were considered as orphan SKs or RRs. Five of the fifteen TCSs genes clusters were well characterized in ATCC17978: BfmRS, AdeRS, BaeSR, PmrAB, and GacSA.

BLAST search against the sequenced genome of AB030 identified seventeen SK genes and sixteen RR genes, and a total of 15 TCSs genes clusters were common between AB030 and AB031 and ATCC17978. In AB031, two additional TCSs gene clusters were identified, these two TCSs cluster were only common between AB031 and AB0057. Results are listed in Table 3.1.

The TCSs cluster *A1S_2937_38* was absent in AB030 and also missing in AB031. In AB031, two more TCSs gene clusters were identified that are homologous to *AB57_2542_43 and AB57_2550_51*, found only in the clinical isolate AB0057 (GenBank accession no. CP001182) (Table. 3.2).

Taken together, comparative genome analysis of the TCSs among these strains provides valuable insights into the MDR and virulence phenotype in *A. baumannii*.

Table 3.1. Summary of the absence and presence of putative two component systems genes in the genomes of *A. baumannii* AB030 and *A. baumannii* AB031. Closest orthologs are indicated in parenthesis. References are provided for gene products characterized in *A. baumannii*

***A. baumannii* ATCC17978**	**Gene name**	***A. baumannii* AB030**	***A. baumannii* AB031**
A1S_0232	response regulator (PilR)	*IX87_15710*	*IX88_03400*
A1S_0235	sensor histidine kinase (PilS)	*IX87_15730*	*IX88_03405*
A1S_0236	GacA (Cerqueira *et a.*, 2013)	*sirA*	*sirA*
A1S_0260	sensor histidine kinase (AlgZ)	*IX87_15850*	*IX88_03525*
A1S_0261	response regulator (AlgR)	*IX87_15855*	*IX88_03530*
A1S_2938	sensor kinase (CusS)	**-**	**-**
A1S_2937	response regulator (CusR)	**-**	**-**
A1S_0574	sensor histidine kinase (BarA)	*IX87_17370*	*IX88_05080*
A1S_0748	BfmS (Tomaras *et al*., 2008)	*IX87_18030*	*IX88_05725*
A1S_0749	BfmR (Tomaras *et al*., 2008)	*IX87_18025*	*IX88_05720*
A1S_1393	sensor histidine kinase	*IX87_03280*	*IX88_08935*
A1S_1394	response regulator	*IX87_03275*	*IX88_08940*
A1S_1753	AdeR (Marchand *et al.*, 2004)	*IX87_20165*	*IX88_10940*
A1S_1754	AdeS (Marchand *et al.*, 2004)	*IX87_20170*	**-***
A1S_1977	sensor histidine kinase (GlnL)	*IX87_21680*	*IX88_12105*
A1S_1978	response regulator (GlnG)	*glnG*	*glnG*
A1S_2006	response regulator (NasT)	**-***	*IX88_12260*
A1S_2137	response regulator (KdpE)	*IX87_06675*	*IX88_13225*
A1S_2138	sensor histidine kinase(KdpD)	*IX87_06680*	*IX88_13230*

A1S_3302	Sensor kinase	*IX87_13525*	*IX88_01290*
A1S_3304	response regulator	*IX87_13540*	*IX88_01300*
A1S_3375	response regulator (PhoB)	*IX87_13940*	*IX88_01695*
A1S_3376	sensor histidine kinase (PhoR)	*IX87_13945*	*IX88_01700*
A1S_2287	sensor histidine kinase (QseC)	*IX87_07540*	*IX88_14435*
A1S_2288	response regulator	*IX87_07545*	*IX88_14440*
A1S_2750	PmrA (Arroyo *et al.*, 2011)	*IX87_10330*	*IX88_16920*
A1S_2751	PmrB (Arroyo *et al.*, 2011)	*IX87_10335*	*IX88_16925*
A1S_2811	sensor histidine kinase	*IX87_10935*	*IX88_17240*
A1S_2814	sensor histidine kinase	*IX87_10950*	*IX88_17255*
A1S_2815	response regulator (Pil)G	*IX87_10955*	*IX88_17260*
A1S_2883	BaeS (Lin *et al.*, 2013)	*IX87_11300*	*IX88_17590*
A1S_2884	BaeR ((Lin *et al.*, 2013)	*IX87_11305*	*IX88_17595*
A1S_2906	sensor histidine kinase	*IX87_11440*	*IX88_17710*
A1S_3229	response regulator (OmpR)	*IX87_13010*	*IX88_00910*
A1S_3230	sensor histidine kinase (EnvZ)	*IX87_13015*	*IX88_00915*

-*, disrupted

-, absent

Table 3.2. The presence of putative two component systems genes in the genomes of *A. baumannii* AB031. Closest orthologs are indicated in parenthesis. References are provided for gene products characterized in *A. baumannii.*

***A. baumannii* AB0057**	**Gene name**	***A. baumannii* AB030**	***A. baumannii* AB031**
AB57_2542	sensor kinase	**-**	*IX88_13575*
AB57_2543	response regulator	**-**	*IX88_13580*
AB57_2550	heavy metal sensor kinase	**-**	*IX88_13615*
AB57_2551	transcriptional activator protein (IrlR)	**-**	*IX88_13620*

-, absent

iii. Identification and analysis of disruption of A1S_2006 in AB030.

Analysis of TCSs in the whole genome of *A. baumannii* AB030 demonstrated that *A1S_2006,* an orphan response regulator, was disrupted with 3902 bp DNA insertion in length to generate *A1S_2006* mutant strain, as shown in Figure 3.7. Blast search of this additional 3902 bp showed similarities (80%-90%) to insertion elements known among *A. baumannii* such as *ISAba1*element, *blaOXA-23* gene, transposon Tn*2006,* and transposon Tn*2008.*

iv. Identification and analysis of disrupted A1S_1754 (adeS) in AB031

A1S_1754 encodes for the sensor kinase of AdeRS system in *A. baumannii*. Analysis of TCSs in the whole genome of *A. baumannii* AB031 obtained an 1189 bp additional DNA insertion in the gene *A1S_1754 (adeS),* as shown in Figure 3.8. BLAST search of this additional 1189 bp additional DNA insertion showed 100% identity to the *ISAba1* insertion element in *A. baumannii* resulted in a disruption in *adeS.*

v. Analysis of the A1S_1753 (adeR) promoter region from A. baumannii AB031.

The promoter regions of five TCSs operons (*A1S_2006, A1S_1753_54, A1S_1977_77, 3229_30, and 2137_38*) from AB030 and AB031 were analyzed with the purpose of establishing possible differences between strains expressing and those not expressing the TCSs gene. Only *A1S_1753_54* (*adeRS*) promoter showed one nucleotide difference in *A. baumannii* AB031 which showed no expression of the *A1S_1753_54*. The alignment of the *A1S_1753_54* promoter sequences indicated that there is a one nucleotide change, as seen in Figure 3.12.

Figure 3.7. Schematic representation of the genetic organization on *A1S_2006* based on RAST server annotation. A) *A. baumannii* AB030 chromosome with the 3902 bp additional DNA insertion. B) *A. baumannii* ATCC17978 chromosome encoding for *A1S_2006* with neighboring genes.

Figure 3.7.

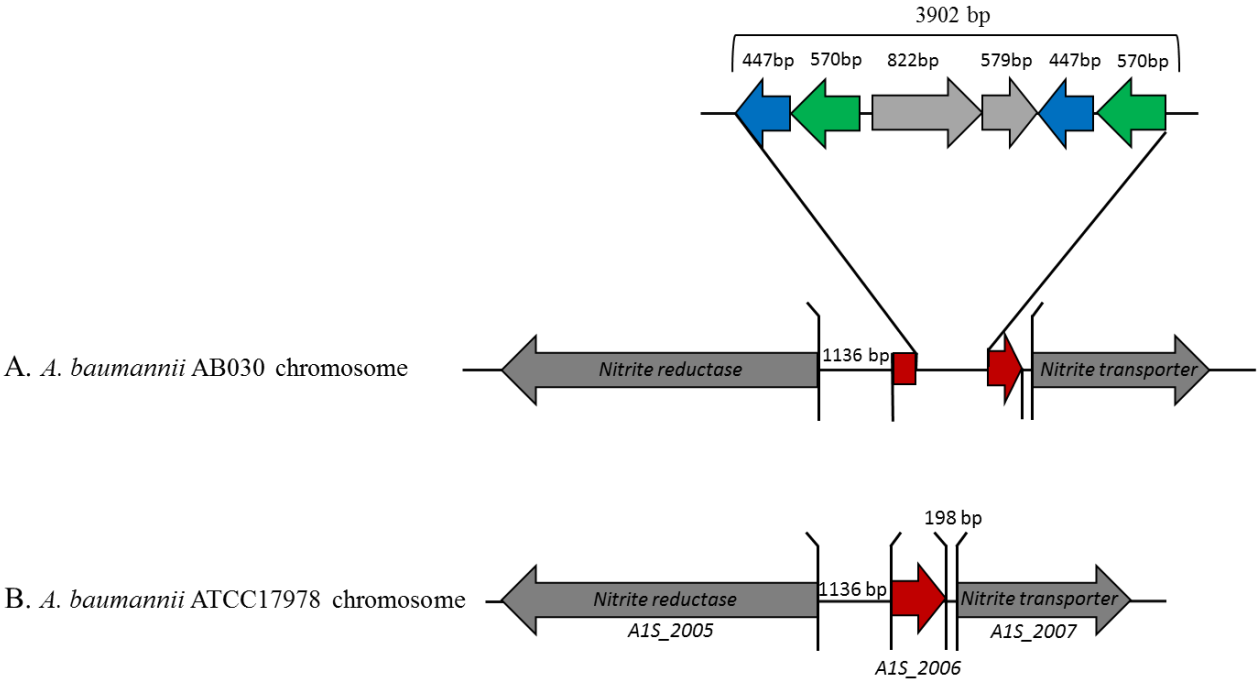

3902 bp
447bp
570bp
822bp
579bp
447bp
570bp
A. A. baumannii AB030 chromosome
Nitrite reductase
1136 bp
Nitrite transporter
198 bp
B. A. baumannii ATCC17978 chromosome
Nitrite reductase
A1S_2005
1136 bp
Nitrite transporter
A1S_2007
A1S_2006

Figure 3.8. Schematic representation of the genetic organization on *A1S_1753* based on RAST server annotation. A) *A. baumannii* AB031 chromosome with the 1189 bp additional DNA insertion. B) *A. baumannii* ATCC17978 chromosome encoding for *A1S_1753* with neighboring genes.

Figure 3.8.

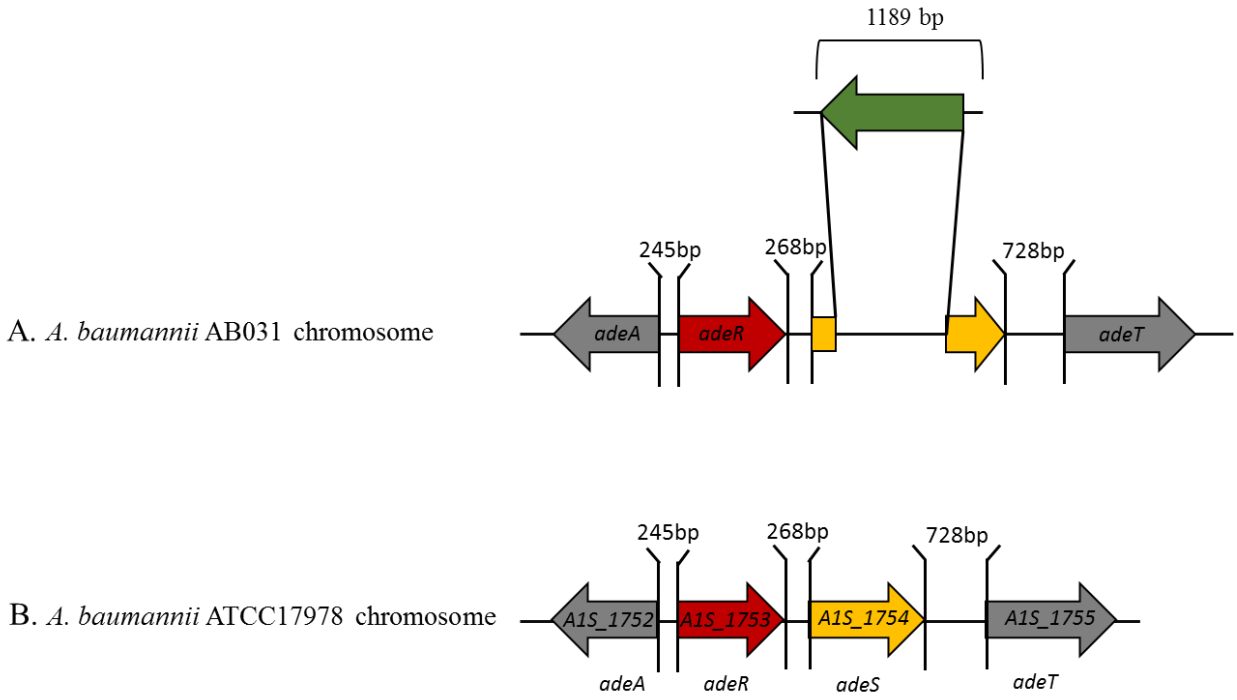
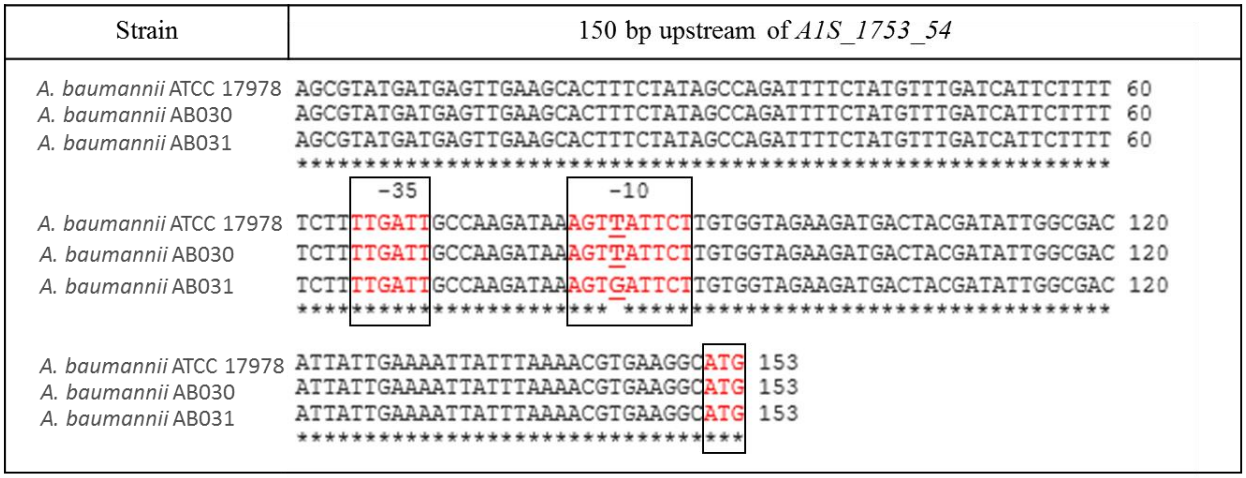

