MOLECULAR CHARACTERIZATION AND COMPARATIVE GENOMICS OF MULTIDRUG RESISTANT *ACINETOBACTER BAUMANNII* FROM MALAYSIA

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FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2015

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DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2015

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **LEAN SOO SUM**

Regisration/Matric No.: **SGR120055**

Name of Degree: **MASTER OF SCIENCE**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

"MOLECULAR CHARACTERIZATION AND COMPARATIVE GENOMICS OF MULTIDRUG RESISTANT *ACINETOBACTER BAUMANNII* FROM MALAYSIA"

Field of Study: **GENETICS AND MOLECULAR BIOLOGY**

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Abstract

Hospital-acquired infections caused by multidrug resistant (MDR) *Acinetobacter baumannii* has become a serious problem due to the bacterium's remarkable ability to develop resistance to all available antibiotics including carbapenems, the drug of choice for *Acinetobacter* infections. To better understand the biology and extent of drug resistance in locally isolated *A. baumannii*, 54 strains from sporadic cases of infection were collected from Hospital Sultanah Nur Zahirah (HSNZ) in Kuala Terengganu throughout 2011 and characterized. The 54 *A. baumannii* strains showed high resistance rates (>50%) to tetracycline, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, levofloxacin, ampicillin-sulbactam, gentamicin, ciprofloxacin, tobramycin, doxycycline and amikacin with 39 strains (or 72.2%) classified as MDR. These MDR *A. baumannii* were then tested for susceptibility to polymyxin B, which is considered as the drug of last resort for the treatment of *A. baumannii* infections. Of concern, 14 of the MDR *A. baumannii* strains were polymyxin resistant and also categorized as extensive drug resistant (XDR). All 54 *A. baumannii* strains were subtyped by pulsed-field gel electrophoresis (PFGE). Dendrogram generated from the different *Apa*I pulsotypes showed that the strains were genetically diverse with carbapenem-resistant strains grouped into four main clusters whereas polymyxin-resistant strains were not clustered. To have a better understanding of the *A. baumannii* resistance mechanisms, one MDR (strain AC29) and two XDR strains (AC12 and AC30) were subjected to whole genome sequencing using the Illumina Genome Analyzer IIx. All three strains had genome sizes of ~3.8 Mbp and analysis of the loci used for multilocus sequence typing (MLST) showed that all three strains belonged to the ST195 lineage. All three strains harboured an approximately 23 kb AbaR4-type resistance island

(RI) that interrupted the *comM* gene and contained the bla_{OXA-23} carbapenase gene within a Tn*2006*-like structure. This RI, designated AC12-RI1 in strain AC12, AC30-RI1 in strain AC30 and AC29-RI1 in strain AC29, also encode determinants for tetracycline and streptomycin resistance. *A. baumannii* AC12 harboured an RI that was not found in either AC29 or AC30: the 10.3 kb AC12RI-2 which contained determinants for aminoglycoside and β-lactam resistance. A smaller RI, the 7 kb Tn*1548*::*armA* which encoded determinants for aminoglycoside and macrolide resistance, was located in the chromosomes of AC12, AC29 and a 16 kb plasmid of AC30 designated pAC30b. Plasmid analysis showed that all three *A. baumannii* strains harboured an 8 kb cryptic plasmid. AC29 and AC30 harboured a similar ~70 kb conjugative plasmid while AC30 harboured the additional pAC30b. Genome analysis also revealed a multitude of efflux pumps and drug transporters in all three strains which could contribute to their multidrug-resistant phenotype. Mutations within lipopolysaccharide (LPS) biosynthesis (*lpxA*, *lpxC*, *lpxD* and *lpsB*) or modification genes (*pmrCAB*) were discovered in the polymyxin-resistant strains. Experimental evidence indicated that disruption in LPS biosynthesis and up-regulation of the LPS modification genes may have contributed to the development of polymyxin resistance. Comparative genome analysis of *A. baumannii* revealed the genetic basis of drug resistance in local clinical strains and the knowledge gained may help in containing the spread of MDR and XDR *A. baumannii*.

(498 words)

Keywords: *Acinetobacter baumannii*, polymyxin B, PFGE, comparative genomics, resistance mechanisms

Abstrak

Jangkitan yang diperolehi daripada hospital yang disebabkan oleh bakteria *Acinetobacter baumannii* yang rintang terhadap pelbagai ubat ("*multidrug resistant*" atau MDR) telah menjadi masalah yang serius disebabkan keupayaan luar biasanya dalam membentuk kerintangan terhadap kebanyakan antibiotik. Ini termasuk antibiotik jenis carbapenem yang merupakan ubat pilihan bagi jangkitan *A. baumannii*. Bagi memahami secara mendalam biologi serta profil rintangan antibiotik *A. baumannii* yang terdapat di Malaysia, 54 strain *A. baumannii* yang diperolehi daripada Hospital Sultanah Nur Zahirah (HSNZ), Kuala Terengganu sepanjang tahun 2011 telah dicirikan. Strain *A. baumannii* daripada HSNZ menunjukkan peratusan kerintangan yang tinggi (>50%) terhadap tetracycline, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, levofloxacin, ampicillin-sulbactam, gentamicin, ciprofloxacin, tobramycin, doxycycline dan amikacin. Daripada 54 strain *A. baumannii* tersebut, 39 (atau 72.7%) dikategorikan sebagai MDR. Menjadi kebimbangan adalah penemuan di mana 14 strain (atau 25.9%) adalah rintang terhadap polymyxin B, yang merupakan ubatan-pilihan-terakhir bagi merawat jangkitan *A. baumannii*. Strain rintang polymyxin B ini juga dikategorikan sebagai rintang-ubatan-meluas (XDR). Kepelbagaian genetik terhadap 54 strain *A. baumannii* telah dikenalpasti melalui kaedah Elektroforesis Gel Medan Denyut (PFGE). Dendogram daripada pembatasan enzim *Apa*I menghasilkan empat gugusan Pulsotype terhadap strain rintang-carbapenem manakala tiada gugusan diperolehi terhadap strain-strain rintang-polymyxin. Untuk memahami dengan lebih mendalam mekanisma kerintangan *A. baumannii*, satu strain MDR (AC29) dan dua strain XDR (AC12 dan AC30) telah dipilih untuk analisis Penjujukan Genom Kesulurahan (WGS) menggunakan sistem Illumina Genome Analyzer GAII. Ketiga-tiga strain mempunyai saiz genom ~3.8 Mbp dan analisis MLST menunjukkan bahawa ketiga-tiga

strain tersebut dikategorikan di bawah garis keturunan ST195. Kesemua strain mempunyai persamaan pada kepulauan rintangan (RI) jenis AbaR4 yang bersaiz ~23 kb yang menggangu gen *comM* dan mengandungi gen carbapenemase $bla_{\text{OXA-23}}$ di dalam struktur transposon Tn*2006*. RI ini yang dinamakan AC12-RI1 bagi strain AC12, AC30-RI1 bagi strain AC30 dan AC29-RI1 bagi strain AC29 juga mengandungi gen-gen yang memberi kerintangan terhadap tetracycline dan streptomycin. *A. baumannii* AC12 mempunyai RI yang unik iaitu AC12-RI2 yang bersaiz 10.3 kb dan mengkodkan kerintangan terhadap antibiotik jenis aminoglycoside dan β-lactam. RI yang lebih kecil, iaitu Tn*1548::armA* yang bersaiz 7 kb dan memberi kerintangan terhadap aminoglycoside serta macrolide, terletak di kromosom AC12 dan AC29 serta di plasmid bersaiz 16 kb di AC30 yang dinamakan pAC30b. Analisa plasmid menunjukkan bahawa ketiga-tiga strain *A. baumannii* mengandungi plasmid samar 8 kb manakala strain AC29 dan AC30 mengandungi plasmid konjugatif ~70 kb. Analisa genom juga menunjukkan bahawa ketiga-tiga strain *A. baumannii* mengandungi pelbagai pam-pam efluks dan pengangkut ubat yang boleh menyumbang terhadap finotip MDR. Mutasi dalam gen-gen biosintesis lapisan lipopolisakarida (LPS) (*lpxA, lpxC, lpxD* dan *lpsB*) dan pengubahsuaian LPS (*pmrABC*) ditemui di dalam strain-strain yang rintang polymyxin. Bukti-bukti eksperimen menunjukkan bahawa gangguan biosintesis LPS serta penzahiran gen-gen pengubahsuaian LPS mungkin menyumbang terhadap pembangunan kerintangan polymyxin dalam strain-strain tersebut. Analisa komparatif genom *A. baumannii* telah mendedahkan asas-asas genetik kerintangan antibiotik pada strain-strain klinikal tempatan dan pengetahuan ini mungkin dapat menolong menangani penyebaran strain-strain MDR dan XDR *A. baumannii* pada masa hadapan.

(474 patah perkataan)

Kata kunci: *Acinetobacter baumannii*, polymyxin B, PFGE, perbandingan genomik, mekanisma kerintangan

Acknowledgements

I would like to thank University of Malaya (UM) and High Impact Research (HIR) grant for funding this research, and to Universiti Sultan Zainal Abidin (UniSZA) for supporting this collaboration. Thanks to Institute of Biological Sciences, Faculty of Science, Dean of faculty and fellow members for appoving this postgraduate study. I am extremely grateful to my supervisor and co-supervisor, Professor Dr Thong Kwai Lin and Associate Professor Dr Yeo Chew Chieng for their supervision, guidance and invaluable advice throughout this period. Also, I am grateful to Mr. Zarizal Suhaili who helped by providing materials and technical support (in UniSZA) for completing this study. My sincere thanks and appreciation goes to my parents for their support, encouragement and financial help. To my research buddies in UM --- Ms Kong Boon Hong and Mr Benacer Douadi, I offer my deepest thanks. Last but not least, to those who gave me a memorable postgraduate experiences in LBSMM --- Thank You.