A. A. baumannii AB031 chromosome
1189 bp
245bp
268bp
728bp
adeA
adeR
adeT
B. A. baumannii ATCC17978 chromosome
245bp
268bp
728bp
A1S_1752
A1S_1753
A1S_1754
A1S_1755
adeA
adeR
adeS
adeT

Figure 3.9. Sequence alignment of *A1S_1753_54* (*adeRS*) promoter region from AB030, AB031, and ATCC17978. Strains AB030 and ATCC17978 express *A1S_1753*, and their sequences showed the presence of a Thymine (T) nucleotide in -10 motif, the nucleotide in this position is showed bold and underlined. AB031 showed expression of A1 gene presented a Guanidine (G) in this position instead. The -35 and -10 motifs of promoters are indicated in boxes, and prediction of promoter region was done using BPROM software.

Figure 3.9.


Strain
150 bp upstream of A1S_1753_54
A. baumannii ATCC 17978 AGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATTTTCTATGTTTGATCATTCTTTT 60
A. baumannii AB030 AGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATTTTCTATGTTTGATCATTCTTTT 60
A. baumannii AB031 AGCGTATGATGAGTTGAAGCACTTTCTATAGCCAGATTTTCTATGTTTGATCATTCTTTT 60
-35
-10
A. baumannii ATCC 17978 TCTTTTGATTGCCAAGATAAAGTTATTCTTGTGGTAGAAGATGACTACGATATTGGCGAC 120
A. baumannii AB030 TCTTTTGATTGCCAAGATAAAGTTATTCTTGTGGTAGAAGATGACTACGATATTGGCGAC 120
A. baumannii AB031 TCTTTTGATTGCCAAGATAAAGTGATTCTTGTGGTAGAAGATGACTACGATATTGGCGAC 120
A. baumannii ATCC 17978 ATTATTGAAAATTATTTAAAACGTGAAGGCATG 153
A. baumannii AB030 ATTATTGAAAATTATTTAAAACGTGAAGGCATG 153
A. baumannii AB031 ATTATTGAAAATTATTTAAAACGTGAAGGCATG 153

3.5 Attempts to create gene-knock out of *A1S_3229_30* in *Acinetobacter baumannii*

i. A1S_3229 expression and sequence analysis

qRT-PCR analysis of *A1S_3229* demonstrated an approximately 2-fold change of expression between *A. baumannii* AB030 and *A. baumannii* AB031 compared to the control strain *A. baumannii* ATCC17978 (Figure 3.4). In addition, *A1S_3229_30* operon shows 73% identity to the AmgRS system of *Pseudomonas aeruginosa* (Figure 3.5), making it a good candidate for this study.

ii. Generation of A1S_3229_30 deletion fragment

A gene-knock out fragment for *A1S_3229_30* operon was created using the technique described by Choi and Schweizer (2005) Annealing of the three fragments 3' and 5' ends from *A1S_3229_30* operon and *aacC1* gene is described in Fig 3.6.A allow the generation of the gene deletion fragment for *A1S_3229_30* operon, Δ*A1S_3229_30::*GmFRT, as seen in Fig. 3.6.C. The pUC18 plasmid was digested with *Sma*I, and ligated with Δ*A1S_3229_30::*GmFRT, to obtain the pPLS125 plasmid and transformed into DH5α competent cells.

Figure 3.10. *A1S_3229_30* sequence analysis. Alignment of amino acids sequences showing similarities of A1S_3229_30 to AmgRS and A1S_3229_30 shows 73% identity to the AmgRS system in *P. aeruginosa* PAO1. The amino acids underlined indicate the predicted phosphorylation site of A1S_3229_30 sensor kinase. The prediction of phosphorylation site was done using NetPhosBac 1.0 Server (Miller *et al.*, 2009).

Figure 3.10.

```
AB030          MDRLLQRELFSLIVLDFMLPVEDGLSICRRLRQSNIDTPIIMLTARGSDSDRIAGLEAGA 60
AB031          MDRLLQRELFSLIVLDFMLPVEDGLSICRRLRQSNIDTPIIMLTARGSDSDRIAGLEAGA 60
ATCC17978      MDRLLQRELFSLIVLDFMLPVEDGLSICRRLRQSNIDTPIIMLTARGSDSDRIAGLEAGA 60
PAO1           MDRLLSRELFQLVVLDLMLPGEDGLTACRRLREQNNQVPIIMLTAKGDEGSRIQGLELGA 60
               *****.****.*:***:*** ****: *****:.* :.*******:*.:..** *** **

AB030          DDYLPKPFNPNELLARIRAVLRRQVREVPGAPSQQVEVVSFGPWSLDLSTRTLTREGQIV 120
AB031          DDYLPKPFNPNELLARIRAVLRRQVREVPGAPSQQVEVVSFGPWSLDLSTRTLTREGQIV 120
ATCC17978      DDYLPKPFNPNELLARIRAVLRRQVREVPGAPSQQVEVVSFGPWSLDLSTRTLTREGQIV 120
PAO1           DDYLAKPFNPRELLARIKAVLRRQAPLVPGAPAGADEVVTFGDYQLFLATRELKKGDEVH 120
               ****.*****.******:******.  *****:   ***:** :.* *:** *.: .::

AB030          TLTTGEFAVLKALVQHPREPLTRDKLMNLARGREWGAMERSIDVQVSRLRRLIEDNPARA 180
AB031          TLTTGEFAVLKALVQHPREPLTRDKLMNLARGREWGAMERSIDVQVSRLRRLIEDNPARA 180
ATCC17978      TLTTGEFAVLKALVQHPREPLTRDKLMNLARGREWGAMERSIDVQVSRLRRLIEDNPARA 180
PAO1           MLTTGEFAVLKALVQHAREPLTRDKLMNLARGREWDALERSIDVQISRLRRLIEPDPSKP 180
                ***************.******************.*:*******:******** :*::.

AB030          RYIQTVWGVGYVFVPDGAEMRTTILVLLVVLFSLFMSLWFFWRTLYLPEIQQHARYLAVE 240
AB031          RYIQTVWGVGYVFVPDGAEMRTTILVLLVVLFSLFMSLWFFWRTLYLPEIQQHARYLAVE 240
ATCC17978      RYIQTVWGVGYVFVPDGAEMRTTILVLLVVLFSLFMSLWFFWRTLYLPEIQQHARYLAVE 240
PAO1           RYIQTVWGVGYVFVPDGNARKAMKTPLWFPQSFFARTLWLVLIVVLFSKALTLVYLLMNE 240
               *****************   ::    * .    :  :**:.  .: :.:    .  *  *

AB030          LELVNNPN-------IRIFHRDSEVDVDTWLRNRVGIEYVTNPKEYPSVRDKIIAEFFTN 293
AB031          LELVNNPN-------IRIFHRDSEVDVDTWLRNRVGIEYVTNPKEYPSVRDKIIAEFFTN 293
ATCC17978      LELVNNPN-------IRIFHRDSEVDVDTWLRNRVGIEYVTNPKEYPSVRDKIIAEFFTN 293
PAO1           DVIVDRQYSHGAALTIRAFWAADE-ESRAAIAKASGLRWVPSSADQPGEQHWPYTEIFQR 299
                 :*:.         ** *   .* :  : : :  *:.:*... : *. :.   :*:* .

AB030          QVEQKLANEIGAKDVTVYFQFKPSPRIWIQTPEMHGNWVREPLKTYANYSPELL--VGWV 351
AB031          QVEQKLANEIGAKDVTVYFQFKPSPRIWIQTPEMHGNWVREPLKTYANYSPELL--VGWV 351
ATCC17978      QVEQKLANEIGAKDVTVYFQFKPSPRIWIQTPEMHGNWVREPLKTYANYSPELL--VGWV 351
PAO1           QMQMELG-----PDTETRLRIHQPSQLWVRAPSLGEGWLAVPLYPHPLRGQRIWSVLGWF 354
               *:: :*.      *. . :::: ..::*:::*.:  .*:  ** .:.  . .:   :**.

AB030          VGVPLVSAIIILILVRQMNRPLRRLQNAANEYSKSGTAPYLDTNHGPLEIRQVNQAFNRM 411
AB031          VGVPLVSAIIILILVRQMNRPLRRLQNAANEYSKSGTAPYLDTNHGPLEIRQVNQAFNRM 411
ATCC17978      VGVPLVSAIIILILVRQMNRPLRRLQNAANEYSKSGTAPYLDTNHGPLEIRQVNQAFNRM 411
PAO1           LGIGLLSTAAAWIFVRQLSQPLKRLVVAARQFGQGRSVRLPLGPETPSEMAEVYRAFNQM 414
               :*: *:*:    *:***:.:**:**  **.::.:. :.      . * *: :* :***:*

AB030          VYTLEQTERERRIMLAGISHDLRTPLTRIRLTAEMLP--DEFLREGLVYDVDDMDAILNQ 469
AB031          VYTLEQTERERRIMLAGISHDLRTPLTRIRLTAEMLP--DEFLREGLVYDVDDMDAILNQ 469
ATCC17978      VYTLEQTERERRIMLAGISHDLRTPLTRIRLTAEMLP--DEFLREGLVYDVDDMDAILNQ 469
PAO1           AEDIEQGGRERELMLAGVSHDLRTPLTRLRLSLELLPESEREMVEDMIRDIEDMDAILDQ 474
               .  :**  ***.:****:**********:**: *:**  :. : *.:: *::******:*

AB030          FISYMRDGSDEELKDTNINILLQELVVQFKP--LDIRFNMQDVPIIPARSLSLKRLIANL 527
AB031          FISYMRDGSDEELKDTNINILLQELVVQFKP--LDIRFNMQDVPIIPARSLSLKRLIANL 527
ATCC17978      FISYMRDGSDEELKDTNINILLQELVVQFKP--LDIRFNMQDVPIIPARSLSLKRLIANL 527
PAO1           FLAFIRDGRDEPVEEGDLTDLVREVVAPFNQTREQVRMALQPVPAMPLRRVSMKRLLGNL 534
               *:::::** ** ::: ::. *::*:*. *:    ::*: :* ** :* * :*:***:.**

AB030          INNAKRYGAEPIELSAKVENE----HILITVADHGEGIPEDQIEELMQPFVRGNSARTIQ 583
AB031          INNAKRYGAEPIELSAKVENE----HILITVADHGEGIPEDQIEELMQPFVRGNSARTIQ 583
ATCC17978      INNAKRYGAEPIELSAKVENE----HILITVADHGEGIPEDQIEELMQPFVRGNSARTIQ 583
PAO1           IDNALNHGGGSVEVASYVAGESAAPYVVLSVLDRGQGIDPAEVDSIFNPFIRGDKARGGK 594
               *:** .:*. .:*::: * .*    :::::* *:*:**   :::.:::**:**:.**  :
```

```
AB030           GSGLGLAIVKRIVDIHQGEIQIHNREQGGLEVIISLPIPKPAPEENTTHNPLEKIKQTLS 643
AB031           GSGLGLAIVKRIVDIHQGEIQIHNREQGGLEVIISLPIPKPAPEENTTHNPLEKIKQTLS 643
ATCC17978       GSGLGLAIVKRIVDIHQGEIQIHNREQGGLEVIISLPIPKPAPEENTTHNPLEKIKQTLS 643
PAO1            GTGLGLAIVKRIAAQHGGSVELRNRDGGGLEARVCLPLGLLLPRGAA------------- 641
                *:**********.  * *.::::**: ****. :.**:    *.  :

AB030           ERF 646
AB031           ERF 646
ATCC17978       ERF 646
PAO1            ---
```