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16S rDNA	16S ribosomal deoxyribonucleic acid
Ab	Acinetobactin
Acb	*Acinetobacter calcoaceticus* complex
AST	Antibiotic Susceptibility Test
BLAST	Basic Local Alignment System Tool
BASys	Bacterial Annotation System
CHDL	Carbapenem Hydrolysing class D Beta-Lactamase
CLSI	Clinical and Laboratory Standard Institute
I	Intermediate
IS	Insertion Sequence
IMP	Imipenem
Mauve	Multiple Alignment of conserved genomic seqeuences
MBL	Metallo-Beta-Lactamase
MDR	Multidrug Resistant
MEM	Meropenem
MIC	Minimum Inhibitory Concentration
NCBI	National Centre of Biotechnology Information

OXA	Oxacillinase
PCR	Polymerase chain reaction
PDR	Pandrug resistant
PFGE	Pulsed-Field Gel Electrophoresis
PI	Pathogenicity Island
qRT-PCR	Quantitative Real-Time PCR
R	Resistant
RAST	Rapid Annotation Server Tool
RE	Restriction Enzyme
RI	Resistance Island
S	Susceptible
SDS-PAGE	Sodium Dideoxyl-Sulphate Polyacrylamide Gel Electrophoresis
Tn	Transposon
WGS	Whole Genome Sequencing
XDR	Extreme Drug Resistant
℃	Degree Celcius
mg/mL	Milligrams per milli litre
mL	Milli Litre

μg/mL	Micrograms per milli litre
μL	Micro Litre

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CHAPTER 1

1.1 INTRODUCTION

Acinetobacter baumannii has been associated with life threatening nosocomial infections such as pneumonia, septicemia, wound sepsis, urinary tract infections, endocarditis and meningitis (Bergogne-Bérézin & Towner, 1996; Nordmann & Poirel, 2008). As an opportunistic pathogen in the hospital, it mainly targets immunocompromised patients, patients quarantined in intensive care units (ICU) and also patients with underlying diseases (Bergogne-Bérézin & Towner, 1996; Nordmann & Poirel, 2008; Camp & Tatum, 2010). Infections due to this pathogen were once easily treated due to its susceptibility to commonly used antibiotics. It was only later discovered in the 1980s that this pathogen had emerged with resistant members, following the introduction of carbapenems as a new therapeutic option (Bergogne-Bérézin & Towner, 1996; Gupta, 2008). Soon, enzymes that could destroy carbapenems emerged and multi-drug resistant (MDR) *A. baumannii* in hospital settings were reported. The emergence and outbreak of MDR *Acinetobacter* infections across different continents of the world marks the success of *A. baumannii* in surviving and adapting to the hospital environment. Clinical strains of *A. baumannii* that are extremely resistant to antibiotics have now been reported worldwide (Hamouda, Evans, Towner, & Amyes, 2010).

According to a report from the United States Centers for Disease Control (CDC) in 2004, *A. baumannii* accounted for 80% of *Acinetobacter* infections worldwide (Camp & Tatum, 2010). *A. baumannii* is also responsible for 10% of hospital-acquired infections and a cause for increase in mortality up to 70% (Zhu et al.,

2013). In Malaysia, *A. baumannii* was the second most common nosocomial Gram-negative organism isolated in the hospital settings (Deris, Harun, Omar, & Johari, 2009). However, it is still the most prevalent nosocomial pathogen in the Intensive Care Units (ICU). Data from the Infection Control Unit, Hospital Universiti Sains Malaysia (HUSM) for the year 2001 showed that *A. baumannii* was among the most common nosocomial pathogen. Prevalence of *Acinetobacter* in HUSM was 6.11% whereas in intensive care units, the prevalence was slightly higher at 8.32% (Deris et al., 2009). Hence, *A. baumannii* has gained notoriety as a nosocomial pathogen and also capable of acquiring and developing resistance to various antimicrobials. Strains that have been characterized as pandrug resistance (i.e., resistant to all known antimicrobial drugs) have been reported and thus pose a serious threat especially in the hospital environment (Ko et al., 2007; Peleg, Seifert, & Paterson, 2008).

With the development and advances in nucleotide sequencing, the latest sequencing platform known as next generation sequencing (NGS) enabled the entire genomes of microbial pathogen including *A. baumannii* to be sequenced (Zhu et al., 2013; Mardis, 2008). Therefore, this technology provides an opportunity to evaluate resistance determinants and mechanisms, pathogenicity and evolutionary studies of *A. baumannii* at a genome-wide level (Zhu et al., 2013; Mardis, 2008). Various NGS platforms have been helpful in providing an in-depth understanding of the genetic content and the differences between strains of *A. baumannii* and have helped to reveal factors that contribute to its adaptation and invasiveness in clinical settings, solutions in controlling its spread and possibly, an effective treatment to its infections.

To date, the genome sequence of several strains of *A. baumannii* have been reported (Fournier et al., 2006; Smith et al., 2007; Adams et al., 2008; Di Nocera, Rocco, Giannouli, Triassi, & Zarrilli, 2011; Snitkin et al., 2011; Huang et al., 2012; Liu et al., 2013; Tan et al., 2013; Zhu et al., 2013; Hamidian & Hall, 2014; Hamidian et al., 2014). The genome size of *A. baumannii* ranges from 3.2 Mb to 3.9 Mb and it is composed of a single chromosome with usually more than one plasmid (Fournier et al., 2006; Vallenet et al., 2008; Imperi et al., 2011). Fournier et al., (2006) reported the presence of two to three plasmids in their strains isolated in France. Besides, genome analysis of *A. baumannii* also revealed multiple genomic islands that have distinct functions in the microorganism. An 86 kb genomic island designated AbaR1 resistance island was discovered in *A. baumannii* AYE from France and this island contained up to 45 genes that could mediate resistance towards various antimicrobials (Fournier et al., 2006). Since the discovery of AbaRI, many other resistance islands have been reported from *A. baumannii* strains from other parts of the globe (Post & Hall, 2009; Post et al., 2010; Nigro & Hall, 2012; Post et al., 2012; Seputiene et al., 2012; Nigro et al., 2013) and these islands serve as convenient reservoirs for the dissemination antimicrobial resistance determinants.

1.2 OBJECTIVES

To date, there has been very little data regarding the extent of multidrug resistance among Malaysin strains of *A. baumannii* and so far, no complete genome sequences of *A. baumannii* from Malaysia or Southeast Asia has been reported. Hence, a complete genome analysis of Malaysian *A. baumannii* hospital strains would provide the tools to better understand the nature and extent of multidrug resistance,

their potential repertoire of virulence and pathogenicity genes and the overall biology of the locally isolated bacterial strains.

Therefore, the objectives of this study are:

1. To determine the antimicrobial susceptibility profiles, in particular the extent of carbapenem and polymyxin resistance in *A. baumannii* strains isolated from a local tertiary hospital in Malaysia;
2. To assess the genetic diversity of the clinical strains of *A. baumannii* ;
3. To determine the genomic content and variation of two extensive-drug resistant *A. baumanii* and one multidrug-resistant *A. baumannii* using comparative genomics approach;
4. To identify the repertoire of genes responsible for various resistance mechanisms, virulence and pathogenicity and their genetic structures from the whole genome sequences of the local *A. baumannii* strains.

CHAPTER 2

2.1 LITERATURE REVIEW

2.1.1 Developing resistance in *Acinetobacter baumannii*

Acinetobacter baumannii is a non-motile, Gram-negative bacterium from the *Acinetobacter* genus. This genus was first described by Beijerinck in 1911 (Gordon & Wareham, 2010) and strains of this genus were discovered to be involved in several nosocomial infections during the 1970s (Bergogne-Bérézin & Towner, 1996). Within a short span of about two decades, this pathogen had acquired the ability to develop resistance to most antimicrobials used in hospitals including β-lactams, aminoglycosides and fluoroquinolones (Diancourt et al., 2010; Di Nocera et al., 2011; Peleg et al., 2012). Emergence of multidrug resistant (MDR) *A. baumannii*, especially carbapenem resistance, soon became a global sentinel event and within a short span of time, outbreaks of carbapenem-resistant *A. baumannii* were reported worldwide (Nordmann and Poirel 2008; Evans et al. 2008). Carbapenem resistance in *A. baumannii* was a critical issue as carbapenems were used as drug of 'last resort' in intensive care units (ICU) for the past decade, before the reintroduction of polymyxins as the last line of antimicrobial therapy for *Acinetoabcter* infections when the remaining antibiotics became ineffective (Diancourt et al., 2010).

2.1.2 Reintroduction of Polymyxins and Impacts

As carbapenems were increasingly compromised by the over production of carbapenemases in *A. baumannii*, a more drastic choice of drug became necessary.

The polymyxins which are a class of drugs that were abandoned for use due to their neurotoxicity and nephrotoxicity, were recruited back for use in hospital as drugs of "last resort" for the treatment of *Acinetobacter* infections (Landman et al., 2008; Park et al., 2011). Considered as an old class of drug, polymyxins were first discovered in the 1940s and were produced by *Paenibacillus (Bacillus) polymyxa* (Landman et al., 2008). These compounds were cyclic, positively-charged peptide antibiotics which were effective against various Gram-negative pathogens (Landman et al., 2008), including *A. baumannii*.

Polymyxins are effective against various nosocomial MDR *A. baumannii* due to its cationic characteristic that will bind to the negatively charged outer wall of the *A. baumannii* cell membrane. Once *A. baumannii* gets in contact with the compound, the membrane integrity will be disrupted by its detergent-like effect (Landman et al., 2008). It is greatly effective in getting rid of the pathogen when used along with hydrophobic antibiotics, such as erythromycins. This is due to the pathogen becoming more susceptible to other antibiotics after the rupture of its membrane. Besides that, polymyxins are also capable of causing damage to the pathogen via cytoplasmic leakage and cationic binding site failure. Through displacing of Mg^{2+} and Ca^{2+} in the cationic binding sites, polymyxins compromises the cell membrane integrity and thus leading to death of *A. baumannii* (Landman et al., 2008).