(-) Gaps/insertions

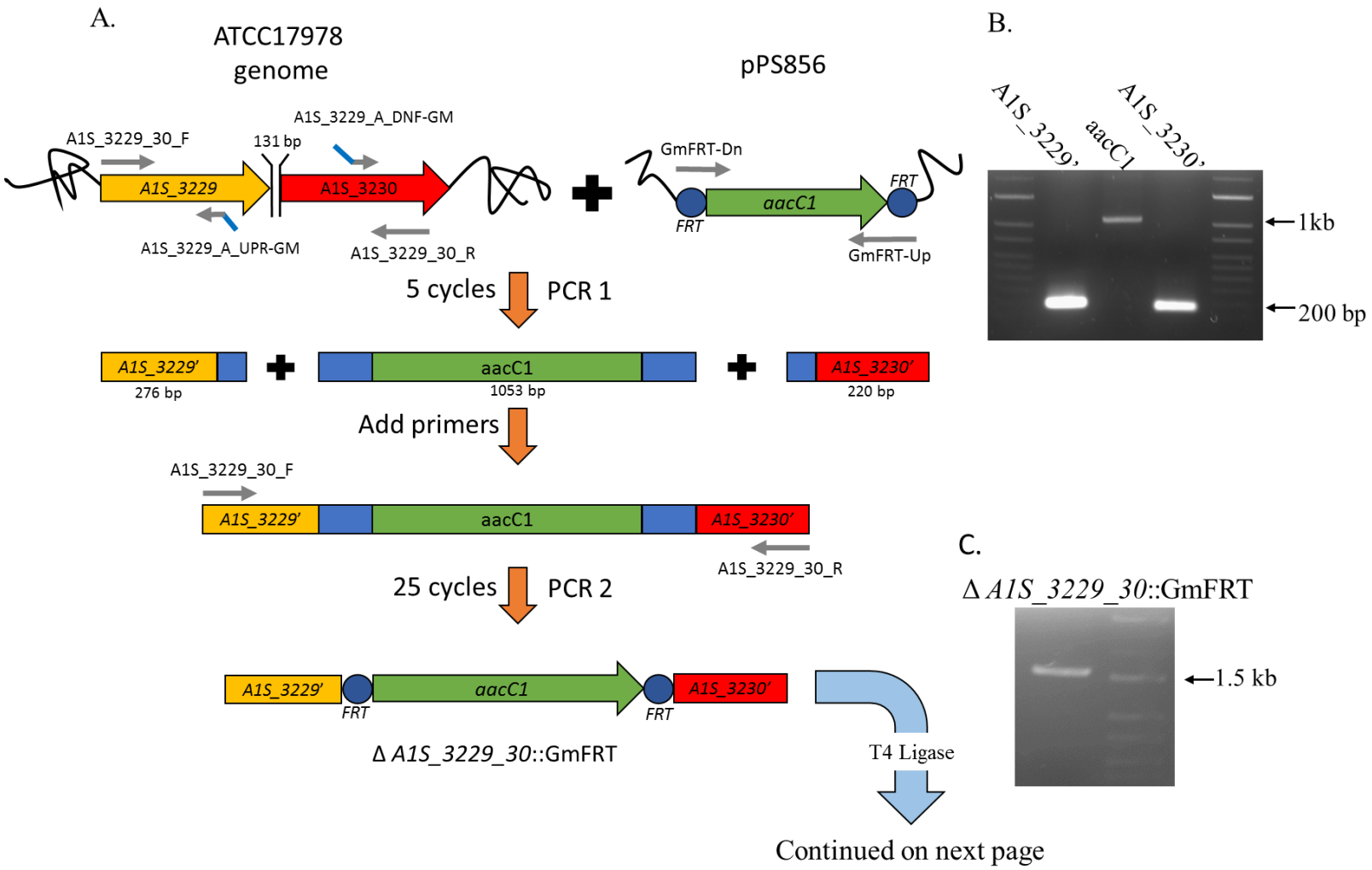
(*) Identical residue

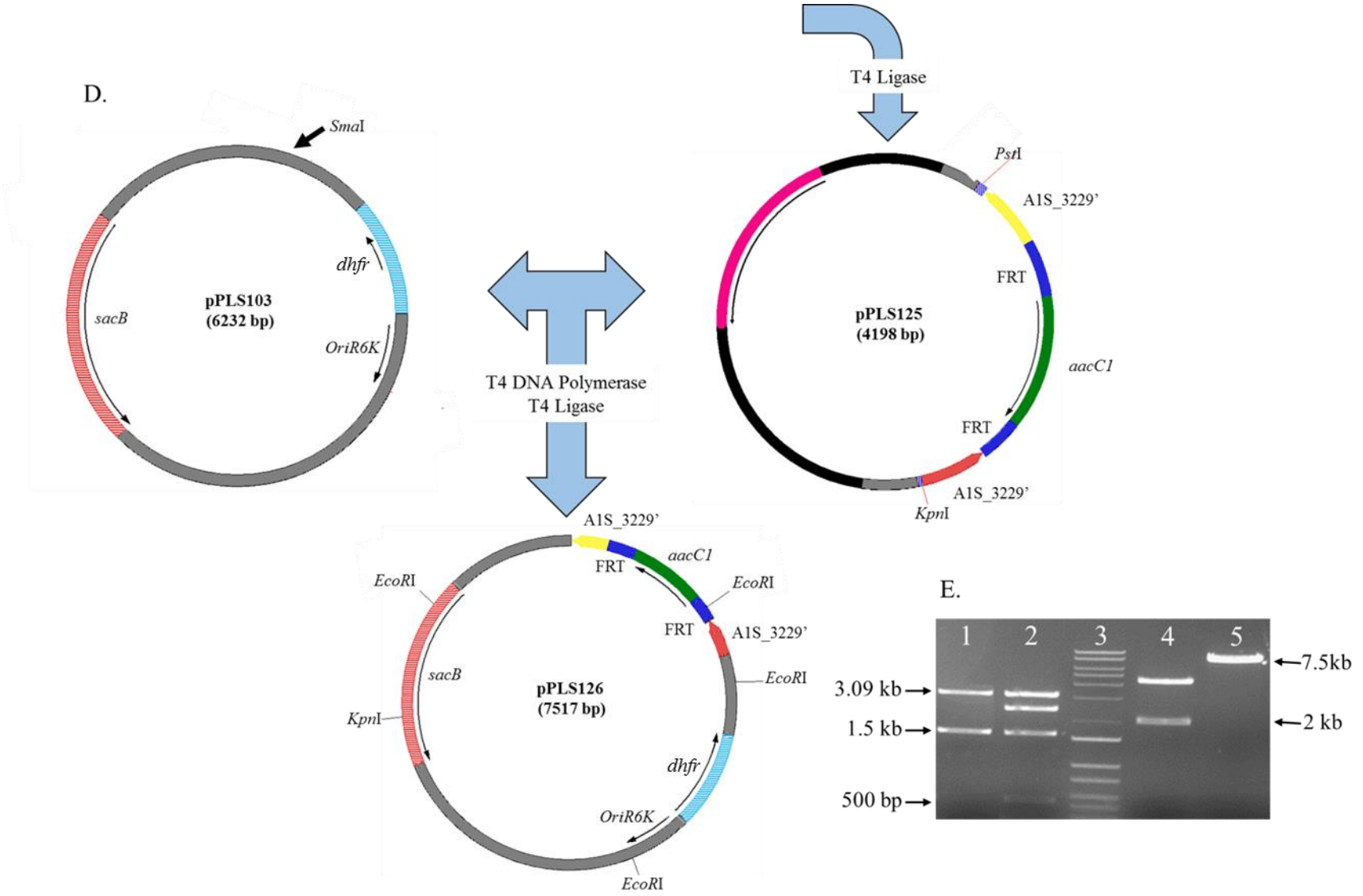
(:) Strongly similar residue (scoring >0.5 in the Gonnet PAM matrix)

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

Figure 3.11. Schematic illustration *A1S_3229_30* knock out fragment generation by overlap extension PCR. During the first round PCR (PCR1), a 276 bp fragment from the 5' end and 220 bp fragment from the 3' ends of *A1S_2339_30* were annealed together with *aacC1* using the splicing overlap extension reaction as described in Figure 3.6.A and B. The 1511 bp assembled fragment was then amplified using full length primers A1S_3229_30_F and A1S_3229_30_R. The *A1S_3229_30* operon deletion fragment obtained from the SOEing PCR reaction shown in Fig. 3.6.C was ligated into pUC18 plasmid digested with *Sma*I restriction enzyme. Δ*A1S_3229_30::*GmFRT operon deletion fragment obtained from pPLS125 was ligated into pPLS103 plasmid digested with *Sma*I restriction enzyme. Verification of the plasmid composition was done by restriction digestion using *EcoR*I resulting in 3097 bp, 2280 bp, 1565 bp, and 575 bp sizes as seen in Fig. 3.6.E. E) Lane 1, pPLS103 digested with EcoRI, and expected sizes are 3097 bp, 1570 bp, and 1565 bp. Lane 2, pPLS126 digested with EcoRI, and expected sizes are 3097 bp, 2280 bp, 1565 bp, and 575 bp. Lane 3, 10 kb DNA marker, Lane 4, pPLS103 digested with *Kpn*I, and expected sizes are 2001 bp, and 4231. Lane 2, pPLS126 digested with *Kpn*I, and expected size is 7517 bp.

Figure 3.11.


A.
ATCC17978
genome
pPS856
A1S_3229_30_F
131 bp
A1S_3229_A_DNF-GM
A1S_3229
A1S_3230
A1S_3229_A_UPR-GM
A1S_3229_30_R
GmFRT-Dn
aacC1
FRT
FRT
GmFRT-Up
5 cycles
PCR 1
A1S_3229'
276 bp
aacC1
1053 bp
A1S_3230'
220 bp
Add primers
A1S_3229_30_F
A1S_3229'
aacC1
A1S_3230'
A1S_3229_30_R
25 cycles
PCR 2
A1S_3229'
aacC1
A1S_3230'
FRT
FRT
Δ A1S_3229_30::GmFRT
T4 Ligase
Continued on next page
B.
A1S_3229'
aacC1
A1S_3230'
1kb
200 bp
C.
Δ A1S_3229_30::GmFRT
1.5 kb


D.
SmaI
dhfr
sacB
pPLS103
(6232 bp)
OriR6K
T4 Ligase
PstI
A1S_3229'
FRT
pPLS125
(4198 bp)
aacC1
FRT
A1S_3229'
KpnI
T4 DNA Polymerase
T4 Ligase
A1S_3229'
aacC1
FRT
EcoRI
EcoRI
FRT
A1S_3229'
sacB
pPLS126
(7517 bp)
EcoRI
KpnI
dhfr
OriR6K
EcoRI
E.
1
2
3
4
5
7.5kb
3.09 kb
2 kb
1.5 kb
500 bp

iii. Construction of pPLS126 the suicide plasmid containing ΔA1S_3229_30::GmFRT

The suicide plasmid pPLS103 was digested with *Sma*I, and the Δ*A1S_3229_30::*GmFRT deletion fragment was obtained from pPLS125 digested by *Kpn*I and *Sph*I and ligated to Δ*A1S_3229_30::*GmFRT, to obtain the pPLS126 plasmid and transformed into MaH1 competent cells. To confirm the identity of the construct screening was performed by restriction enzymes using *EcoR*I in a single digestion and the product of this digestion was a 3097 bp, 2280 bp, 1565 bp, and 575 bp. Another single digestion was performed by *Kpn*I, and the product of this digestion was a 7517 bp.

4. DISCUSSION AND CONCLUSIONS

4.1 Expression of two component systems in clinical isolates of *Acinetobacter baumannii* AB030 and AB031.

It has been reported in different pathogens that a variety of two component systems regulate complex antibiotic resistance and virulence processes (Gooderham and Hancock, 2009; Merighi *et al.,* 2006; Stephenson and Hoch, 2002). Therefore, identification and development of new inhibitors of TCSs as potential targets, especially considering the fact they are not present in mammalian cells, provide new potentially therapeutic options for the treatment of drug-resistant bacterial infections. A few studies have characterized TCSs and their role in MDR *A. baumannii* (Tomaras *et al.,* 2008; Marchand *et al.,* 2004; Lin *et al.,* 2013; Cerqueira *et al.,* 2013; Arroyo *et al.,* 2011); however, a number of putative two component systems encoded in the genome of *A. baumannii* that await detailed characterization. It is likely that some of these uncharacterized systems have global regulatory mechanisms that regulate antibiotic resistance and virulence processes in MDR *A. baumannii*. For this purpose, this research analyzed the expression of TCSs response regulators (characterized as well as yet uncharacterized) in two clinical isolates from Canadian hospitals, which display very different antibiotic susceptibility profiles (Fernando *et al.,* 2013). The analysis observed differential expressions of six TCSs response regulators in both clinical isolates AB030 and AB031, these genes are *bfmR* (*A1S_0748_49*), *adeRS* (*A1S_1753*), *A1S_1978*, *A1S_2137*, *A1S_2006*, and *A1S_3229*. *bfmR* has been shown to play a role in *A. baumannii* virulence (Tomaras *et al.,* 2008), and *adeRS* was reported to play a major role in antibiotic resistance (Marchand *et al.,* 2004), suggesting the involvement of TCSs in the pathogenicity and antibiotic resistance of *A. baumannii*. By analyzing the expression of as yet uncharacterized TCSs in two blood isolates AB030 and AB031, we aim to identify and characterize novel TCSs that act as global regulators of antibiotic resistance and virulence in the MDR *A. baumannii*.

The ability of *A. baumannii* to generate biofilms represents an important virulence factor as it contributes to the pathogen's ability to survive in the host and the environment, and develop resistance to antibiotics (Roca *et al.,* 2012). Biofilm formation is controlled by BfmRS in *A. baumannii* ATCC19606. It has been reported that inactivation of the TCSs BfmRS abolishes the biofilm formation (Tomaras *et al.,* 2008). Interestingly, *bfmR* showed a higher expression in the clinical isolate AB030 than in AB031; however, preliminary virulence phenotypic analysis of AB030 and AB031 revealed that AB031 forms more biofilm than AB030 (Kumar, unpublished). In addition, genomic analysis showed that BfmRS was highly conserved among ATCC17978, AB030 and AB031 genomes as seen in Appendix 1. Therefore, our data suggest that other factors are involved in the formation of biofilm and regulation of other virulence factors.

The expression of AdeABC efflux pump is tightly regulated by the two component systems AdeRS in *A. baumannii*. The *adeRS* (*A1S_1753_54*) operon is located upstream of *adeABC* operon and is transcribed divergently. The efflux pump AdeABC is responsible for resistance to aminoglycosides, tetracycline, erythromycin, chloramphenicol, trimethoprim, and fluoroquinolones. Disruption of *adeR* and *adeS* genes by insertional mutagenesis resulted in increased susceptibility of the mutant strains of *A. baumannii* to aminoglycosides and other substrates of the pump (Marchand *et al.*, 2004), indicating that the AdeRS system is a positive regulator of the AdeABC pump.

Differences in the expression of the pump AdeABC were reported in both clinical isolates AB030 and AB031, and it was found that the expression level of AdeABC was about 4-fold higher in AB030 than in AB031 (Fernando *et al.,* 2013). Overexpression of the response regulator *adeR* was observed in AB030, suggesting that overexpression of AdeRS system results in overexpression of AdeABC efflux pump which may at least partly explain the MDR

phenotype in AB030. In addition to the overexpression of the AdeRS system, mutations in *adeS* or *adeR* also contribute to AdeABC overexpression (Marchand *et al.,* 2004). We found novel mutations in *adeR* gene that encodes for AdeR (response regulator) in AB030 as seen in Appendix 2, and it is possible that these novel mutations played a role in the observed overexpression of *adeR (A1S_1753)* in AB030 but still; however, further analysis of these point mutations is required in order to validate any of these explanations.

On the other hand, *adeR* expression was not observed in AB031 and it likely to be reason for the lower expression of AdeABC efflux pump in comparison to AB030. However, the expression of AdeABC in AB031 is still higher than that in ATCC17978 suggesting that the regulation of *adeABC* operon expression is complicated, and not merely dependent on the AdeRS system. The analysis of the predicted sigma70 promoter region of *adeR* (*A1S_1753*) in AB031 showed a G→T substitution in the predicted -10 motif (Figure 3.9), this could affect the binding of the DNA-dependent RNA-polymerase to DNA modulating the gene transcription of *adeR* (*A1S_1753*). In addition, analysis of *adeRS* (*A1S_1753_54*) sequence in AB031 showed 1189 bp additional DNA insertion in the gene *A1S_1754* (*adeS*) as seen in Figure 3.8, and it is likely that this additional DNA insertion could result in a truncated, putatively non-functional, *adeS* sensor kinase. Further studies are necessary to determine the role of AdeRS system in the regulation of the AdeABC efflux pump in AB031, and possibly characterize regulator(s) that play a role in regulation of AdeABC efflux pump other than AdeRS system.

In addition to the AdeRS system, overexpression of the orphan TCSs response regulator *A1S_2006* was observed in AB030. However, the whole-genome sequencing analysis in of TCSs in AB030 revealed the presence of 3902 bp DNA insertion in length to generate *A1S_2006* mutant strain, as seen in Figure 3.7. It is evident that this insertion renders *A1S_2006* non-

functional. Interestingly, we still observed an overexpression of *A1S_2006* in AB030 and it is because the primer binding sites for the qRT-PCR are present downstream of the insertion element, and that the qRT-PCR was detecting the truncated mRNA. However, this finding also suggests that *A1S_2006* is likely to be an interesting candidate to further characterize in order to study its role in antibiotic resistance and virulence of *A. baumannii*.

Since both AB030 and AB031 are clinical isolates and prior to this study no information was available about their genomes, we decided to sequence both strains in order to characterize the TCSs in these strains. Comparative whole-genome analysis is an effective way to understand the genetic features that have been acquired, modified, or lost, and helped *A. baumannii* to evolve and adapt to specific environmental niches. Thus, the whole-genome analysis of TCSs in the clinical isolates AB030 and AB031 can provide insights about the mechanisms that are responsible for their adaptations and pathogenesis. Our data revealed the presence of fifteen TCSs genes clusters, these clusters were found to be common between AB030 and AB031 and ATCC17978, and the TCSs cluster *A1S_2937_38* was absent from AB030 and AB031. Two more TCSs gene clusters were identified *AB57_2542_43* and *AB57_2550_51* and these two TCSs were only common between a previously sequenced bloodstream isolate AB0057 (NCBI: NC_011586) and AB031. This comparative genomic analysis allowed the identification of a core of complete TCSs protein orthologs among these two clinical isolates. The whole-genome sequencing analysis of AB030 and AB031 identified point mutations in *A1S_2137_38* operon (Appendix. 3), *A1S_1977_78* operon (Appendix. 4), and *A1S_1573_74* operon in AB030 (Appendix. 2) that could alter the activities of these TCSs in AB030 and AB031. Further genomic investigation of these point mutations is important in order to clarify the role of TCSs in MDR *A. baumannii*.

Little is known about TCSs in *A. baumannii*, and the results obtained during this study will be helpful in understanding the multi-drug resistance phenotype in *A. baumannii*, and uncover the essential role of TCSs in regulating antibiotics resistance and virulence in *A. baumannii*.

4.2 Identification and characterization of the two component system *A1S_3229_30.*

Expression analysis of TCSs from the clinical isolates AB030 and AB031 observed differential expressions of five TCSs response regulators. BLAST search in other bacterial species did not show any significant homology for these four genes (*A1S_1753_54*, *A1S_1977_78*, *A1S_2006*, and *A1S_2137_38)*, suggesting that these genes are only unique to *Acinetobacter* species. On the other hand, A1S_3229_30 showed 73% identity to the AmgRS operon that encodes the characterized AmgRS system of *P. aeruginosa* as seen in Figure 3.5.

The TCSs AmgRS in the pathogen *P. aeruginosa* confers resistance to aminoglycoside antibiotics and is required for the virulence of the problematic pathogen *P. aeruginosa*. Screening for small molecules to inhibit AmgRS system is expected to provide the dual benefit of compromising infection directly as well as enhancing antibiotic sensitivity (Lee *et al.,* 2009). This finding is crucial and supports the idea of identifying a novel TCSs for a new drug discovery, this also indicates that A1S_3229_30 system in *A. baumannii* is an excellent candidate for further characterization, since that the sequence of *A1S_3229_30* was highly conserved among the wild-type ATCC17978, AB030, and AB031.

4.3 Creation of a gene-knock out of the two component systems operon *A1S_3229_30.*

Genetic characterization of the A1S_3229-30 system in *A. baumannii* will require the creation of gene knock-out as the first step. One of the challenges with creating knock-outs in *A.*

baumannii in general is the lack of proper genetic tools. In addition to this, creation of knock-outs in clinical isolates has its own problems because of their high resistance to antibiotics that are used to selection. For example, the plasmid pKNG101 (Kaniga *et al.,* 1991), which is widely used for creating knock-outs in Gram-negative bacteria, cannot be used in ATCC17978, AB030, or AB031 because of their resistance to streptomycin which is the selection marker present on this plasmid. In order to investigate of the role *A1S_3229_30* a creation of a deletion mutant of *A1S_3229_30* is essential. Since, there are relatively few proper genetic tools for manipulation of chromosomal genes in *A. baumannii,* construction of two suicide plasmids (trimethoprim-resistant, pPLS103 and tetracycline-resistant, pPLS109) was carried for allelic replacement in *A. baumannii*. There are various advantages associated with the use of these plasmids; it contains multiple cloning sites and the presence of *sacB* gene that works as a counterselection marker enabling the curing of the plasmid (Choi and Schweizer, 2005).

A gene deletion construct for *A1S_3229_30* was prepared using the PCR-based method (SOEing) as shown in Figure 2.1. This product was cloned into the suicide vector pPLS103 (Figure 3.8) to be used as delivery plasmid. However, repeated attempts to delete *A1S_3229_30* in the chromosome of the wild-type strain ATCC17978 were not successful. It is possible that the plasmid gets incorporated into another region of the chromosome other than targeted region. In addition, the whole genome sequencing showed the presence of aminoglycoside resistance gene *aac(3)-IIa* was detected in the chromosome of AB030, thus explaining the failure to isolate conjugants with the plasmid insertion at the desired site of *A1S_3229_30*. This problem can be alleviated by using a different antibiotic resistance marker, for example kanamycin or by using an unmarked truncated gene for gene deletion.

In summary, the purpose of this study was to identify and characterize TCSs in *A. baumannii* that are likely to act as global regulators of antibiotic resistance and/or virulence. This study resulted in the successful cloning of thirteen different TCSs-encoding genes. Using two clinical isolates and comparative genomics and transcriptomics, this study was able to identify five novel TCSs in *A. baumannii* that may be involved in regulating virulence and antibiotic resistance. Also of interest are *A1S_2006,* which was found to contain an insertion sequence in AB030 but not in AB031. This gene would be a good candidate for further characterization. Also of note is the data generated on AdeRS system in combination with the previously published data on the expression of AdeABC pump (Marchand *et al.*, 2004), which is regulated by the AdeRS system. This study shows that the expression of this efflux pump can be regulated independent of the AdeRS system as seen in AB031. Another important candidate identified in this study is the A1S_3229_30, a close homolog of the AmgRS system in *P. aeruginosa,* which is known to regulate antibiotic resistance and virulence in this organism.

While the confirmation of the role of these five TCSs identified in this study can only be achieved by creating gene knock-outs, this study also resulted in the creation of new and versatile plasmids that will facilitate these studies.

6. FUTURE DIRECTIONS

The creation of a deletion mutant of *A1S_3229_30* is important in order to characterize the role of this system by using an alternative strategy to delete *A1S_3229_30* operon by disrupting the operon with different antibiotic resistance markers such as kanamycin or neomycin. This could increase the ability of the construct to recombine into the chromosome, and enable for better selection of the single recombination event in both the wild-type strain ATCC17978, AB030, and AB031. Followed by expression analysis experiment such as qRT-PCR to clarify if this system is playing a role in the regulation of RND efflux pumps, porins, or if this system is involved in the regulation of characterized virulence factors like OmpA, PhosD. Further virulence phenotypic analysis is required such as biofilm formation assay, and motility assay. Minimum inhibitory concentration assay using clinical antibiotics such as aminoglycosides is essential to determine if the deletion mutant strain of *A1S_3229_30* exhibit susceptibility phenotype to these drugs.

The whole-genome analysis of AB030 observed 3902 bp DNA insertion in length to generate AB030 *A1S_2006* mutant strain. The full length gene with its native promoter is cloned into pGEMT-Easy commercial vector, and the recombinant plasmid is named pPLS105. Cloning the full length gene with its own promoter into an *A. baumannii* overexpression vector and introducing it to AB030 to complement the *A1S_2006* mutant in AB030 is an interesting experiment. Followed by phenotypic experiments such as MIC and qRT-PCR, biofilm formation assay, this possibly can give a better insight of the role of *A1S_2006* in AB030 MDR phenotype.

Furthermore, AB030 and AB031 also provide an excellent model to understand seemingly complex regulation of the AdeABC pump. Since, AB031 overexpresses the AdeABC pump in spite of an insertion element in the *adeR* gene, it would be interesting to complement the AB031 strain with the intact copy of the *adeRS* genes in order to determine the extent of role

AdeRS system plays in the overexpression of the AdeABC pump. Similarly, deletion of the AdeRS system in AB030 will show whether the overexpression of the AdeABC system in this strain solely due to the activity of the AdeRS system.

To summarize, more research is required in order to understand the global mechanisms of TCSs in the regulation of MDR and virulence phenotypes. All this information is essential to understand and resolve the role of TCSs in increasing resistance and virulence in MDR *A. baumannii*. This research has provided exciting results to understand the role of TCSs in *A. baumannii*, nevertheless more research is required before this information can be interpreted into development of treatments against MDR *A. baumannii*.

7. LITERATURE CITED

Adams MD, Goglin K, Molyneaux N, Hujer KM, Lavender H, Jamison JJ and Gill SR. 2008. Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *Journal of Bacteriology*, *190*:8053–64

Afzal-Shah M, Woodford N and Livermore DM. 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

Alsan M and Klompas M. 2010. *Acinetobacter baumannii*: an emerging and important pathogen. *Journal of Clinical Outcomes Management, 17*:363-369

Antunes LCS, Imperi F, Carattoli A and Visca P. 2011. Deciphering the multifactorial nature of *Acinetobacter baumannii* pathogenicity. *PloS One*, *6*:e22674

Aranda J, Pozal M, Pardo BG, Rumbo S, Rumbo C, Parreira JR, Rodríguez-Velo P and Bou G. 2010. A rapid and simple method for constructing stable mutants of *Acinetobacter baumannii. BMC Microbiology, 10*:279

Arroyo L, Herrera CM, Fernandez L, Hankins JV, Trent MS and Hancock REW. 2011. The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrobial Agents and Chemotherapy*, *55*:3743–51

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A and Zagnitko O. 2008. The RAST Server: rapid annotations using subsystems technology. *BMC Genomics, 9*:75

Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M and Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of

Acinetobacter baumannii mediated by the *pmrAB* two-component regulatory system. *Antimicrobial Agents and Chemotherapy*, *55*:3370–9

Beier D and Gross R. 2006. Regulation of bacterial virulence by two-component systems. *Current Opinion in Microbiology*, *9*:143–152

Magnet S, Courvalin P and Lambert T. 2001. Resistance-Nodulation-Cell Division-Type Efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrobial Agents and Chemotherapy*, *45:*3375-3380

Catel-Ferreira M, Coadou G, Molle V, Mugnier P, Nordmann P, Siroy A, Jouenne T and Dé E. 2011. Structure-function relationships of CarO, the carbapenem resistance-associated outer membrane protein of *Acinetobacter baumannii*. *The Journal of Antimicrobial Chemotherapy*, *66*:2053–6

Catel-Ferreira M, Nehméa R, Molle V, Aranda J, Bouffartigues E, Chevalier S, Bouc G, Jouennea T and Dé E. 2012. Deciphering the function of the outer membrane protein OprD homologue of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, *56*:3826–32

Cayo R, Rodríguez MC, Espinal P, Fernández-Cuenca F, Ocampo-Sosa A, Pascual A, Ayala AA, Vila J and Martínez-Martínez L. 2011. Analysis of genes encoding penicillin-binding proteins in clinical isolates of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, *55*:5907–13

Cerqueira GM, Kostoulias X, Khoo C, Aibinu I, Qu Y, Traven A, and Peleg AY. 2014. A global virulence regulator in *Acinetobacter baumannii* and its control of the phenylacetic acid catabolic pathway. *The Journal of Infectious Diseases*, *210*:46-55

Centers for Disease Control and Prevention (CDC). 2013. Antibiotic resistance threats in the United States, 2013<http://www.cdc.gov/drugresistance/threatreport-2013/pdf/ar-threats-2013-508.pdf>, accessed May 23, 2014