Although the re-use of polymyxins as a therapeutic option led to successes in controlling carbapenem-resistant *Acinetobacter* infections, it was later discovered that acquired resistance to polymyxin was becoming increasingly prevalent (Arroyo et al, 2011; Park et al, 2011). Exposure to polymyxins led to the modification of the outer membrane in *A. baumannii*, resulting in reduced charge to the membrane,

thereby rendering the drug ineffective in destroying the pathogen. Modification of the outer membrane charge was caused by changes in the lipid-A moieties 4'-phosphate and the glycosidic diphosphate (Landman et al., 2008; Arroyo et al., 2011: Park et al., 2011). It was even reported that in some polymyxin-resistant *A. baumannii*, the development of polymyxin resistance was due to the complete loss of the outer membrane lipopolysaccharide (LPS) (Arroyo et al., 2011; Park et al., 2011).

2.1.3 Molecular Subtyping of *A. baumannii*

The genus *Acinetobacter* has a complex taxonomic history. Since the 1970s, bacteria of this genus suffered a long history of taxonomic changes before it was classified. Although it has been known that Gram-negative and non-fermentative are among the basic features to phenotypically classify bacteria into the genus *Acinetobacter*, *Acinetobacter* species had been previously recognized by various nomenclatures. Among the different "generic" names given to bacteria of this genus were *Bacterium anitratum, Herellea vaginicola, Mima polymorpha, Achromobacter, Alcaligenes, Micrococcus calcoaceticus* and "B5W", *Moraxella glucidolytica* and *Moraxella lwoffii* (Bergogne-Bérézin & Towner, 1996). The appearance of these different names had given rise to further detailed and often confusing identification of the *Acinetobacter* species (Bergogne-Bérézin & Towner, 1996).

Early delineation and identification of members of the *Acinetobacter* genus revealed that they were heterogeneous, and this became a problem in classifying them. It was only in 1986 that *Acinetobacter* species were classified by a more detailed phenotypic system, which was proven useful in identifying most of the

Acinetobacter species (Bergogne-Bérézin & Towner, 1996; Dijkshoorn et al., 2007). Nevertheless, there were still *Acinetobacter* species which were inseparable using this phenotypic system. Therefore, DNA hybridization and sequencing were developed to introduce a more accurate and reliable determination of these unidentified *Acinetobacter* species. These species were later known as *A. calcoaceticus*, *A. baumannii*, genospecies 3, genospecies 13 TU, "close to TU13" and "between 1 and 3" (Bergogne-Bérézin & Towner, 1996; Misbah et al., 2005; Dijkshoorn et al., 2007). Despite their heterogeneity, DNA hybridization of these bacterial species revealed that they shared high similarity in their DNA-DNA relatedness. Hence, they were conveniently grouped as the *A. calcoaceticus-A. baumannii* (Acb) complex.

Since *A. baumannii* is widely involved in hospital infections and has been identified as multidrug, and even pan-drug resistant pathogens, it is important to be able to identify and distinguish *A. baumannii* from other species within the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (Acb) complex (Dijkshoorn et al, 2007). This could provide a better understanding on the mode of transmission and possibly, better control, especially during nosocomial outbreaks (Kong et al, 2011). Hence, various molecular identification methods for *A. baumannii* have been developed. These includes pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), amplified ribosomal DNA restriction analysis (ARDRA), 16S DNA identification, REP-PCR and multilocus sequence typing (MLST) (Dessel et al., 2004; Misbah et al, 2005; Dijkshoorn et al., 2007).

In order to distinguish strains of the same species from different sources, time and space; a more discriminating typing method was developed, which is PFGE (Dijkshoorn et al., 2007; Karah et al, 2012). PFGE applies the macrorestriction method to differentiate closely related bacteria, designated as clones under the same species. This typing method has been considered as a 'gold standard' in molecular typing of *A. baumannii* and most other bacterial pathogens (Hamouda et al, 2010; Karah et al., 2012). This macrorestriction-based typing method generates distinct patterns of chromosomal DNA fragments, via digestion using rare-cutting enzymes. The resulting large fragments were then separated in an altering electric field (i.e. pulsed field) and bands were sorted according to their various molecular sizes (Dijkshoorn et al., 2007). Being considered as the most discriminatory method for *A. baumannii*, PFGE is able to infer the mode of transmission (i.e. intra- and inter- hospitals) and it is also a well-suited method to investigate local short-term outbreaks (Hamouda et al., 2010; Villalón et al., 2011; Karah et al., 2012). Despite its highly discriminative nature (Hamouda et al., 2010), PFGE also has its drawbacks; the main drawback being the data produced by PFGE cannot be transferred across different labs and it is considered costly as compared to other typing methods (Bartual et al, 2005; Karah et al., 2012).

Another recently developed typing method, multilocus sequence typing (MLST), has been increasingly used for molecular typing of *A. baumannii*, especially since the introduction of whole genome sequencing. MLST function by comparing sequences from seven different housekeeping genes to the sequences in MLST databases. To date, there are two MLST schemes that apply seven housekeeping genes into typing *A. baumannii*; the Bartual scheme and the Pasteur scheme (Jolley

et al., 2004; Bartual et al., 2005; Karah et al., 2012). The seven housekeeping genes used in the Bartual scheme are *gltA, gyrB, gdhB, recA, cpn60, gpi* and *rpoD* (Bartual et al., 2005) whereas housekeeping genes used in the Pasteur scheme are *gltA, pyrG, rplB, recA, cpn60, fusA* and *rpoB* (Jolley et al., 2004). These two schemes compare DNA sequences of different housekeeping genes and classify them accordingly; combinations of the seven housekeeping genes' classes will yield the appropriate sequence type (ST) of different *A. baumannii* strains. Besides the Bartual and Pastuer schemes that are well recognized and widely accepted in publications, there is also another recently introduced typing scheme which only requires DNA sequences from three different genes. This recent scheme was designated the tri-locus sequence typing (3LST). 3LST was developed by the Health Protection Agency of the United Kingdom (UK-HPA) and it requires only the *csuE, ompA* and $bla_{\text{OXA-51}}$ gene sequences to complete the typing of *A. baumannii* (Giannouli et al., 2009; Turton et al., 2011; Gogou et al., 2011). MLST opens the possibility of transferring data across labs and the results can be compared, however, it could be costly to perform Sanger dideoxy sequencing for all seven housekeeping genes when there are large number of samples.

2.1.4 *A. baumannii* Genomes and Next Generation Sequencing (NGS)

Epidemic *A. baumannii* strains mainly belonged to ST1 or ST2, especially strains that were characterized as MDR (Imperi et al., 2011). Lineages of these ST types were also referred to as Clonal Complex (CC) or global clonal (GC) I or II since the 1980s (Imperi et al., 2011; Tan et al., 2013b). Over the years, *A. baumannii* from these lineages have greatly imposed serious health care issues in hospitals and are difficult to treat due to its MDR phenotype (Imperi et al., 2011; Poirel et al., 2011).

However, there are still gaps in our understanding of its pathogenicity, adaptability, virulence and even invasiveness (Imperi et al., 2011; Sahl et al., 2011a; Peleg et al., 2012). When the first whole genome sequence of *A. baumannii* ATCC17978 was compared to the non-pathogenic soil bacterium *A. baylyi* ADP1, it led to the analysis and comparison of *Acinetobacter* genomes to highlight differences that reflect the adaptation and survival of the organism in a specific environment (Vallenet et al., 2008; Imperi et al., 2011; Gordon & Wareham, 2010). This is especially reflected in the ability of *A. baumannii* to survive in the human host and the hospital environment (Vallenet et al., 2008; Gordon & Wareham, 2010).

With the rapid advances in DNA sequencing and assembly approaches in the past decade, next generation sequencing (NGS) platforms such as Roche/454 FLX, Applied Biosystem SoLiD and Illumina were introduced (Mardis, 2008). Besides fast and high thorughput sequencing, these platforms enable genome explorations and provided answers to genome-wide biological questions (Mardis, 2008; Almeida & Araujo, 2013). Simultaneous sequencing of crucial genes involved in the biological functions of pathogens, such as drug resistance and virulence, generate informative data and help in revealing its detailed function in the genome (Almeida & Araujo, 2013). Previously used traditional epidemiology and/or concepts were clearly redefined with NGS, and combination of both will definitely provide a more in-depth understanding of *A. baumannii* (Sahl et al., 2011; Almeida & Araujo, 2013). Currently, there are more than 100 completely seqeunced *A. baumannii* genomes available, making higher possibilities in comparing closely related strains (Imperi et al., 2011; Almeida & Araujo, 2013).

In general, the GC content of *Acinetobacter* species is approximately 40% (Fournier et al., 2006; Vallenet et al., 2008; Sahl et al., 2011). Analysis on *Acinetobacter* genomes revealed the presence of a single chromosome and several plasmids present in strains AYE and SDF (Vallenet et al., 2008). However, these multiple copies of plasmids were not found in the environmental strain, *Acinetobacter baylyi* (Fournier et al., 2006; Adams et al., 2008; Vallenet et al., 2008). *A. baumannii* strains also feature unique genomic islands, such as those found in *A. baumannii* AYE, ACICU, AB0057 and 3208, which were designated AbaR1 (AbaR for *Acinetobacter baumannii* Resistance Island), R2, R3 and R5 respectively. Among these genomic islands, it was reported that the 86 kb AbaR1 genomic island was by far the largest (Fournier et al., 2006; Adams et al., 2008; Vallenet et al., 2008; Sahl et al., 2011). In the genome of the MDR clinical strain AYE from France, 52 genes related to antibiotic resistance were discovered and 45 of these resistance genes were clustered into this 86 kb genomic island which has been designated as a resistance island (Fournier et al., 2006).

Within the AbaR1 resistance island, the antibiotic resistance genes were found tightly clustered and the island had disrupted a putative ATPase open reading frame (designated *comM*) in the *A. baumannii* AYE genome (Fournier et al., 2006; Nordmann & Poirel, 2008). The insertion site was flanked at both extremities by a five nucleotide direct repeat (Vallenet et al., 2008; Nordmann & Poirel, 2008). Repetition that flanks the insertion site was probably due to duplication, which suggests a transposition mechanism for the insertion (Vallenet et al., 2008; Nordmann & Poirel, 2008).

To date, most of the *A. baumannii* genomes that have been reported were from the global clone (GC) 1 and 2 lineages which were more disseminated worldwide among the three GC (i.e. GC1, GC2 and GC3; previously known as European Clone (EC) I, II and III during the 1990's) (Kim et al., 2013). These reports of *A. baumannii* include the discovery of novel structures of resistance islands (RIs), of which AbaR-type resistance islands were the most prevalent across different strains. Most of these AbaR-type RIs interrupt the *comM* gene which is likely a hotspot for the insertion of these AbaR RIs. Among the different AbaR-type RIs, AbaR4-like RIs were found to be widely distributed, and untill now, the AbaR4-like islands can be subdivided into eight subtypes (Kim et al., 2013). Comparisons between the AbaR4-like RIs showed the simplest structure under its lineage was Tn*6022*. The Tn6022 structure carries only nine genes and is the backbone of most AbaR4-like RIs. The AbaR4-like RIs continues to evolve across different strains. AbaR4-D36 and AbaR4-AB210 were examples of extended AbaR4-like resistant islands, whereby both islands were formed from the basic backbone of Tn*6022* with an extra $bla_{\text{OXA-23}}$ embedded within Tn*2006* (Kim et al., 2013). Further expansion of this lineage includes Tn*6166*, which can be further divided into three subtypes (i.e. Tn*6166*-I type, Tn*6166*-II type and Tn*6166*-III type). Tn*6166* itself was an expansion from AbaR4-AB210 added with genes such as Δ*tetA*, *tetR*, *CR2*, *strB*, *strA* and *orf4b*. Another RI which belonged to the lineage of AbaR4-like RI was the AbaR25-type. AbaR25-type RIs can be subtyped into AbaR25-I and AbaR25-II, and these two subtypes were the largest RIs under the lineage. This AbaR25-type was an expansion from Tn*6166*, with an addition of an extra copy of Tn*2006* (Kim et al., 2013).

Comparisons between the lineages of AbaR4-like RIs revealed changes in the RIs due to occasions of addition, deletion and recombination. For example, the AbaR4-D36 type and AbaR4-AB210 type shared the same Tn*2006*, however, their differences lies within the transposons (Kim et al., 2013). AbaR4-D36 was known to carry a complete set of transposons, spanning from *tniC* to *tniE* (i.e. which includes *tniC*, *tniA*, *tniB*, *tniD* and *tniE* in the region). However, in AbaR4-AB210, *tniD* was found missing from the set and, only partial *tniB* and *tniE* was present instead of the complete gene. Transposons were a common feature in AbaR4-like RIs, this was proven again in Tn*6166*-II and Tn*6166*-III types. Although the traditional type of Tn*6166*-I harbors Tn*2006* along with the presence of $bla_{\text{OXA-23}}$, this feature, was absent from its subtypes – i.e., the Tn*6166*-II and Tn*6166*-III types. Reports also showed that comparison between the subtypes of Tn*6166* revealed the presence of *tniB, tniD* and *tniE* in Tn*6166*-II but not in Tn*6166*-I and Tn*6166*-III (Kim et al., 2013). Similar to the Tn*6166*-I type, AbaR25-I type carries a copy of Tn*2006*. Major differences between the AbaR25-I and AbaR25-II type lies within the presence and absence of Tn*2006*, even though both types were likely the result of recombination of an AbaR4-D36 type and a fragment from AbaR25 (Kim et al., 2013).

Although the GC content across *Acinetobacter* species may be similar (Fournier et al., 2006; Vallenet et al., 2008; Sahl et al., 2011), differences in the genomic content were still present. Comparison of different *Acinetobacter* genomes showed two distinct types of gene pool, namely the core genome and flexible gene pool. The core genome varies across *Acinetobacter* species (i.e., strain AYE, SDF and *A. baylyi*), ranging from 57.2% to 70.7% of the DNA sequences. Whereas for the

flexible gene pool, each of the *Acinetobacter* species contains approximately 800 genes, but only 361 genes were reported to be shared between strain AYE and *A. baylyi*. This provides evidence that there were genomic differences that were associated with adaptation in various changing environments.

2.1.5 Antibiotic Resistance and Resistance Mechanisms

The remarkable ability and adaptability of *A. baumannii* to the harsh hospital environment where the bacteria is constantly bombarded by disinfectants and antimicrobial drugs led to the rapid development of multidrug resistant (MDR), extensive drug resistant (XDR) and even pandrug resistant (PDR) *A. baumannii* (Camp and Tatum, 2010) in which the bacteria is resistant to all classes of antimicrobials. *A. baumannii* likely emerged as a notorious pathogen in the hospital settings as it is in close association with other Gram negative bacteria, which enables *A. baumannii* to acquire an impressive array of resistance mechanisms, on top of its own intrinsic ability (Poole, 2004; Camp and Tatum 2010). Besides acquisition of new mobile genetic elements, the most common yet powerful mechanism to repel most effects from antibiotics is the production of enzymes in *A. baumannii*. The most common antimicrobial enzyme produced is β-lactamase, which hydrolyses and breakdown the antibiotic molecules, resulting in *A. baumannii* that are resistant towards β-lactams and even carbapenems (Poole, 2004; Nordmann and Poirel 2008; Camp and Tatum 2010). Genes encoding for β-lactamases in *A. baumannii* can be over expressed and/or acquired, they can be passed down from parent to the next generation of pathogen. Hence, with continual exposure to antibiotics, *A. baumannii* will become increasingly resistant and almost

invulnerable in the hospital settings. (Poole 2004; Nordmann and Poirel 2008; Camp and Tatum 2010; Poirel et al., 2011).

The β-lactamases produced from *A. baumannii* can be classified by their different functional groups. Briefly, β-lactamases are divided into two major functional groups, which are the serine group and the metallo group (Figure 1). Members of the serine group are metal-independent enzymes whereas the metallo group members are metal-dependent enzymes (Poole, 2004). The serine group includes Class A (carbapenemase, i.e. KPC), C (Cephalosporinases, i.e. AmpC) and D (oxacillinase, i.e. OXAs) β-lactamases; whereas Class B β-lactamases belong to the metallo group (Figure 1). β-lactamases that are classified as carbapenemases are either from Class A, Class D or Class B (Nordmann & Poirel, 2008). The major mechanism of defense in *A. baumannii* against β-lactam antibiotics is through the production of β-lactamase enzymes. Other defense mechanisms include diminished expression of outer membrane proteins, mutated topoisomerase and also up-regulation of efflux pumps (Bonomo & Szabo, 2006).

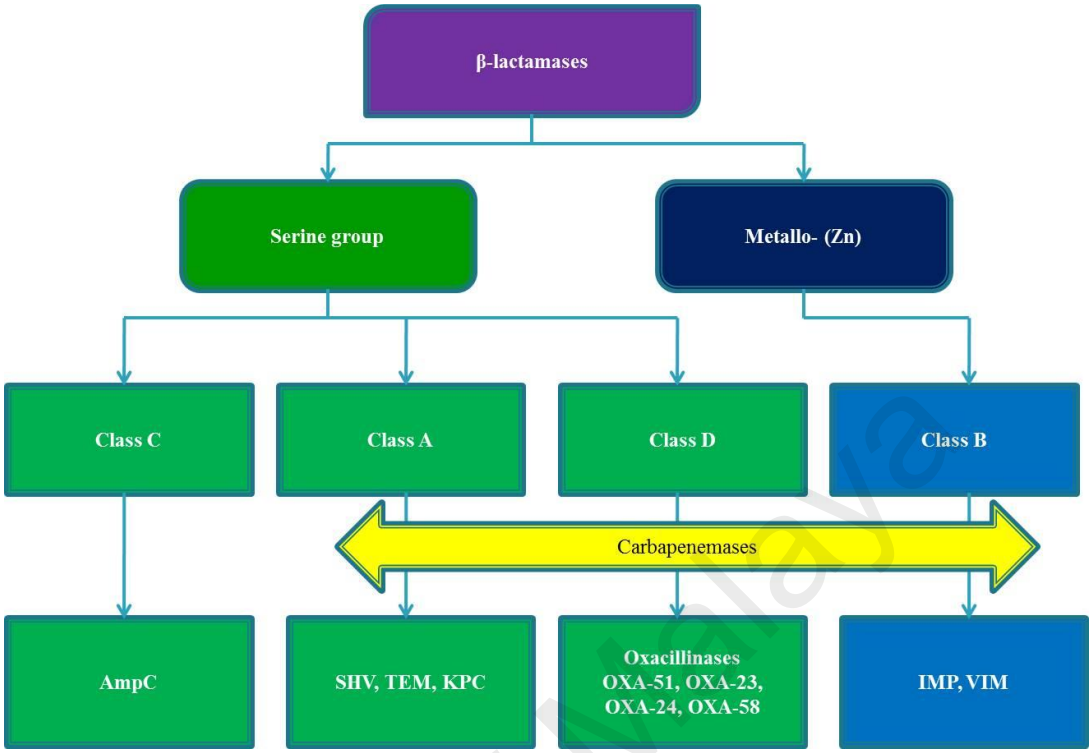


Figure 1: Classification of the various β-lactamases produced in *Acinetobacter baumannii*.