Choi, KH and Schweizer HP. 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiology*, *5*:30-41

Cisneros JM, Reyes MJ, Pachón J, Becerril B, Caballero FJ, García-Garmendía JL, Cobacho R. 1996. Bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical findings, and prognostic features. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *22*:1026–32

Clark RB. 1996. Brief reports imipenem resistance among *Acinetobacter baumannii* : association with reduced expression of a 33-36 kDa outer membrane protein. *Journal of Antimicrobial Chemotherapy*, *38*:245-251

Dalebroux ZD and Miller SI. 2014. Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity. *Current Opinion in Microbiology*, *17*:106–13

Damier-Piolle L, Magnet S, Brémont S, Lambert T and Courvalin P. 2008. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii. Antimicrobial Agents and Chemotherapy*, *52*:557–62

Del Mar Tomas M, Cartelle M, Pertega S, Beceiro A, Llinares P, Canle D and Bou G. 2005. Hospital outbreak caused by a carbapenem-resistant strain of *Acinetobacter baumannii*: patient prognosis and risk-factors for colonization and infection. *Clinical Microbiology and Infection*, *11*:540–6

Dijkshoorn L, Nemec A and Seifert H. 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nature Reviews. Microbiology*, *5*:939–51

Dorsey CW. 2003. Genetic organization of an *Acinetobacter baumannii* chromosomal region harbouring genes related to siderophore biosynthesis and transport. *Microbiology*, *149*:1227–1238

Drawz SM, Bethel CR, Doppalapudi VR, Sheri A, Pagadala SRR, Hujer A. Skalweit MJ, Anderson VE, Chen SG, Buynak JD and Bonomo R. 2010. Penicillin sulfone inhibitors of class D beta-lactamases. *Antimicrobial Agents and Chemotherapy*, *54*:1414–24

Fernández-Reyes M, Rodríguez-Falcón M, Chiva C, Pachón J, Andreu D and Rivas L. 2009. The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. *Proteomics*, *9*:1632–45

Fernando D, Zhanel GG and Kumar A. 2013. Antibiotic resistance and expression of resistance-nodulation-division pumps and outer membrane porins in *Acinetobacter* spp isolated from Canadian hospitals. *Canadian Journal of Infectious Diseases and Medical Microbiology*, *24*: 17-21

Gaddy JA and Actis LA. 2009. Regulation of *Acinetobacter baumannii* biofilm formation. *Future Microbiology*, *4*:273-278

Galdiero S, Falanga A, Cantisani M, Tarallo R, Elena M, Pepa D, D'Oriano V and Galdiero M. 2012. Microbe-Host interactions : structure and role of gram-negative bacterial porins. *Current Protein and Peptide Science*, *13*:843–854

Gaynes R and Edwards JR. 2005. Overview of nosocomial infections caused by gram-negative bacilli. *Clinical Infectious Diseases*, *41*:848–54

Gooderham WJ and Hancock REW. 2009. Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. *FEMS Microbiology Reviews*, *33*:279–94

Gordon N and Wareham D. 2010. Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *International Journal of Antimicrobial Agents*, *35*:219–226

Gotoh Y, Eguchi Y, Watanabe T, Okamoto S, Doi A and Utsumi R. 2010. Two-component signal transduction as potential drug targets in pathogenic bacteria. *Current Opinion in Microbiology*, *13*:232–9

Grant JR and Stothard P. 2008. The CGView server: a comparative genomics tool for circular genomes. *Nucleic Acids Research*, *36*:W181–4

Harris TL, Worthington RJ, Hittle LE, Zurawski DV, Ernst RK and Melander C. 2014. Small molecule downregulation of PmrAB reverses lipid A modification and breaks colistin resistance. *ACS Chemical Biology, 9*:122–127

Hujer KM, Hujer AM, Hulten E. Bajaksouzian S, Adams JM, Donskey CJ and Bonomo R. 2006. Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrobial Agents and Chemotherapy*, *50*:4114–23

Inoue H, Nojima H and Okayama H. 1990. High efficiency transformation *of Escherichia coli* with plasmids. *Gene, 96*:23-8

Kaniga K, Delor I and Cornelis GR. 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene, 109*:137–41

Ko KS, Suh JY, Kwon KT, Jung SI, Park KH, Kang CI, Chung DR, Peck KR and Song JH. 2007. High rates of resistance to colistin and polymyxin B in subgroups *of Acinetobacter baumannii* isolates from Korea. *Antimicrobial Chemotherapy, 60:*1163-1167

Krell T, Lacal J, Busch A, Silva-Jiménez H, Guazzaroni ME and Ramos JL. (2010). Bacterial sensor kinases: diversity in the recognition of environmental signals. *Annual Review of Microbiology*, *64*:539–59

Kvitko BH, Bruckbauer S, Prucha J, McMillan I, Breland EJ, Lehman S, Mladinich K, Choi KH, Karkhoff-Schweizer R and Schweizer HP. 2012. A simple method for construction of *pir+* Enterobacterial hosts for maintenance of R6K replicon plasmids. *BMC Research Notes, 5*:157

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ and Higgins DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics, 23*:2947-8

Leblanc SKD, Oates CW and Raivio TL. 2011. Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*. *Journal of Bacteriology*, *193*:3367–75

Lee S, Hinz A, Bauerle E, Angermeyer A, Juhaszova K, Kaneko Y, Singh PK and Manoil C. 2009. Targeting a bacterial stress response to enhance antibiotic action. *Proceedings of the National Academy of Sciences, 106*:14570–14575

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

Limansky AS, Mussi MA and Viale AM. 2002. Loss of a 29-Kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. *Journal of Clinical Microbiology, 40*:4776–4778

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

Lin MF, Lin YY, Yeh HW, Lan CY. 2014. Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii adeAB* genes and its correlation with tigecycline susceptibility. *BMC Microbiology, 14:*119

Livak K and Schmittgen T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. *Methods, 25*:402-408

Mak JK, Kim MJ, Pham J, Tapsall J and White P. 2009. Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*. *The Journal of Antimicrobial Chemotherapy*, *63*:47–54

Mammeri H, Poirel L, Mangeney N and Nordmann P. 2003. Chromosomal integration of a cephalosporinase gene from *Acinetobacter baumannii* into *Oligella urethralis* as a source of acquired resistance to β –Lactams. *The Journal of Antimicrobial Chemotherapy, 47*:1536

Maragakis LL and Perl TM. 2008. *Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options. *Clinical Infectious Diseases*, *46*:1254–63

Marchand I, Damier-Piolle L, Courvalin P and 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

Mark JK, MJ Kim, Pham A, Tapsall J and White PA. 2009 Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*. *The Journal of Antimicrobial Chemotherapy, 63*:47-54

McConnell MJ, Domínguez-Herrera J, Smani Y, López-Rojas R, Docobo-Pérez F and Pachón J. 2011. Vaccination with outer membrane complexes elicits rapid protective immunity to multidrug-resistant *Acinetobacter baumannii*. *Infection and Immunity, 79*:518–26

Miller ML, Soufi B, Jers C, Blom N, Macek B and Mijakovic I. 2009. NetPhosBac - a predictor for Ser/Thr phosphorylation sites in bacterial proteins. *Proteomics, 9*:116-25

Merighi M, Carroll-portillo A, Septer, AN, Bhatiya A and Gunn J S. 2006. Role of *Salmonella enterica* serovar typhimurium two-component system PreA/PreB in modulating PmrA-Regulated gene transcription. *Journal of Bacteriology, 188*:141-149

Michalopoulos A and Falagas ME. 2010. Treatment of *Acinetobacter* infections. *Expert Opinion on Pharmacotherapy*, *11*:779–88

Lin MF, Lin YY, Yeh HW and Lan CY. 2014. Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii adeAB* genes and its correlation with tigecycline susceptibility, *BMC microbiology, 14*:119

Mitrophanov AY and Groisman E. 2008. Signal integration in bacterial two-component regulatory systems. *Genes & Development, 22*:2601–11

Morita Y, Tomida J and Kawamura Y. 2012. MexXY multidrug efflux system of *Pseudomonas aeruginosa*. *Frontiers in Microbiology*, *3*:408

Mussi MA, Limansky AS and Viale AM. 2005. Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumannii* : natural insertional inactivation of a gene encoding a member of a novel family of β -barrel outer membrane proteins. *Antimicrobial Agents Chemotherapy, 49*:1432

Peleg A, Seifert H and Paterson D. 2008. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clinical Microbiology Reviews*, *21*: 538–582

Piddock L. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical Microbiology Infections*, *19*:382–402

Poole K. 2004. Efflux-mediated multiresistance in Gram-negative bacteria. *Clinical Microbiology and Infection, 10*:12–26

Rasko DA, Moreira CG, Li de R, Reading NC, Ritchie JM, Waldor MK, Williams N, Taussig R, Wei S, Roth M, Hughes DT, Huntley JF, Fina MW, Falck JR and Sperandio V. 2008. Targeting QseC signaling and virulence for antibiotic development. *Science, Science, 321*:1078–1080

Roca I, Espinal P, Martí S and Vila J. 2011. First identification and characterization of an AdeABC-like efflux pump in *Acinetobacter genomospecies* 13TU. *Antimicrobial Agents and Chemotherapy*, *55*:1285–6

Roca I, Espinal P, Vila-Farrés X and Vila J. 2012. The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. *Frontiers in Microbiology*, *3*:148

Rosenfeld N, Bouchier C, Courvalin P and Périchon B. 2012. Expression of the resistance-nodulation-cell division pump AdeIJK in *Acinetobacter baumannii* is regulated by AdeN, a TetR-type regulator. *Antimicrobial Agents and Chemotherapy*, *56*:2504–2510

Solovyev V and Salamov A. 2011. Automatic annotation of microbial genomes and metagenomic sequences. In metagenomics and its applications in agriculture. *Biomedicine and Environmental Studies*, 61-78

Stephenson K and Hoch J. 2002. Virulence- and antibiotic resistance-associated two-component signal transduction systems of Gram-positive pathogenic bacteria as targets for antimicrobial therapy. *Pharmacology & Therapeutics, 93*:293–305

Stock M, Robinson VL and Goudreau PN. 2000. Two-component signal transduction. *Annual Review of Biochemistry*, *69*:183–215

Stothard P and Wishart DS. 2005. Circular genome visualization and exploration using CGView. *Bioinformatics, 21*:537-9

Sun JR, Perng CL, Chan MC, Morita Y, Lin JC, Su CM, Wang WY, Chang TY and Chiueh TS. 2012. A truncated AdeS kinase protein generated by ISAba1 insertion correlates with tigecycline resistance in *Acinetobacter baumannii*. *PloS One*, *7*:e49534

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

Tomaras AP, Flagler MJ, Dorsey CW, Gaddy JA and Actis LA. 2008. Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology, 154*:3398–3409

Vaara M, Siikanen O, Apajalahti J, Fox J, Frimodt-Møller N, He H, Poudyal A, Li J, Nation RL and Vaara T. 2010. A novel polymyxin derivative that lacks the fatty acid tail and carries only three positive charges has strong synergism with agents excluded by the intact outer membrane. *Antimicrobial Agents and Chemotherapy, 54*:3341–6

Valencia R, Arroyo LA, Conde M, Aldana JM, Torres MJ, Fernández-Cuenca F, Garnacho-Montero J, Cisneros JM, Ortíz C, Pachón J and Aznar J. 2009. Nosocomial

outbreak of infection with pan-drug-resistant *Acinetobacter baumannii* in a tertiary care university hospital. *Infection Control and Hospital Epidemiology*, *30*:257–63

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

Vila J, Martí S and Sánchez-Céspedes J. 2007. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii. Journal of Antimicrobial Chemotherapy, 59:*1210-1215

Watanabe T, Igarashi M, Okajima T, Ishii E, Kino H, Hatano M, Sawa R, Umekita M, Kimura T, Okamoto S, Eguchi Y, Akamatsu Y and Utsumi R. 2012. Isolation and characterization of signermycin B, an antibiotic that targets the dimerization domain of histidine kinase WalK. *Antimicrobial Agents and Chemotherapy*, *56*:3657–63

Zordan S, Prenger-Berninghoff E, Weiss R, Reijden T, Broek P, Baljer G and Dijkshoorn L. 2011. Multidrug-resistant *Acinetobacter baumannii* in veterinary clinics, Germany. *Emerging Infectious Diseases, 17*:1751–1754