Carbapenems such as imipenem and meropenem were dubbed as the drug of last resort for *Acinetobacter* infections in the past two decades. Antibiotics of this class were important β-lactams due to their activity against β-lactamases, it is especially effective in treating infections caused by extended spectrum β-lactamase (ESBL) producing *A. baumannii*. Nevertheless, this class of antibiotics has been increasingly compromised by the production of specific class A, B and D β-lactamases from *A. baumannii* termed carbapenemases that were not inhibited by EDTA and can occur naturally and/or acquired across clinical pathogens. Of particular, class B and D carbapenemase have been widely disseminated and associated with high resistance against carbapenems. For instance, IMP and VIM

were carbapenem-hydrolyzing metallo-β-lactamases that belonged to class B β-lactamase. The IMP carbapenemase was first reported in Japan and has now evolved into a vast diversity (such as IMP1-9, IMP-10, IMP-11, IMP-12, IMP-13, etc.) (Poole, 2004). IMP carbapenemases function to hydrolyze almost all β-lactams except monobactams. Similar to IMP carbapenamase, VIM hydrolyzes most β-lactams except monobactams. VIM was first discovered in Italy and until now, there are at least seven VIM-type carbapenemases described (Poole, 2004; Bonomo & Szabo, 2006). Production of these class B carbapenemase in *A. baumannii* became one of its vital resistance mechanisms against currently used antibiotics, as well as new-generation of antibiotics.

Another class of β-lactamases that is of public health concern is the class D oxacillinases (OXAs) and β-lactam resistance in *A. baumannii* is often related to the expression of OXA genes (Feizabadi *et al*., 2008; Thapa *et al.*, 2010). There is a large variety in the OXA lineage, and in *A. baumannii* some of them can be naturally occurring whereas others can be acquired by horizontal gene transfer. Generally, there are eight subgroups of OXA enzymes identified in *A. baumannii*, the most frequently reported were the OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like (Feizabadi *et al.*, 2008). Among these four identified subgroups, OXA-23-like and OXA-51-like carbapenemases were acquired and naturally occurring, respectively. These OXAs were reported mostly in *A. baumannii* ranging from Europe to Asia, and exert activity against various β-lactams. The emergence of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ genes in mediating carbapenem resistance in *A. baumannii* is due to the over-expression of these genes (Nordmann & Poirel, 2008). Expression of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ can be further enhanced by the insertion of the IS*Aba*1

insertion sequence element upstream of the carbapenemase encoding gene (Zavascki *et al*., 2010). This is due to the presence of an outward directing promoter at the end of the IS element. Acquisition of the IS*Aba*1 and IS*Aba*9 elements upstream of the OXA-type carbapenemase encoding genes may cause the increased rate of imipenem hydrolysis (Zavascki *et al*., 2010). Other than these OXA genes, there are also several other acquired genes that produce β-lactamases such as bla_{IMP} and bla_{VIM} (Poole, 2004).

Another class of β-lactamases produced by *A. baumannii* that is of great concern are the AmpC enzymes (class C cephalosporinases) that target extended spectrum β-lactams especially the cephalosporins (Poole, 2004; Nordmann & Poirel, 2008). This class C cephalosporinase plays an important role in the development of multiple β-lactam resistance in *A. baumannii*, as it demonstrates resistance to most penicillin, cephalosporins, oxyminocephalosporins, cephamycins as well as monobactams (Poole, 2004). Although AmpC usually retain its susceptibility against carbapenems and fourth-generation cephalosporins, effectiveness of these antibiotics can be compromised by the overexpression of AmpC (Poole, 2004; Nordmann & Poirel, 2008). Resistance to fourth-generation cephalosporin has been recently reported due to the presence of mutant AmpC enzymes in *A. baumannii*, which greatly induce the production of AmpC (Bou & Martínez-Beltrán, 2000; Rodríguez-Martínez et al., 2010). Reports from Rodríguez-Martínez et al (2010) also showed that the hyper-expression of bla_{AmpC} can be the result of the insertion of upstream IS elements which provide the bla_{AmpC} with a strong promoter. Hence, the upregulation of mutant bla_{AmpC} contributed resistance to many of the newer generation of β-lactams.

Genes encoding for aminoglycoside-modifying enzymes such as acetyltransferase, phosphotransferase and nucleotidyl transferase are commonly found within class 1 integrons in *A. baumannii* (Camp and Tatum, 2010). Another aminoglycoside resistance determinant, mediated by the *armA* gene which leads to 16S rRNA methylation thereby preventing the binding of aminoglycoside to its target site, is commonly found within Tn*1548* and is plasmid-borne (Dolejska et al., 2013).

Mutations in the *gyrA* (encoding DNA gyrase) and *parC* (encoding DNA topoisomerase) genes are well known in *A. baumannii* to mediate resistance towards fluoroquinolones by interfering with target site binding. Many quinolones are also substrates for efflux pumps such as AdeABC and AdeM (Nordmann & Poirel, 2008; Camp & Tatum, 2010; Poirel et al., 2011; McConnell et al. , 2013).

Porins, efflux pumps and membranes are part of the *A. baumannii* intrinsic resistance mechanisms (Poole, 2004; Nordmann & Poirel, 2008; Camp et al., 2010; Poirel et al., 2011). These form the secondary resistance mechanisms when *A. baumannii* is exposed to antibiotics in its environment. When porins (i.e. outer membrane protein, OMP) function to allow the passage of metabolites, antibiotic molecules will be transported via these porins as well (Camp & Tatum, 2010; Poirel et al., 2011). Hence, as larger numbers and sizes of porins are present on the cell membrane, these will directly cause the cell membrane to be more permeable and the pathogen susceptible to antibiotics. However, *A. baumannii* has been reported to have fewer porins on its outer membrane. Besides that, its small sized porins also made *A. baumannii* a good fit in developing antibiotic resistance (Camp & Tatum, 2010). In order to protect itself from the effects of antibiotics, *A. baumannii* carries another powerful mechanism - efflux pumps. Efflux pumps provide the ability to

pump out antibiotic molecules from the cell, and therefore, these became a source of resistance to various antibiotics (Poole, 2004; Nordmann & Poirel, 2008; Camp & Tatum, 2010). For instance, TetA and TetB are one of the resistance determinants carried in *A. baumannii*, and these determinants function as specific efflux proteins for tetracyclines (Nordmann & Poirel, 2008; McConnell et al., 2013). The presence of these pumps will prevent ribosome from binding with tetracycline, thereby protecting the cell from being disrupted by the antibiotic (Nordmann & Poirel, 2008).

The rapid development of resistance in *A. baumannii* has led to decreasing therapeutic options. The wide dissemination of acquired carbapenemases marked the end of carbapenems as the drug of choice for *A. baumannii* infections. Now, XDR *A. baumannii* is mostly only susceptible to polymyxins; however, the intensive use and lack of optimal dosing had created an opportunity for *A. baumannii* to develop resistance towards drugs from the polymyxins group (Arroyo et al., 2011). As polymyxins work to bind with the polyanionic lipopolysaccharide (LPS) layer of *A. baumannii* causing disorganization of the outer membrane, this pathogen developed resistance through covalent modification of the lipid A (i.e. lipid anchor) moiety in the LPS layer (Hood et al., 2010; Arroyo et al., 2011; Park et al., 2011; Hood et al., 2013). This modification (the addition of phosphoethanolamine to lipid A) enables *A. baumannii* to reduce its net negative charge on the surface of the outer membrane, hence decreasing the affinity of polymyxins. Phosphoethanolamine modification of lipid A in *A. baumannii* is mediated by the *pmrCAB* operon and mutations in these genes have been found in polymyxin-resistant strains (Adams et al., 2009; Arroyo et al., 2011; Beceiro et al.,

2011; Park et al., 2011). *pmrAB* encode a two-component regulatory system and upregulation of both *pmrB*, which encode the sensor kinase, and *pmrA*, which encode the response regulator, have been observed in polymyxin-resistant strains. How changes in the expression levels of *pmrAB* affect *pmrC* which catalyzes the phosphoethanolamine addition to lipid A is currently unknown. Resistance to poymyxins in *A. baumannii* was also found to be mediated by a complete loss in LPS production, likely due to mutations in the LPS biosynthesis genes *lpxA*, *lpxC*, *lpxD* and *lpsB* (Moffatt et al., 2011; Hood et al., 2013).

The combinations of mechanisms involving acquisition of resistance genes, overexpression of β-lactamases and efflux pumps, mutations in resistance determinants, loss of porins and modification of the LPS outer membrane in *A. baumannii* are indeed a nightmare for clinicians and the healthcare industry (Camp & Tatum, 2010; Hood et al., 2010). This is an especially worrisome phenomenon, as heteroresistance and complete resistance to the polymyxins group of antimicrobials are increasingly reported (Hood et al., 2010; Arroyo et al., 2011; Park et al., 2011; Hood et al., 2013). With very few therapeutic options left to treat XDR *A. baumannii*, the clonal spread of pandrug resistant (PDR) strains would be of utmost concern as this scenario would leave us with no treatment options from our current antimicrobial arsenal.

CHAPTER 3

3.1 METHODOLOGY

3.2 MOLECULAR SUBTYPING

3.2.1 Bacterial Strains

Fifty-four non-repeat clinical strains of *A. baumannii* were collected throughout 2011 from sporadic cases of infection in Hospital Sultanah Nur Zahirah (HSNZ), the main tertiary hospital in Kuala Terengganu, the capital of the state of Terengganu located in the east coast of Penisular Malaysia. These *A. baumannii* strains were isolated from patients admitted to HSNZ (Appendix 1) by the hospital laboratory staff. There was no specific selection criteria and the strains were obtained from the hospital laboratory as and when they were available. The strains were isolated and initially identified by standard biochemical methods at the clinical microbiology laboratory. The strains were subsequently validated as *A. baumannii* by PCR-amplification and sequencing of the 16S rRNA gene. All 54 strains showed >99% sequence identity with *A. baumannii* 16S rRNA gene, thus confirming their strain identity (Lean et al., 2014). Single colonies of *A. baumanii* were then cultured into Nutrient Broth and incubated at 37 ^{0}C for 24 hours for subsequent experiments.

3.2.2 Antibiotic susceptibility tests (AST)

The Kirby-Bauer disc diffusion method was used to test the susceptibility of the *A. baumannii* strains to the following 12 antibiotics (Oxoid Ltd., Basingstoke, UK): gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), piperacillin-tazobactam (100/10 μg), cefotaxime (30 μg), ceftazidime(30 μg), cefepime (30 μg), ampicillin-sulbactam(10/10 μg), tetracycline (30 μg), and doxycycline (30 μg). The antibiotic susceptibility test was carried out on Mueller Hinton (Hi media) Agar swabbed with 0.5 McFarland standard suspension of *A. baumannii* and incubated at 37 $^{\circ}$C for 24 hours. All the tests were conducted in triplicates. Guidelines from the Clinical and Laboratory Standard Institute (CLSI) (Clinical and Laboratory Standards Institute, 2013) were used to interpret the diameters of the inhibition zones produced.

3.2.3 MIC determination for carbapenems

To determine the susceptibility of these *A. baumannii* strains towards carbapenems, their minimum inhibitory concentration (MIC) values were measured. Bacterial cultures of *A. baumannii* adjusted to 0.5 McFarland standard were used to determine the MIC to imipenem and meropenem using M.I.C. Evaluator (M.I.C.E.) strips (Oxoid Ltd., Basingstoke, UK). The surface of Mueller Hinton (Hi media) Agar was swabbed uniformly with the bacteria culture using a sterile cotton bud. The agar surface was dried for approximately 15 minutes before applying the M.I.C.E. stripes. Inoculated media was incubated at 35 $\pm$ 2 ℃ for 16 – 20 hours. The MIC value of the respective inhibition ellipses that interacts with the stripes were taken and

recorded. Data were analyzed according to the guidelines given for M.I.C.E. strips and the MIC was determined.

3.2.4 MIC determination for polymyxin B

MIC values for polymyxin B were determined by the agar dilution method (Andrews, 2001). Agar dilution plates were prepared by using Mueller Hinton (Hi media) Agar added with concentrations of 2, 4, 8, 16, 32, 64 and 128 μg/mL polymyxin B sulfate (Sigma-Aldrich, St. Louis, Missouri, USA). A multipoint inoculator was used to deliver bacterial suspensions adjusted to 0.5 McFarland Standard onto the agar surface and the agar subsequently incubated at 35 ± 2 ℃ for 16 – 20 hours. The MIC value for polymyxin B was determined as the lowest concentration of the antibiotic in which the bacteria was susceptible. The standard breakpoint for resistance according to the CLSI guideline (CLSI, 2013) was ≥ 4 μg/mL.

3.2.2 Genotyping of *Acinetobacter baumannii*

Strain typing of *A. baumannii* was carried out by Pulsed-Field Gel Electrophoresis (PFGE), as previously described (Kong et al., 2011). Briefly, chromosomal DNA for PFGE analysis was prepared in agarose gel blocks, digested with restriction endonuclease *Apa*I (Promega, Madison, Wis., USA). Restriction fragments were separated by using the CHEF Mapper (Bio-Rad, Hercules, Calif., USA) with

0.5×TBE buffer for 26 hours at 14 ℃ with pulse times of 2 – 40 seconds. *Xba*I-digested *Salmonella enterica* ser. Braenderup H9812 was used as the molecular size standard (Kong et al, 2011). The PFGE profiles were analyzed using BioNumerics version 6.0 software (Applied Maths, Kortrijk, Belgium). The unweighted pair group method with averages (UPGMA), with a position tolerance for comparison of 1.5%, was used to produce the dendrogram (Kong et al, 2011).

3.3 WHOLE GENOME SEQUENCING

3.3.1 Strain selection and DNA extraction

Two extensive drug resistant (XDR), polymyxin-resistant strains (AC12 and AC30) and one multidrug resistant (MDR), polymyxin-susceptible strain (AC29) were chosen for whole genome sequencing. All three selected strains of *A. baumannii* were grown overnight under 37 ℃ in Nutrient Broth. Genomic DNA was extracted from these overnight cultures of *A. baumannii* using the Wizard Genomic DNA Purification Kit from Promega.

DNA extraction was carried out using 1 mL of the bacterial culture which was harvested by centrifugation for 2 minutes at 14000 rpm. The cell pellet formed was re-suspended in 600 μL of lysis solution and incubated at 80 °C for 5 minutes. Following that, 3 μL of RNase solution was added to the re-suspended pellet, mixed and incubated at 37 ℃ for 15 – 60 minutes. This was followed by the addition of 200 μL of protein precipitation solution and the tube was vortexed to mix. Then, it was placed on ice for 5 minutes prior to centrifugation in a microcentrifuge for 3 minutes at room temperature. The resulted supernatant was transferred into a new microcentrifuge tube containing 600 μL of isopropanol at room temperature, and then followed by centrifugation at 14000 rpm for 2 minutes to recover the precipitated DNA. The supernatant was discarded and 600 μL of room temperature 70% ethanol were added to wash the DNA. The solution was centrifuged for another 2 minutes at 14000 rpm, and ethanol was discarded before the DNA pellet was air-dried. The dried DNA pellet was resuspended in 100 μL sterile distilled

water and stored at 4℃ for short term storage or at -80 ℃/-20 ℃ for long term storage.

Extracted DNA was quantified using the spectrophotometer at OD_{260} and the purity was determined by OD_{260}/OD_{280} ratio (Ausubel et al., 2002). Pure preparations of DNA will have OD_{260}/OD_{280} values of 1.8 to 2.0 (Ausubel et al., 2002).

3.3.2 Genome sequencing, assembly and annotation

Whole genome sequencing of *A. baumannii* strains AC12, AC30 and AC29 were carried out by a commercial vendor using the Illumina Genome Analyzer IIx platform. CLC Bio software package was used to assemble the DNA sequencing data *de novo*. The sequences were also assembled by mapping to a reference genome, *A. baumannii* BJAB0715 (accession no. NC_021733.1), particularly in assembling the sequences for the genomic islands. Open reading frame (ORF) prediction and gene functional assignments were done by using Prodigal 2.60 (downloadable from http://prodigal.ornl.gov/) (Hyatt et al., 2010), RNAmmer 1.2 (http://www.cbs.dtu.dk/services/RNAmmer/) (Lagesen et al., 2007) and tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe & Eddy, 1997; Schattner et al., 2005). Functional annotation of the genome was performed by using Blast2Go (downloadable from http://www.blast2go.com/b2ghome) and Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/) (Aziz et al., 2008).

3.3.3 Genome analyses and comparative studies

Multilocus sequence typing (MLST) was performed *in silico* from the whole genome sequence data according to the two schemes available. The sequence types were determined by accessing the PubMLST database hosted at Oxford University (http://pubmlst.org/) which follows the Bartual scheme (Bartual et al., 2005) and the Institute Pasteur MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/) (Jolley et al., 2004). The phyologenetic tree was constructed based on the data from the published genomes of *A. baumannii* in the NCBI along with the three sequenced isolates in this study. The tree was constructed by using composition vector tree version 2 (Xu & Hao, 2009) and the Molecular Evolutionary Genetic Analysis (MEGA 5) program (downloadable from http://www.megasoftware.net/) (Tamura et al., 2011). Contigs arrangements and comparative assembly were identified using related reference based on contig arrangement tool (r2cat) (downloadable from http://bibiserv.techfak.uni-bielefeld.de/cg-cat/r2cat.html) (Husemann & Stoye, 2010). Circular chromosome and plasmid map of this strain was constructed using CGView Server developed by the Stothart Research Group (Grant & Stothard, 2008). Linear comparison figures were constructed using Easyfig 2.1 software (Sullivan et al., 2011). Genome sequences of *A. baumannii* AC12, AC30 and AC29 were compared using Mauve (Darling et al., 2010) to the completed *A. baumannii* genomes that were downloaded from the NCBI FTP site. To screen for resistance determinants, genes for efflux pumps and virulence genes, a local database of *A. baumannii* genomes including the three sequenced strains was first created using NCBI C++ toolkit. Local BLAST was then used to search for known resistance

determinants, efflux pumps and virulence genes which were obtained from Fournier et al (2006) and Peleg et al (2012).

3.3.4 GenBank accession number

The *A. baumannii* AC12, AC30 and AC29 genome sequences were deposited in GenBank under accession numbers CP007549, CP007577 and CP007535, respectively. In addition, plasmid sequences of pAC12, pAC30a, pAC30b, pAC30c, pAC29a and pAC29b have been deposited in the GenBank under accession numbers CP007550, CP007578, CP007579, CP007580, CP007536 and CP007537, respectively.

3.4 RESISTANCE MECHANISMS

3.4.1 Amplification of polymyxin resistant determinants and *bla*$_{\text{OXA-23}}$

Genetic determinants that have been implicated in conferring polymyxin resistance in *A. baumannii*, namely the *pmrCAB*, *lpxA*, *lpxC*, *lpxD* and *lpsB* genes were PCR-amplified using specific primers and conditions as shown in Appendix 2 on all polymyxin-resistant strains (14 strains) and 3 polymyxin-susceptible strains as controls. The 54 *A. baumannii* strains were also screened for the presence of the carbapenemase-encoding *bla*$_{\text{OXA-23}}$ by PCR using primers listed in Appendix 2. Conventional PCR was performed on a Veriti® Thermal Cycler (Applied Biosystems). Following PCR amplification, a 2 µL aliquot from each PCR reaction was electrophoretically separated on a 1% (w/v) agarose gel to ascertain the presence and size of the PCR products. PCR products of the expected size were purified using Wizard SV Gel and PCR Clean up System from Promega. The purified PCR products were then sequenced by automated Sanger dideoxy sequencing.