8. APPENDICES

Appendix 1. Amino acids alignment of BfmRS in *A. baumannii* ATCC17978, AB030, and AB031.

```
AB030           MLPGADGLTVCREVRPHYHQPILMLTARTEDMDQVLGLEMGADDYVAKPVQPRVLLARIR 60
AB031           MLPGADGLTVCREVRPHYHQPILMLTARTEDMDQVLGLEMGADDYVAKPVQPRVLLARIR 60
ATCC17978       MLPGADGLTVCREVRPHYHQPILMLTARTEDMDQVLGLEMGADDYVAKPVQPRVLLARIR 60
                ************************************************************

AB030           ALLRRTDKTVEDEVAQRIEFDDLVIDNGGRSVTLNGELVDFTSAEYDLLWLLASNAGRIL 120
AB031           ALLRRTDKTVEDEVAQRIEFDDLVIDNGGRSVTLNGELVDFTSAEYDLLWLLASNAGRIL 120
ATCC17978       ALLRRTDKTVEDEVAQRIEFDDLVIDNGGRSVTLNGELVDFTSAEYDLLWLLASNAGRIL 120
                ************************************************************

AB030           SREDIFERLRGIEYDGQDRSIDVRISRIRPKIGDDPENPKRIKTVRSKGYLFVKETNGLM 180
AB031           SREDIFERLRGIEYDGQDRSIDVRISRIRPKIGDDPENPKRIKTVRSKGYLFVKETNGLM 180
ATCC17978       SREDIFERLRGIEYDGQDRSIDVRISRIRPKIGDDPENPKRIKTVRSKGYLFVKETNGLM 180
                ************************************************************

AB030           KALPIFVLDYLMFYPGQEQEYLAKIQKHFSYPINIQNIQDVNLDSEQIGRLRQDQSVMLY 240
AB031           KALPIFVLDYLMFYPGQEQEYLAKIQKHFSYPINIQNIQDVNLDSEQIGRLRQDQSVMLY 240
ATCC17978       KALPIFVLDYLMFYPGQEQEYLAKIQKHFSYPINIQNIQDVNLDSEQIGRLRQDQSVMLY 240
                ************************************************************

AB030           KDSATVRGTTISIVSPIPNHPAQVLVLGPVPMFNWMPLQLSAGITLFSLFLLSLGVYGLI 300
AB031           KDSATVRGTTISIVSPIPNHPAQVLVLGPVPMFNWMPLQLSAGITLFSLFLLSLGVYGLI 300
ATCC17978       KDSATVRGTTISIVSPIPNHPAQVLVLGPVPMFNWMPLQLSAGITLFSLFLLSLGVYGLI 300
                ************************************************************

AB030           LPLERKIRQVRYALNRMKSGDLSLRVPIEGSDEMANLASSYNNMSDHIQRLIEAQRELMR 360
AB031           LPLERKIRQVRYALNRMKSGDLSLRVPIEGSDEMANLASSYNNMSDHIQRLIEAQRELMR 360
ATCC17978       LPLERKIRQVRYALNRMKSGDLSLRVPIEGSDEMANLASSYNNMSDHIQRLIEAQRELMR 360
                ************************************************************

AB030           AVSHELRTPVARIRFGTEMLAEEDDYNHRMHQVDMIDKDIEALNTLIDEIMTYAKLEQGT 420
AB031           AVSHELRTPVARIRFGTEMLAEEDDYNHRMHQVDMIDKDIEALNTLIDEIMTYAKLEQGT 420
ATCC17978       AVSHELRTPVARIRFGTEMLAEEDDYNHRMHQVDMIDKDIEALNTLIDEIMTYAKLEQGT 420
                ************************************************************

AB030           PSLDFAEIVLFEVLDQVAVETEALKTQKEIELIPPPLYVKVDAERRYLHRVVQNLVGNAV 480
AB031           PSLDFAEIVLFEVLDQVAVETEALKTQKEIELIPPPLYVKVDAERRYLHRVVQNLVGNAV 480
ATCC17978       PSLDFAEIVLFEVLDQVAVETEALKTQKEIELIPPPLYVKVDAERRYLHRVVQNLVGNAV 480
                ************************************************************

AB030           RYCDNKVRITGGIHSDGMAFVCVEDDGPGIPEQDRKRVFEAFARLDDSRTRASGGYGLGL 540
AB031           RYCDNKVRITGGIHSDGMAFVCVEDDGPGIPEQDRKRVFEAFARLDDSRTRASGGYGLGL 540
ATCC17978       RYCDNKVRITGGIHSDGMAFVCVEDDGPGIPEQDRKRVFEAFARLDDSRTRASGGYGLGL 540
                ************************************************************

AB030           SIVSRIAYWFGGEIKVDESPSLGGARFIMTWPAHRFKQPPLKTNKKAPA 589
AB031           SIVSRIAYWFGGEIKVDESPSLGGARFIMTWPAHRFKQPPLKTNKKAPA 589
ATCC17978       SIVSRIAYWFGGEIKVDESPSLGGARFIMTWPAHRFKQPPLKTNKKAPA 589
                *************************************************
```

(-) Extra residues.

(*) Identical residue

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

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

Appendix 2. Amino acids alignment of AdeRS in *A. baumannii* ATCC17978 and AB030.

```
ATCC17978       MSVIRAMNGKQAIELHASQPIDLILLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDI 60
AB030           MSVIRAMNGKQAIELHASQPIDLILLDIKLPELNGWEVLNKIRQKAQTPVIMLTALDQDI 60
                ************************************************************

ATCC17978       DKVMALRIGADDFVVKPFNPNEVVARVQAVLRRTQFANKATNKNKLYKNIEIDTDTHSVY 120
AB030           DKVMALRIGADDFVVKPFNPNEVIARVQAVLRRTQFANKATNKNKIYKNIEIDTDTHSVY 120
                **********************:**********************:**************

ATCC17978       IHSENKKILLNLTLTEYKIISFMIDQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRK 180
AB030           IHSENKKILLNLTLTEYKIISFMIDQPHKVFTRGELMNHCMNDSDALERTVDSHVSKLRK 180
                ************************************************************

ATCC17978       KLEEQGIFQMLINVRGVGYRLDNPLAVKDDAMRLAKRFIVPINFLAEAAKKISHGDLSAR 240
AB030           KLEEQGIFQMLINVRGVGYRLDNPLAVKDDAMRLAKRFIVPINFLAEAAKKISHGDLSAR 240
                ************************************************************

ATCC17978       AYDNRIHSAEMSELLYNFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDG 300
AB030           AYDNRIHSAEMSELLYKFNDMAQKLEVSVKNAQVWNAAIAHELRTPITILQGRLQGIIDG 300
                ****************:*******************************************

ATCC17978       VFK**L**DEVLFKSLLNQVEGLSHLVEDLRTLSLVENQQLRLNYELFDFKAVVEKVLKAFEDR 360
AB030           VFK**P**DEVLFKSLLNQVEGLSHLVEDLRTLSLVENQQLRLNYELFDFKAVVEKVLKAFEDR 360
                *** ********************************************************

ATCC17978       LDQAKLVPELDLTSTPVYCDRRRIEQVLIALIDNAIRYSNAGKLKISSEVVSQNWILKIE 420
AB030           LDQAKLVPELDLTSTPVYCDRRRIEQVLIALIDNAIRYSNAGKLKISSEVVSQNWILKIE 420
                ************************************************************

ATCC17978       DEGPGIATEFQDDLYKPFFRLEESRNKEFGGTGLGLAVVHAIIVALK**G**TIQYSNQGSKSV 480
AB030           DEGPGIATEFQDDLFKPFFRLEESRNKEFGGTGLGLAVVHAIVVALK**S**TIQYSNQGSKSI 480
                **************:***************************:****.***********:

ATCC17978       FTIKISMGHEEIG 493
AB030           FTIKISM------ 487
                *******
```

(-) Extra residues.

(*) Identical residue

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

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

Appendix 3. Amino acids alignment of A1S_2137_38 in *A. baumannii* ATCC17978, AB030, and AB031.

```
ATCC17978       MTKPFSVQELLARIRVILRNQPIQQEAHIYDDGYLKVDVTQRLVWIEQQPITLTRKEFQL 60
AB030           MTKPFSVQELLARIRVILRNQPIQQEAHVYDDGYLKVDVTQRLLWVEQQPITLTRKEFQL 60
AB031           MTKPFSVQELLARIRVILRNQPIQQEAHIYDDGYLKVDVTQRLVWIEQQPITLTRKEFQL 60
                ****************************:**************:*:**************

ATCC17978       LALLMRYQGQLLTQPQLLKELWGPTHQEDTHYLRILVGKLRSKLGDNAIQPRYIATEPGV 120
AB030           LTLLMRYQGQLLTQPQLLKELWGPTHQEDTHYLRILVGKLRSKLGDNAIQPRYIATEPGV 120
AB031           LTLLMRYQGQLLTQPQLLKELWGPTHQEDTHYLRILVGKLRSKLGDNAIQPRYIATEPGV 120
                *:************************************************************

ATCC17978       GLRFLAKQKNHMLARAKELYQQGTDVVVGIVETHGRIETLKILEGLPQIARKEMQYQGHT 180
AB030           GLRFLAKQKNHMLARAKELFQQGTDVVVGIVETHGRIETLKILEGLPQIARKEMQYQGHT 180
AB031           GLRFLAKQKNHMLARAKELFQQGTDVVVGIVETHGRIETLKILEGLPQIARKEMQYQGHT 180
                *******************:****************************************

ATCC17978       LEEMDLDAILLRHPQIVLVDELAHRNVPNSRHERRWQDVNELLDAGIDVFTTINIQHLES 240
AB030           LEEMDLDAILLRHPQIVLVDELAHRNVPNSRHERRWQDVNELLDAGIDVFTTINIQHLES 240
AB031           LEEMDFDAILLRHPEIVLVDELAHRNIPNSRHERRWQDVNELLDAGIDVFTTINIQHLES 240
                *****:*********:***********:********************************

ATCC17978       LNDVVYQITGIRVNETVPDRVFDRIRDIRLIDLPVSELIERLHQGKVYVPEQANLALQGF 300
AB030           LNDVVYQITGIRVNETVPDRVFDRIRDIRLIDLPVSELIERLHQGKVYVPEQANLALQGF 300
AB031           LNDVVHQITGIRVNETVPDRVFDRIRDIRLIDLPVSELIERLHQGKVYVPEQANLALQGF 300
                *****:************************************************************

ATCC17978       FSISNLTALRELAMQCVAEHVDSDLKESYASKGLKSISLQNELMIAIDGQGSSEYLVRAG 360
AB030           FSISNLTALRELAMQCVAEHVDSDLKESYASKGLKSISLQNELMIAIDGQGSSEYLVRAG 360
AB031           FSISNLTALRELAMQCVAEHVDSDLKESYASKGLKSISLQNELMIAIDGQGSSEYLVRAG 360
                ************************************************************

ATCC17978       CRLAERNGATWTVVNVAKSLDFGQSSVSSYKKEYIEIDRAFELARQLGGRTEVLYGPRVA 420
AB030           CRLAERNGATWTVVNVAKSLDFGQSSVSSSKKEYIEIDRAFELARQLGGRTEVLYGPRVA 420
AB031           CRLAERYGATWTVVNVAKSLDFGQSSRNSYKKEYIEIDRAFELARQLGGRTEVLYGHQVA 420
                ****** ******************* .* ************************* :**

ATCC17978       SVLMDAAVDRGISNLVIGKSISPWWLKLFKKNLAQQLLNQENSIALTILHPEQGTKKINQ 480
AB030           SVLMDAAVDRGISNLVIGKSISPWWLKLFKKNLAQQLLNQENSIALTILHPEQGTKKINQ 480
AB031           SVLMDAAVDRGISNLVIGKSISPWWLKLFKKNLAQQLLNQENSIALTILHPEQRTKKINQ 480
                ************************************************ ******

ATCC17978       LKKPSFLSLKESVFVLAVTCGSIFIAHFAEVLFGIEDFSVIFIISVLIVATKTRMLAAVV 540
AB030           LKKPSFLSLKESVFVLAVTCGSIFLAHFAEVLFGIEDFSVIFIISVLIVATKTRMLAAVV 540
AB031           LEKPSFLSLKESVFVLAVTCASIFIAHFAEVLFGIEDFSVIFIISVLIVATKTRMLAAVV 540
                *:******************.***:************************************

ATCC17978       AALICFLAYNFFFIAPRYTFQISAHQGVVTVVAFFAAALIAGRLASQLRQQVLSLKAANA 600
AB030           AALICFLAYNFFFIAPRYTFQISAHQGVVTVVAFFAAALIAGRLASQLRQQVLSLKAANA 600
AB031           AALICFLAYNFFFIAPRYTFQISAHQGVVTVVAFFAAALIAGRLASQLRQQVLSLKAANA 600
                ************************************************************

ATCC17978       YTTVMQDLARKLSSAVNLEEVMQTGRMTLETQLQTKVWISIRDKIISSDIELNDKEKVAA 660
AB030           YTMVMQDLARKLSSAVNLEEVMQTGRMTLETQLQTKVWISIRDKVISSDIELNDKEKVAA 660
AB031           YTTVMQDLARKLSSAVNLEEVMQTGRMTLETQLQTKVWISIQDKVISSDIELNDKEKVAA 660
                ** ************************************:**:**************

ATCC17978       EWCLKHQQPCGRFTDTLSQSNWWFLPLLEQKNSLGIVGIYFKDEVVSLNFEQKKLTESVI 720
AB030           EWCLKHKQPCGRFTDTLSQSNWWFLPLLEQKNSLGIVGIYFKDEVVSLNFEQKKLTESVI 720
AB031           EWCLKHKQPCGRFTDTLSQSNWWFLPLLEQKNSLGIVGIYFKDEVVSLNFEQKKLTESVI 720
```

```
                ******:**************************************************

ATCC17978       EYIAQAALRTQLVNELEQAKVTSETERLRSALLSSVSHDLRSPLASIIGAADTLANFKAE 780
AB030           EYIAQAVLRTQLVNELEQAKVTSETERLRSALLSSVSHDLRSPLASIIGAADTLANFKAE 780
AB031           EYIAQAVLRTQLVNELEQAKVTSETERLRSALLSSVSHDLRSPLASIIGVADTLANFKAE 780
                ******.****************************************.**********

ATCC17978       MTEQDQQDLLETIHLEGERLDRYIQNLLDMTRLGHEGLTLKRDWIGVDELIGSATRRLKR 840
AB030           MTEQDQQDLLETIHLEGERLDRYIQNLLDMTRLGHEGLTLKRDWIGVDELIGSATRRLKR 840
AB031           MSEQDQQDLLETIHLEGERLDRYIQNLLDMTRLGHEGLTLKRDWIGVDELIGSATRRLKR 840
                *:**************************************************

ATCC17978       YKPDTQVVVQLPEQPISLYVHPALVEQAIFNVLENAANFSPPDESVMIRAQLLSEDEVKI 900
AB030           YKPDTQVVVQLSEQPISLYVHPALVEQAIFNVLENAANFSPPDEPVMIRTHLVSEDEVKI 900
AB031           YKPDTQVAVQLPEQPISLYVHPALVEQAIFNVLENAANFSPPDEPVMIRTHLVSEDEVKI 900
                *******.***.*********************************.****::*:*******

ATCC17978       EIEDKGVGIPEDERHRIFDMFYTMERGDRGKFGTGLGLTIVKAIIGAHMGTIEAFSGRQN 960
AB030           EIEDKGAGIPEDERHRIFDMFYTMERGDRGKFGTGLGLTIVKAIIGAHMGTIEAFSGCQN 960
AB031           EIEDKGVGIPEDERHRIFDMFYTMERGDRGKFGTGLGLTIVKAIIGAHRGTIEAFSGRQN 960
                ******.*************************************** ******** **

ATCC17978       KGTLIQIKLPLRPVKE 976
AB030           KGTLIQIKLPLHPVKE 976
AB031           KGTLIQIKLPLHPVKE 976
                ***********:****
```

(-) Extra residues.