3.4.2 DNA Sequencing and Sequence Analysis

Sequencing of purified PCR products was carried out by automated Sanger dideoxy sequencing at a commercial facility (First Base Sdn. Bhd., Malaysia). DNA sequences obtained were edited using BioEdit v7.0.9 (downloadable from http://www.mbio.ncsu.edu/bioedit/page2.html) and analyzed with BLAST at the National Center of Biotechnology Information website

(http://www.ncbi.nlm.nih.gov/BLAST). Sequence analysis was also done by using ExPASy translate tool at the Swiss Institute of Bioinformatics website (http://web/expasy.org/translate/) and ClustalW2 multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3.4.3 Quantitative Real-time reverse transcriptase-PCR for determination of *pmrAB* transcript levels

Transcript levels for *pmrAB* were determined for the polymyxin-resistant *A. baumannii* AC12 and AC30. *A. baumannii* ATCC 19606 and AC29 were used as polymyxin-susceptible controls. *A. baumannii* strains were grown to log phase in LB broth and total RNA was extracted using Qiagen RNeasy Mini Kit. Firstly, 1 mL of the bacterial culture was pelleted. To the resulting cell pellet, 350 µL of buffer RLT was added and vortexed. The lysate was then mixed well with 350 µL of 70% ethanol by pipetting. All 700 µL of the sample was immediately transferred to an RNeasy Mini spin column, which was already placed in a 2 mL collection tube. RNeasy Mini spin column was centrifuged at ≥ 8000 × *g* for 15 seconds and the flow-through was discarded. The spin column was then subjected to DNase treatment by on-column DNase digestion (Qiagen). A total of 350 µL buffer RW1 was added into the spin column and centrifuged for 15 seconds at ≥ 8000 × *g*. The flow-through was discarded before adding 80 µL DNase I incubation mix directly to the spin column membrane which was then placed on the benchtop at room temperature for 15 minutes. Following this, 350 µL buffer RW1 was added to the column, which was then centrifuged again for 15 seconds at ≥ 8000 × *g* and the

resulting flow-through discarded. This step was repeated with 700 µL of buffer RW1, followed by washing with 500 µL buffer RPE twice. Finally, the spin column was placed in a new 1.5 mL collection tube and the purified RNA was eluted by adding 30 µL RNase-free water directly to the spin column membrane and subjecting the column to centrifugation for 1 minute at ≥ 8000 × *g*.

Extracted RNA is now ready for reverse transcription which was performed in a T-Gradient PCR machine from Biometra. Prior to reverse transcription, the extracted RNA was subjected to a genomic DNA elimination step using RNase-Free DNase (Qiagen). The reaction was prepared on ice according to the Table 1 below. The reaction was incubated for 2 minutes at 42 ℃ and placed onto ice upon completion.

Table 1: Components in genomic DNA (gDNA) elimination reaction

Component	**Volume/reaction**
gDNA wipeout Buffer, 7x	2 µL
Template RNA (100 ng/µL)	2 µL
RNase-free water	10 µL
Total volume	14 µL

After genomic DNA elimination, RNA was subjected to reverse-transcription using the Quantitect Reverse Transcription Kit (Qiagen). The master mix for reverse transcription was prepared on ice as in Table 2. When all the components were mixed, the reaction was incubated at 42 ℃ for 15 minutes. This is followed by a 3 minute inactivation of Quantitect Reverse Transcriptase at 95 ℃. Then, the reaction was placed on ice before proceeding to real-time PCR. The cDNA produced from this reverse transcription can be stored at -20 ℃ for long-term storage.

Table 2: Master Mix setup for reverse-transcription

Component	Volume/reaction
Quantiscript Reverse Transcriptase	1 µL
Quantiscript RT Buffer, 5×	4 µL
RT Primer Mix	1 µL
Template RNA (100 ng/µL)	14 µL
Total reaction volume	**20 µL**

Reaction master mix for real-time PCR (SYBR Green PCR Kit, Qiagen) was setup according to Table 3. The template used for each reaction was 2 µL (100 ng) of cDNA and primers designed for the amplification of *pmrAB* (Appendix 3). Components of the master mix were mixed thoroughly and aliquoted into PCR tubes prior to addition with template cDNA. The reaction was then placed into a Rotor-Gene 6000 Real-Time PCR Machine to start the programmed cycling condition. Real-time cycling condition for the reactions was started with 5 minutes PCR initial heat activation at 95 ℃, then the 2-step cycling process which includes 10 seconds of denaturation at 95 ℃ and then annealing at 60 ℃ for 30 seconds. Total number of cycles programmed for this real-time PCR was 35 cycles. During the experiment, melting curve profile for each amplification reaction and the relevant C_T value was automatically generated, using the software provided with the Rotor-Gene 6000 Real-Time PCR Machine (Corbett Life Science/Qiagen). The *rpoB* gene was used as the housekeeping gene for normalization with the primer sequences listed in Appendix 3 (Park et al., 2011). Relative quantification using the $\Delta\Delta C_T$ (Pfaffl, 2001) was then applied to quantify the expression of *pmrAB*.

Table 3: Real-time PCR Master Mix components

Components	Volume/reaction
2x Quantifast SYBR Green PCR Master Mix	12.5 µL
Forward Primer	0.3 µL
Reverse Primer	0.3 µL
RNase-free water	9.9 µL
Template cDNA (50 ng/µL)	2 µL
Total reaction volume	25 µL

3.4.4 LPS analysis

Modification of lipopolysaccharide (LPS) is one of the crucial features in the development of polymyxin resistance in *A. baumannii* (Moffatt et al., 2010; Arroyo et al., 2011; Moffatt et al., 2011; Hood et al., 2013). In order to investigate if there are any differences in the LPS of the sequenced polymyxin-resistant strains AC12 and AC30, LPS was extracted and analysed. *A. baumannii* AC29 and ATCC19606 were used as polymyxin-susceptible controls. LPS extraction was carried out according to Hood et al. (2013). *A. baumannii* strains that were subjected to LPS analysis were AC12 and AC30 (polymyxin resistant strains) with AC29 and ATCC19606 as polymyxin susceptible controls. Bacteria were swabbed from overnight LB agar plates into 154 mM NaCl, normalized to an OD_{600} of 1.5, pelleted by centrifugation and resuspended in lysis buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl, pH 6.8). Samples were then boiled for 10 min, cooled to 60 ℃ and treated with proteinase K for 1 hour. These samples were electrophoresed through a 15 % sodium dodecyl sulphate-polyacrylamide gel

(SDS-PAGE) and stained with Pro-Q Emerald 300 LPS stain according to the manufacturer's recommendations (Invitrogen).

SDS-PAGE apparatuses were prepared and assembled as follows. The glass plate sandwich of the electrophoresis apparatus was assembled using two clean glass plates and 0.75 mm spacers, according to the manufacturer's instruction (BioRad). The sandwich was then locked to the casting stand before casting the minigel. Separating gel solution was first prepared for the minigel, 15% resolving gel was chosen to separate the LPS sample. A total of 15 mL of the resolving gel was prepared, which consists of 3.4 mL of distilled water, 7.5 mL of 30% acrylamide mix, 3.8 mL of 1.5 M Tris (pH 8.8), 0.15 mL 10% SDS, 0.15 mL 10% ammonium persulfate and 0.006 mL TEMED. The resolving gel was pipetted into the sandwich along the edge of the spacers until the height of the resolving gel reached almost half of the glass plates. Immediately, distilled water was gently layered against the edge of one spacer to cover the top of resolving gel and it was left to polymerize at room temperature.

After the resolving gel has polymerized, the distilled water on top was discarded and the stacking gel solution was pipetted into the center of the sandwich. The 5% stacking gel solution prepared was 3 mL in volume, consisting 2.1 mL of distilled water, 0.5 mL 30% acrylamide mix, 0.38 mL 1.0 M Tris (pH8.8), 0.03 mL 10% SDS, 0.03 mL ammonium persulfate and 0.003 mL TEMED. This stacking gel solution was applied to the sandwich until its height is about 1 cm from the top of the plates. A 0.75 mm Telfon comb was immediately inserted into the sandwich and left to polymerize at room temperature. After polymerization, the Teflon comb was carefully removed without tearing the edges. The gel sandwich was then attached to

the buffer chamber and the chamber was filled with 1 × SDS electrophoresis buffer. 10 μL of molecular weight marker, control and samples, added with 5 μL of loading dye each, were then loaded into wells. Molecular weight marker used in the experiment was Precision Plus Protein Dual Xtra Stndards (BioRad). The minigel was then run at 100 V until the bromophenol blue has reached the bottom of resolving gel.

Following electrophoresis, the minigel was immersed into 100 mL of fix solution provided with the Pro-Q Emerald 300 LPS stain kit (Invitrogen). The solution and minigel were incubated in room temperature and agitated at 50 rpm for 45 minutes. When the SDS was fully washed out from the minigel, the minigel was rinsed with 100 mL of the wash solution provided. For this washing step, which was repeated twice, the minigel was incubated at room temperature for 10-20 minutes each wash and with slow agitation at 50 rpm. Before staining the SDS-PAGE minigel, the Pro-Q Emerald 300 staining solution was freshly prepared by diluting its stock solution 50 × with Pro-Q Emerald 300 staining buffer. Hence, a total of 500 μL Pro-Q Emerald 300 stock solution was diluted into 25 mL of staining buffer for each individual minigel. Together with 25 mL of the staining solution, the minigel was incubated and gently agitated in the dark for 90 – 120 minutes. Following this, the minigel was incubated with 100 mL of wash solution at room temperature for 15 – 20 minutes. The wash solution was discarded after each wash and this final washing step was repeated twice. Stained minigel was then viewed and photographed under UV light using a UV Gel Imager (Alpha-Innotech).