(*) Identical residue

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

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

Appendix 4. Amino acids alignments of A1S_1977_78 in *A. baumannii* ATCC17978, AB030, and AB031.

```
AB030           MLKISKEENLIQQHQVARQLVRGVAHEIKNPLAGIRGATQLLARSLNDESYAEFTDIIIN 60
AB031           MLKISKEENLIQQHQVARQLVRGVAHEIKNPLAGIRGATQLLARSLNDESYAEFTDIIIN 60
ATCC17978       MLKISKEENLIQQHQVARQLVRGVAHEIKNPLAGIRGATQLLARSLNDESYAEFTDIIIN 60
                ************************************************************

AB030           EVDRLTNLADTMLGSRQLPSYENVNVHEPLERVRSLIANQTKKKIKITRDYDLSLPDVKA 120
AB031           EVDRLTNLADTMLGSRQLPSYENVNVHEPLERVRSLIANQTKKKIKITRDYDLSLPDVKA 120
ATCC17978       EVDRLTNLADTMLGSRQLPSYENVNVHEPLERVRSLIANQTKKKIKITRDYDLSLPDVKA 120
                ************************************************************

AB030           DRDQLIQVMLNISVNAIQAITENKSFFTDQEPELILRTRIQRLVTINGVLNRSAVRVDIE 180
AB031           DRDQLIQVMLNISVNAIQAITENKSFFTDQEPELILRTRIQRLVTINGVLNRSAVRVDIE 180
ATCC17978       DRDQLIQVMLNISVNAIQAITENKSFFTDQEPELILRTRIQRLVTINGVLNRSAVRVDIE 180
                ************************************************************

AB030           DNGPGIPESILESVFYPLVTGRAKGTGLGLSIAQNIMHQHNGMIECQSVPGKTMFSLYLP 240
AB031           DNGPGIPESILESVFYPLVTGRAKGTGLGLSIAQNIMHQHNGMIECQSVPGKTMFSLYLP 240
ATCC17978       DNGPGIPESILESVFYPLVTGRAKGTGLGLSIAQNIMHQHNGMIECQSVPGKTMFSLYLP 240
                ************************************************************

AB030           WESDRVAKMRWVLEKTFKEEGFDVTNFEEAQTALERLHHDAPDVILTDIRMPGIDGLTFL 300
AB031           WESDRVAKMRWVLEKTFKEEGFDVTNFEEAQTALERLHHDAPDVILTDIRMPGIDGLTFL 300
ATCC17978       WESDRVAKMRWVLEKTFKEEGFDVTNFEEAQTALERLHHDAPDVILTDIRMPGIDGLTFL 300
                ************************************************************

AB030           SKVKNSHPDLPVIIMTAHSDLESAVSSYQTGAFEYLPKPFDIDEALALVNRAILHINKLQ 360
AB031           SKVKNSHPDLPVIIMTAHSDLESAVSSYQTGAFEYLPKPFDIDEALALVNRAILHINKLQ 360
ATCC17978       SKVKNSHPDLPVIIMTAHSDLESAVSSYQTGAFEYLPKPFDIDEALALVNRAILHINKLQ 360
                ************************************************************

AB030           QQEATKTASPLQSTEIIGESPAMQEVFRAIGRLSQSHITVLINGESGTGKELVAHALHKH 420
AB031           QQEATKTASPLQSTEIIGESPAMQEVFRAIGRLSQSHITVLINGESGTGKELVAHALHKH 420
ATCC17978       QQEATKTASPLQSTEIIGESPAMQEVFRAIGRLSQSHITVLINGESGTGKELVAHALHKH 420
                ************************************************************

AB030           SPRRAKPFIALNMAAIPKDLIETELFGHEKGAFTGANTQHQGRFEQANGGTLFLDEIGDM 480
AB031           SPRRAKPFIALNMAAIPKDLIETELFGHEKGAFTGANTQHQGRFEQANGGTLFLDEIGDM 480
ATCC17978       SPRRAKPFIALNMAAIPKDLIETELFGHEKGAFTGANTQHQGRFEQANGGTLFLDEIGDM 480
                ************************************************************

AB030           PFETQTRLLRVLADGEFYRVGGHIPVKVDVRIVAATHQDLEKLVNEGRFREDLYHRLNVI 540
AB031           PFETQTRLLRVLADGEFYRVGGHIPVKVDVRIVAATHQDLEKLVNEGRFREDLYHRLNVI 540
ATCC17978       PFETQTRLLRVLADGEFYRVGGHIPVKVDVRIVAATHQDLEKLVNEGRFREDLYHRLNVI 540
                ************************************************************

AB030           RIHIPKLAHRSEDIPMLAQHFLARAGKELGVSPKILHTETTDYMQQLPWPGNVRQLENTC 600
AB031           RIHIPKLAHRSEDIPMLAQHFLARAGKELGVSPKILRTETTDYMQQLPWPGNVRQLENTC 600
ATCC17978       RIHIPKLAHRSEDIPMLAQHFLARAGKELGVSPKILRTETTDYMQQLPWPGNVRQLENTC 600
                ***********************************:************************

AB030           RWLTVMITGREVYPEDLPSELKQVPLQKSSETSQPAPSFERISLHHWDELLSQWAIQKLK 660
AB031           RWLTVMITGREVYPEDLPSELKQVPLQKSSETSQPAPSFERISLHHWDELLSQWAIQKLK 660
ATCC17978       RWLTVMITGREVYPEDLPSELKQVPLQKSSETSQPTPSFERISLHHWDELLSQWAIQKLK 660

                ***********************************:************************
```

```
AB030           NGEMKILDIATPMFERTLINAALQQTRGRKRHAAELLGWGRNTLTRKLKELGMDSADDDD 720
AB031           NGEMKILDIATPMFERTLINAALQQTRGRKRHAAELLGWGRNTLTRKLKELGMDSADDDD 720
ATCC17978       NGEMKILDIATPMFERTLINAALQQTRGRKRHAAELLGWGRNTLTRKLKELGMDSADDDD 720
                ************************************************************

AB030           EDEHKATLSEA 731
AB031           EDEHKATLSEA 731
ATCC17978       EDEHKATLSEA 731
                ***********
```

(-) Extra residues.

(*) Identical residue

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

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

Appendix 5. Nucleotide alignments *of A1S_2006* in *A. baumannii* ATCC17978, andAB030. The nucleotides underlined indicates the sequence of the additional DNA insertion in AB030.

```
17978          ATGGATCATCCCCATCGTGACATTATTGAAAGTTGTGTGAGTAGCTATGACCTACCAACT 60
AB030          ATGGATCATCCCCATCGTGACATTATTGAAAGTTGTGTGAGTAGCTATGACCTACCAACT 60
               ************************************************************

17978          GTTCTATTCACTAAAAATTCAGATAAAGACACCATCAAACAAGCTATAGATGCAGGCGTA 120
AB030          GTTCTATTCACTAAAAATTCAGATAAAGACACCATCAAACAAGCTATAGATGCAGGCGTA 120
               ************************************************************

17978          ACGGCTTATATTGTAGATGGCATAGACCCCGCCCGCTTACATACCATTTTAGAGATTTCA 180
AB030          ACGGCTTATATTGTAGATGGCATAGACCCCGCTCGCTTACATACCATTTTAGAGATTTCA 180
               ******************************* ************************

17978          ATTGAGCAATATAAAAAACAT---------------------------------------- 201
AB030          ATTGAGCAATATAAAAAACATCTCTGTACACGACAAATTTCACAGAACCCTTATCCTATC 240
               **********************

17978          ------------------------------------------------------------
AB030          AGGATTCTGCCTTCTTAAAATGACACATCTCAATGAGTTATATCTTATCTTAAACAAATC 300

17978          ------------------------------------------------------------
AB030          TCTAAAATGGAACAAGTCACATTTAAAGTGCTTTGCGCTCATCATGCTTGTGATTATTTT 360

17978          ------------------------------------------------------------
AB030          AAAGCAAACATGTAATCTTTCTTCTGCATCTAAAGCCTTGCCCATCAAGTGCTTACCACA 420

17978          ------------------------------------------------------------
AB030          ATCATTTTATCGACGTATGCAGCGCTTCTTTGCAGGTCAGTATTTTGATTATCGTCAAAT 480

17978          ------------------------------------------------------------
AB030          TTCTCAGTTGATTTTCAATATGTTTTCATTCGACCAAGTGCAACTGACTTTAGATAGAAC 540

17978          ------------------------------------------------------------
AB030          CAATTGGAAATGGGGAAAACGAAATATTAATATCCTGATGCTCGCAATCGTTTATCGTGG 600

17978          ------------------------------------------------------------
AB030          AATAGCGATACCTATCCTTTGGACATTGCTTAATAAACGTGGAAATTCAGATACGAAAGA 660

17978          ------------------------------------------------------------
AB030          GCGCATTGCTTTGATTCAACGCTTTATAGCCATTTTTGGTAAAGACCGTATTGTGAATGT 720

17978          ------------------------------------------------------------
AB030          GTTCGCAGACAGAGAGTTTATCGGTGAGCAGTGGTTTACATGGTTAATTGAACAAGACAT 780
```

```
17978           ------------------------------------------------------------
AB030           CAACTTCTGCATTCGTGTTAAAAAAACTTCATTGTCACCAATCATTTAGATGAATAAATA 840

17978           ------------------------------------------------------------
AB030           TTTTACTTGCTATGTGGTTGCTTCTCTTTTTCTTTCTGGTTGTACGGTTCAGCATAATTT 900

17978           ------------------------------------------------------------
AB030           AATAAATGAAACCCCGAGTCAGATTGTTCAAGGACATAATCAGGTGATTCATCAATACTT 960

17978           ------------------------------------------------------------
AB030           TGATGAAAAAAACACCTCAGGTGTGCTGGTTATTCAAACAGATAAAAAAATTAATCTATA 1020

17978           ------------------------------------------------------------
AB030           TGGTAATGCTCTAAGCCGCGCAAATACAGAATATGTGCCAGCCTCTACATTTAAAATGTT 1080

17978           ------------------------------------------------------------
AB030           GAATGCCCTGATCGGATTGGAGAACCAGAAAACGGATATTAATGAAATATTTAAATGGAA 1140

17978           ------------------------------------------------------------
AB030           GGGCGAGAAAAGGTCATTTACCGCTTGGGAAAAAGACATGACACTAGGAGAAGCCATGAA 1200

17978           ------------------------------------------------------------
AB030           GCTTTCTGCAGTCCCAGTCTATCAGGAACTTGCGCGACGTATCGGTCTTGATCTCATGCA 1260

17978           ------------------------------------------------------------
AB030           AAAAGAAGTAAAACGTATTGGTTTCGGTAATGCTGAAATTGGACAGCAGGTTGATAATTT 1320

17978           ------------------------------------------------------------
AB030           CTGGTTGGTAGGACCATTAAAGGTTACGCCTATTCAAGAGGTAGAGTTTGTTTCCCAATT 1380

17978           ------------------------------------------------------------
AB030           AGCACATACACAGCTTCCATTTAGTGAAAAAGTGCAGGCTAATGTAAAAAATATGCTTCT 1440

17978           ------------------------------------------------------------
AB030           TTTAGAAGAGAGTAATGGCTACAAAATTTTTGGAAAGACTGGTTGGGCAATGGATATAAA 1500

17978           ------------------------------------------------------------
AB030           ACCACAAGTGGGCTGGTTGACCGGCTGGGTTGAGCAGCCAGATGGAAAAATTGTCGCTTT 1560

17978           ------------------------------------------------------------
AB030           TGCATTAAATATGGAAATGCGGTCAGAAATGCCGGCATCTATACGTAATGAATTATTGAT 1620

17978           ------------------------------------------------------------
AB030           GAAATCATTAAAACAGCTGAATATTATTTAAATGCAAATAACTCTAGAAATCAAATGTCC 1680

17978           ------------------------------------------------------------
AB030           AACCTGCCTCAGTGACAGTATAAAGAAAAATGGCATCAAAGTAGATGGGAAACAAAACTA 1740
```

```
17978           ------------------------------------------------------------
AB030           CCAATGCAAAGACTGCAAACGTCAGTTTATTGGTGACCATGCTCTGAGCTATCTAGGATG 1800

17978           ------------------------------------------------------------
AB030           TAATTCTGGCATTACTCGTAAAATATTACAGTTAATGGTCAGAGGCAGCGGTATACGAGA 1860

17978           ------------------------------------------------------------
AB030           TATCGCTGAAGTTGAGCGCATTAGTATCGGTAAAGTCTTACGGACTTTAACTGAATCGGC 1920

17978           ------------------------------------------------------------
AB030           CTATCAAATTCAGCCTAAACAAAGTCATTATGAATCTCTCGAAGTAGATGAATTCTGGAC 1980

17978           ------------------------------------------------------------
AB030           TTTTGTTGGAAATAAAAATAATAAACAATGGCTTATTTACGCCTACCATCGAGAAACAGG 2040

17978           ------------------------------------------------------------
AB030           TGAGATTGTTGCTTATGTTTGGGGTAAGAGAGATTTAGCTACAGTCCAAAGGTTGAAGAC 2100

17978           ------------------------------------------------------------
AB030           AAAGCTTAAACAATTAGGTATTCACTACACCCGAATTGCAAGTGATCATTGGGACAGTTT 2160

17978           ------------------------------------------------------------
AB030           CATCACTGCTTTTCTCTGTACACGACAAATTTCACAGAACCCTTATCCTATCAGGATTCT 2220

17978           ------------------------------------------------------------
AB030           GCCTTCTTAAATGACACATCTCAATGAGTTATATCTTATCTTAAACAAATCTCTAAAATG 2280

17978           ------------------------------------------------------------
AB030           GAACAAGTCACATTTAAAGTGCTTTGCGCTCATCATGCTTGTGATTATTTTAAAGCAAAC 2340

17978           ------------------------------------------------------------
AB030           ATGTAATCTTTCTTCTGCATCTAAAGCCTTGCCCATCAAGTGCTTACCACAATCATTTTA 2400

17978           ------------------------------------------------------------
AB030           TCGACGTATGCAGCGCTTCTTTGCAGGTCAGTATTTTGATTATCGTCAAATTTCTCAGTT 2460

17978           ------------------------------------------------------------
AB030           GATTTTCAATATGTTTTCATTCGACCAAGTGCAACTGACTTTAGATAGAACCAATTGGAA 2520

17978           ------------------------------------------------------------
AB030           ATGGGGAAAACGAAATATTAATATCCTGATGCTCGCAATCGTTTATCGTGGAATAGCGAT 2580

17978           ------------------------------------------------------------
AB030           ACCTATCCTTTGGACATTGCTTAATAAACGTGGAAATTCAGATACGAAAGAGCGCATTGC 2640

17978           ------------------------------------------------------------
AB030           TTTGATTCAACGCTTTATAGCCATTTTTGGTAAAGACCGTATTGTGAATGTGTTCGCAGA 2700
```

```
17978          ------------------------------------------------------------
AB030          CAGAGAGTTTATCGGTGAGCAGTGGTTTACATGGTTAATTGAACAAGACATCAACTTCTG 2760

17978          ------------------------------------------------------------
AB030          CATTCGTGTTAAAAAAACTTCATTGTCACCAATCATTTAGATGAGTTATCTATTTTTGTC 2820

17978          ------------------AAAAAGCTCGAAGGTGACTTAAAAGAAGCTCAAACCAAACT 242
AB030          GTGTACAGAGAAAAAACATAAAAAGCTCGAAGGTGACTTAAAAGAAGCTCAAACCAAACT 2880
                                  ****************************************

17978          GGCAGACCGTAAAGATGTTGAAAAGGCCAAAGTGCTACTCATGCAACTGCATGGTTTACC 302
AB030          GGCAGATCGTAAAGATGTTGAAAAGGCCAAAGTGCTACTCATGCAACTGCATGGCTTACC 2940
               ****** ********************************************** *****

17978          TGAAGACACAGCTTTTCAATTACTCAGAAAAAATGCCATGAGTCACCGTATAACGATTGG 362
AB030          TGAAGACACAGCTTTTCAATTACTCAGAAAAAATGCCATGAGTCACCGTATAACGATTGG 3000
               ************************************************************

17978          AGAAATGGCACGGCGTTTACTTGACGCTCAAAAATTACTAAATGATCAACTAAAGGATGA 422
AB030          AGAAATGGCACGGCGTTTACTTGACGCTCAAAAATTACTAAATGATCAACTAAAGGATGA 3060
               ************************************************************

17978          ATAA 426
AB030          ATAA 3064
               ****
```

Appendix 6. Nucleotide alignment of *A1S_1754* in *A. baumannii* ATCC17978, andAB031. The nucleotides underlined indicates the sequence of the additional DNA insertion in AB031.

```
AB031          ATGAGTGTTATTCGGGCCATGAATGGAAAGCAAGCGATTGAATTGCACGCTAGCCAACCC 60
17978          ATGAGTGTTATTCGGGCCATGAATGGAAAGCAAGCAATTGAATTGCATGCGAGCCAACCC 60
               ********************************** *********** ** *********

AB031          ATCGATTTAATCTTACTTGATATTAAATTACCCGAATTAAACGGTTGGGAAGTATTAAAT 120
17978          ATCGATTTAATCTTACTTGATATTAAATTACCCGAATTAAACGGTTGGGAAGTGTTAAAT 120
               ************************************************** ******

AB031          AAAATACGCCAAAAAGCTCAGACTCCCGTGATCATGTTGACGGCGCTAGATCAAGATATT 180
17978          AAAATACGCCAAAAAGCTCAGACTCCCGTGATCATGTTGACGGCGTTAGATCAAGATATT 180
               ******************************************** **************

AB031          GATAAAGTTATGGCATTACGCATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCA 240
17978          GATAAAGTTATGGCATTACGCATAGGTGCAGATGACTTTGTGGTGAAGCCTTTTAACCCA 240
               ************************************************************

AB031          AATGAAGTCATCGCTAGAGTTCAGGCAGTCTTAAGACGTACTCAGTTTGCAAACAAGGCA 300
17978          AATGAAGTCGTCGCTAGAGTTCAGGCAGTCTTAAGACGTACTCAGTTTGCAAACAAAGCA 300
               ********* ********************************************** ***

AB031          GCTAATAAAAATAAACTCTATAAAAATATTGAAATTGATACCGACACTCATAGCGTTTAT 360
17978          ACTAATAAAAATAAACTCTATAAAAATATTGAAATTGATACCGACACTCATAGCGTTTAT 360
                ************************************************************

AB031          ATACTCTCAGAGAACAAGAAGATCTTGCTTAATCTGACGCTAACTGAATATAAAATTATT 420
17978          ATACACTCTGAGAATAAGAAGATCTTGCTTAATCTGACGCTGACTGAATATAAAATTATT 420
               **** *** ***** ************************** ******************

AB031          TCATTCATGATTGACCAGCCTCATAAAGTTTTTACGCGTGGAGAACTTATGAATCACTGC 480
17978          TCATTCATGATTGATCAGCCTCATAAAGTTTTTACGCGCGGAGAGCTTATGAATCACTGC 480
               ************** ********************** ***** ***************

AB031          ATGAATGATAGCGATGCACTAGAGCGAACCGTAGATAGCCATGTGAGTAAGCTAAGAAAA 540
17978          ATGAATGATAGCGATGCACTAGAGCGAACCGTAGATAGCCATGTAAGTAAGCTGAGAAAA 540
               ****************************************** ******** ******

AB031          AAACTAGAAGAACAAGGCATATTTCAAATGTTAGTTAATGTGCGTGGCGTGGGATATAGA 600
17978          AAACTAGAAGAACAAGGCATATTTCAAATGTTAATTAATGTGCGTGGCGTGGGATATAGG 600
               ********************************** ************************

AB031          CTAGATAATCCCGTAGCTATAAAAGATGATGCCTAATTGGTTGGTCGGGTGAAACTATAT 660
17978          CTAGATAATCCCCTAGCTGTAAAAGATGACGCCTAA---------------------- 636
               ************ ***** ********** ******

AB031          ATAAGTGCACTACAGTTAGAAAATGGAGAGCTTTTACTCGTCGTTTCTCCTCAGTTTAAT 720
17978          ------------------------------------------------------------

AB031          GCCAATGCTATTCAGGATTATGCATTACGCTGGGAAATTGAAACCTTATTCAGTTGTCTC 780
17978          ------------------------------------------------------------

AB031          AAAGGACGCGGGTTTAATCTTGAAAATACGCGCTTGACAGACCCTAGACGAGTGAAAAAA 840
17978          ------------------------------------------------------------
```

```
AB031          TTGATTGCGGTGTTAGCTATAAGCTTCTGTTGGTGTTACTTAACGGGTGAATGGCAACAT 900
17978          ------------------------------------------------------------

AB031          AATCAAAAAAAAGCGATAAAAATAAAGAAGCATGGACGACTCTCAATGAGTTTATTTCGC 960
17978          ------------------------------------------------------------

AB031          TATGGTTTAGACTATGTTCAAATGGCGATTCAGCGTTTAATTGGTTTTGGGAAAAAAGAA 1020
17978          ------------------------------------------------------------

AB031          GAGTTTAAGGAAATTTTGGCAATTTTAAGAAAGCAGAATCCTGATAGGATAAGGGTTCTG 1080
17978          ------------------------------------------------------------

AB031          TGATTGGTTGGTCGGGTGAAACTATATATAAGTGCACTACAGTTAGAAAATGGAGAGCTT 1140
17978          ------------------------------------------------------------

AB031          TTACTCGTCGTTTCTCCTCAGTTTAATGCCAATGCTATTCAGGATTATGCATTACGCTGG 1200
17978          ------------------------------------------------------------

AB031          GAAATTGAAACCTTATTCAGTTGTCTCAAAGGACGCGGGTTTAATCTTGAAAATACGCGC 1260
17978          ------------------------------------------------------------

AB031          TTGACAGACCCTAGACGAGTGAAAAAATTGATTGCGGTGTTAGCTATAAGCTTCTGTTGG 1320
17978          ------------------------------------------------------------

AB031          TGTTACTTAACGGGTGAATGGCAACATAATCAAAAAAAAAGCGATAAAAATAAAGAAGCAT 1380
17978          ------------------------------------------------------------

AB031          GGACGACTCTCAATGAGTTTATTTCGCTATGGTTTAGACTATGTTCAAATGGCGATTCAG 1440
17978          ------------------------------------------------------------

AB031          CGTTTAATTGGTTTTGGGAAAAAAGAAGAGTTTAAGGAAATTTTGGCAATTTTAAGAAAG 1500
17978          ------------------------------------------------------------

AB031          CAGAATCCTGATAGGATAAGGGTTCTGTGAATGCGCCTCGCAAAGCGTTTTATTGTGCCA 1560
17978          ----------------------------ATGCGCCTCGCAAAGCGTTTTATTGTGCCA 666
                                           ****************************

AB031          ATTAACTTCTTAGCCGAAGCAGCAAAAAAAATTAGTCACGGCGACCTCTCTGCTAGAGCT 1620
17978          ATTAACTTCTTAGCCGAAGCAGCAAAAAAAATTAGTCACGGCGACCTCTCTGCTAGAGCT 726
               ************************************************************

AB031          TACGATAACCGAATTCACTCCGCCGAAATGTCGGAGCTTTTATATAATTTTAATGATATG 1680
17978          TACGATAACCGAATTCACTCCGCCGAAATGTCGGAGCTTTTATATAATTTTAATGATATG 786
               ************************************************************

AB031          GCTCAAAAGCTAGAGGTTTCCGTTAAAAATGCGCAGGTTTGGAATGCAGCCATCGCACAT 1740
17978          GCTCAAAAGCTAGAGGTTTCCGTTAAAAATGCGCAGGTTTGGAATGCAGCCATCGCACAT 846
               ************************************************************

AB031          GAGTTAAGAACGCCTATAACGATATTACAAGGTCGTTTACAGGGAATTATTGATGGCGTT 1800
17978          GAGTTAAGAACGCCTATAACGATATTACAAGGTCGTTTACAGGGAATTATTGATGGCGTT 906
               ************************************************************
```

```
AB031          TTTAAACTTGATGAAGTTCTATTTAAAAGTCTTTTAAATCAAGTTGAAGGTTTATCTCAC 1860
17978          TTTAAACTTGATGAAGTTCTATTTAAAAGTCTTTTAAATCAAGTTGAAGGTTTATCTCAC 966
               ************************************************************

AB031          TTAGTCGAAGACTTACGGACTTTAAGCTTAGTAGAGAACCAGCAACTCCGGTTAAATTAT 1920
17978          TTAGTCGAAGACTTACGGACTTTAAGCTTAGTAGAGAACCAGCAACTCCGGTTAAATTAT 1026
               ************************************************************

AB031          GAATTGTTTGACTTTAAGGCGGTAGTTGAAAAAGTTCTTAAAGCATTTGAAGATCGTTTG 1980
17978          GAATTGTTTGACTTTAAGGCGGTAGTTGAAAAAGTTCTTAAAGCATTTGAAGATCGTTTG 1086
               ************************************************************

AB031          GATCAAGCTAAGCTAGTACCAGAACTTGACCTAACGTCCACTCCTGTATATTGCGACCGC 2040
17978          GATCAAGCTAAGCTAGTACCAGAACTTGACCTAACGTCCACTCCTGTATATTGCGACCGC 1146
               ************************************************************

AB031          CGTCGTATTGAGCAAGTTTTAATTGCTTTAATTGATAATGCGATTCGCTATTCAAATGCA 2100
17978          CGTCGTATTGAGCAAGTTTTAATTGCTTTAATTGATAATGCGATTCGCTATTCAAATGCA 1206
               ************************************************************

AB031          GGCAAACTTAAAATTTCCTCGGAAGTGGTATCACAAAACTGGATATTAAAAATTGAGGAT 2160
17978          GGCAAACTTAAAATTTCCTCGGAAGTGGTATCACAAAACTGGATATTAAAAATTGAGGAT 1266
               ************************************************************

AB031          GAAGGCCCTGGCATTGCAACCGAGTTCCAAGACGATTTATATAAGCCTTTCTTTAGATTA 2220
17978          GAAGGCCCTGGCATTGCAACCGAGTTCCAAGACGATTTATATAAGCCTTTCTTTAGATTA 1326
               ************************************************************

AB031          GAAGAATCAAGGAATAAAGAATTTGGCGGCACAGGTTTAGGTCTTGCTGTTGTACATGCA 2280
17978          GAAGAATCAAGGAATAAAGAATTTGGCGGCACAGGTTTAGGTCTTGCTGTTGTACATGCA 1386
               ************************************************************

AB031          ATTATTGTGGCACTGAAAGGTACTATTCAATATAGCAACCAAGGCTCGAAAAGTGTTTTC 2340
17978          ATTATTGTGGCACTGAAAGGTACTATTCAATATAGCAACCAAGGCTCGAAAAGTGTTTTC 1446
               ************************************************************

AB031          ACCATAAAAATTTCTATGGGTCATGAAGAAATAGGGTAA 2379
17978          ACCATAAAAATTTCTATGGGTCATGAAGAAATAGGGTAA 1485
               ***************************************
```