3.4.5 Cloning and Expression of the Novel *bla*$_{ampC}$ Gene from *A. baumannii* AC12, AC29 and AC30

PCR amplification of *bla*$_{ampC}$ genes from AC12, AC29 and AC30 were carried out using specific primers and conditions stated in Appendix 5. Purified PCR products were then cloned into pGEM-T Easy (Promega, USA), according to the manufacturer's instructions. Briefly, the ligation reaction of the *bla*$_{ampC}$ amplified products into pGEM-T Easy Vector was set up as in Table 4 and the reaction mixture was prepared on ice. The reaction was mixed by pipetting and then incubated at room temperature for an hour. After incubation, the ligation reaction was briefly centrifuged and 2 µL of the ligation reaction was transferred to a sterile 1.5 mL tube on ice. This procedure was followed by adding 100 µL of just thawed *E. coli* JM109 competent cells; the tube was gently flicked and incubated on ice for 20 minutes. The cells were heat-shocked for 45 seconds in water bath of 42 ℃, it was then immideately returned on ice for 2 minutes. A total of 900 µL of the SOC medium was added to the ligation reaction transformation and followed by incubation at 37 °C with shaking (~150 rpm) for 1.5 hours. 100 µL of the transformation culture was plated onto LB agar supplemented with 100 mM ampicillin (Amp), 100 mM IPTG and 50 mg/mL X-gal. Positive control and background control were also prepared as manufacturer's instructions. Plating of each transformation culture was duplicated. The plates were incubated overnight at 37 ℃. Selected white colonies was re-streaked onto LB + Amp Agar and grown for 16 hours at 37 ℃. Cells were then harvested for plasmid extraction using the Plasmid Miniprep Kit from Promega following the manufacturer's protocol (Promega, USA). Extracted recombinant plasmids were digested with restriction

enzyme *Eco*RI at 37 ℃ for 2 hours. Digested plasmid was electrophoresed using 1% (w/v) agarose gel in 1 X TBE as the running buffer to determine their presence and size. Plasmids extracted from successful pGEM-T Easy recombinant clones were sequenced by automated Sanger dideoxy sequencing for validation and the sequences obtained analyzed by BLAST for confirmation.

Table 4: Reagents used in ligation of pGEM-T Easy Vector

Reagents	**Standard Reaction**	**Positive Control**	**Background Control**
2 x Rapid Ligation Buffer	5 µL	5 µL	5 µL
pGEM-T Easy Vector (50 ng/µL)	1 µL	1 µL	1 µL
PCR product (100 ng/ µL)	2 µL	-	-
Control Insert DNA (4 ng/µL)	-	2 µL	-
T4 DNA Ligase (100 U)	1 µL	1 µL	1 µL
Deionised water	1 µL	1 µL	3 µL
Total Reaction Volume	10 µL	10 µL	10 µL

Once the pGEM-T recombinant plasmids were validated to contain the bla_{ampC} genes from AC12, AC29 and AC30, they were subcloned into pET30a expression vector for expression in *E. coli* BL21(DE3)(pLysS). The pGEM-T recombinant plasmid was digested with *Bam*HI and *Hin*dIII, which cut at the restriction sites that have been incorporated into the primers used for bla_{ampC} amplification. Similarly, the pET30a vector was also digested with *Bam*HI and *Hin*dIII. The digested pET30a vector and the bla_{ampC} fragment were purified from agarose gels using Wizard SV Gel and PCR Clean up System (Promega) and subjected to ligation using T4 DNA ligase overnight at 4 ^{o}C. The ligated products were initially transformed into *E. coli* JM109 and transformants selected on LB plates supplemented with 50 µg/ml kanamycin. Colonies on the selection plate were screened by plasmid extraction and digesting the extracted plasmid with *Bam*HI and *Hin*dIII prior to separation by

electrophoresis on 1% agarose gels. The validated pET30a-*bla*$_{ampC}$ recombinant plasmids were then transformed into *E. coli* BL21 (DE3/pLysS). Expression of the cloned *bla*$_{ampC}$ gene in *E. coli* BL21 was carried out by inducing the cells with 0.1 mM IPTG. After that, the *E. coli* BL21 clones were tested for their MIC values for extended spectrum cephalosporins (i.e., ceftazidime and cefepime), as well as aztreonam and imipenem at concentrations of 2, 4, 8, 16 and 32 μg/mL by using the agar dilution method.

CHAPTER 4

4.1 RESULTS

4.2 MOLECULAR SUBTYPING

4.2.1 Antimicrobial resistance profiles of the *A. baumannii* strains

The antimicrobial resistance profiles of 54 *A. baumannii* strains isolated from patients warded at Hospital Sultanah Nur Zahirah, Kuala Terengganu throughout 2011 were determined. Antibiotic susceptibility tests (AST) carried out indicated that the strains were highly resistant to the antibiotics tested (Table 5; Appendix 1). High levels of resistance were observed for the following antibiotics: tetracycline (87%), piperacillin-tazobactam (72.2%), cefotaxime (72.2%), ceftazidime (72.2%), cefepime (72.2%), levofloxacin (70.4%), ampicillin-sulbactam (68.5%), gentamicin (66.7%), ciprofloxacin (66.7%), tobramycin (64.8%), doxycycline (61.1%) and amikacin (57.4%). The strains also showed high levels of resistance to carbapenems with 77.8% of the strains resistant to meropenem and 74.1% resistant to imipenem. Of the 54 strains, 39 (or 72.2%) could be categorized as multidrug resistance (MDR) – i.e., resistant to more than three classes of antibiotics. All MDR strains were resistant to meropenem and imipenem.

The strains showed highest resistance levels to tetracycline (87%) whereas the lowest resistance was to amikacin (57.4%). Thus, the resistance rates to all the antibiotics tested were more than 50%, which is a cause for concern as it indicates that conventional antibiotics used for treating *A. baumannii* infections were losing their effectiveness against the pathogen.

The *A. baumannii* strains were further tested for susceptibility towards polymyxin B, the drug of "last resort" for *A. baumannii* infections. Polymyxin resistance was assessed by determining the MIC values for polymyxin B using the agar dilution method. Of the 54 strains, 14 (or 25.9%) were categorized as polymyxin resistant (MIC $\geq$ 4 μg/ml; CLSI, 2013). Four of these strains (AC12, AC27, AC30 and AC61) had MIC values of > 128 μg/ml for polymyxin B (Table 6). All 14 polymyxin-resistant strains could also be categorized as extensive-drug resistant (XDR) strains based on the criteria proposed by Magiorakos et al. (2011) (i.e., resistant towards all except one or two antimicrobials).

Table 5: Antibiotic susceptibility patterns of the 54 *A. baumannii* strains isolated from Hospital Sultanah Nur Zahirah (HSNZ) in 2011 according to disc diffusion method.

Antibiotics	Concentration (µg/disk)	Percentage (%)		
		R	I	S
CARBAPENEMS				
Meropenem	10	63	0	37
Imipenem	10	61.1	1.9	37
AMINOGLYCOSIDES				
Gentamycin	10	66.7	1.9	31.5
Amikacin	30	57.4	0	42.6
Tobramycin	10	64.8	0	35.2
ANTIPSEUPODIAL FLUOROQUINOLONES				
Ciprofloxacin	5	66.7	1.9	31.5
Levofloxacin	5	70.4	0	29.6
ANTIPSEUPODIAL PENICILLIN + β-LACTAMS				
Piperacillin-tazobactam	100/10	72.2	7.4	20.4
EXTENDED SPECTRUM β-LACTAMS				
Cefotaxime	30	72.2	25.9	1.9
Ceftazidime	30	72.2	11.1	16.7
Cefepime	30	72.2	0	27.8
PENICILLINS + BETA-LACTAMASE INHIBITORS				
Amplicillin-Sulbactam	10	68.5	3.7	27.8
TETRACYCLINES				
Tetracycline	30	87	1.9	11.1
Doxycycline	30	61.1	7.4	31.5

Table 6: Minimal inhibitory concentration (MIC) of 14 extensive-drug resistant (XDR) *A. baumannii* strains to Polymyxin B.

Strain	**Concentration of polymyxin B**							**Interpretation (R/S)**
	2 μg/mL	4 μg/mL	8 μg/mL	16 μg/mL	32 μg/mL	64 μg/mL	128 μg/mL	
AC3	+	+	+	-	-	-	-	R
AC12	+	+	+	+	+	+	+	R
AC13	+	+	+	+	+	-	-	R
AC16	+	+	+	+	+	-	-	R
AC17	+	+	+	+	+	-	-	R
AC18	+	+	+	+	+	-	-	R
AC20	+	+	+	+	+	-	-	R
AC21	+	+	+	+	+	-	-	R
AC22	+	+	+	+	+	-	-	R
AC27	+	+	+	+	+	+	+	R
AC30	+	+	+	+	+	+	+	R
AC38	+	+	+	+	+	-	-	R
AC51	+	+	+	+	+	+	-	R
AC61	+	+	-	-	-	+	+	R

'+' indicates growth on agar containing the stated concentration of polymyxin B, '-' indicates no growth.

4.2.2 Pulsotypes of *Acinetobacter baumannii*

DNA sequence analyses of the 16S rRNA amplicons previously showed ≥99% sequence identity with *A. baumannii* 16S rRNA genes validating that all 54 strains in this study were truly *A. baumannii* (Lean et al., 2014). These strains were then further characterized and subtyped using pulsed field gel electrophoresis (PFGE). PFGE analysis of the 54 *ApaI*-digested *A. baumannii* (Appendix 4) gave 40 reproducible profiles (pulsotypes) with a Dice coefficient, *F*, ranging from 0.90 to 1.00. Cluster analysis of the *Apa*I pulsotypes grouped the 54 *A. baumannii* strains into 6 clusters with a cut-off point at 80% similarity (Figure 2). Carbapenem-resistant strains were grouped into four main clusters, A – D, while carbapenem-susceptible strains were grouped into two clusters, E and F.

PCR was used to determine the presence of $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23}}$, the two most common carbapenemase-encoding genes in *A. baumannii*. The $bla_{\text{OXA-51-like}}$ gene is intrinsic in *A. baumannii* whereas $bla_{\text{OXA-23}}$ is usually acquired (Feizabadi et al., 2008; Thapa et al., 2010; Zavascki et al., 2010).

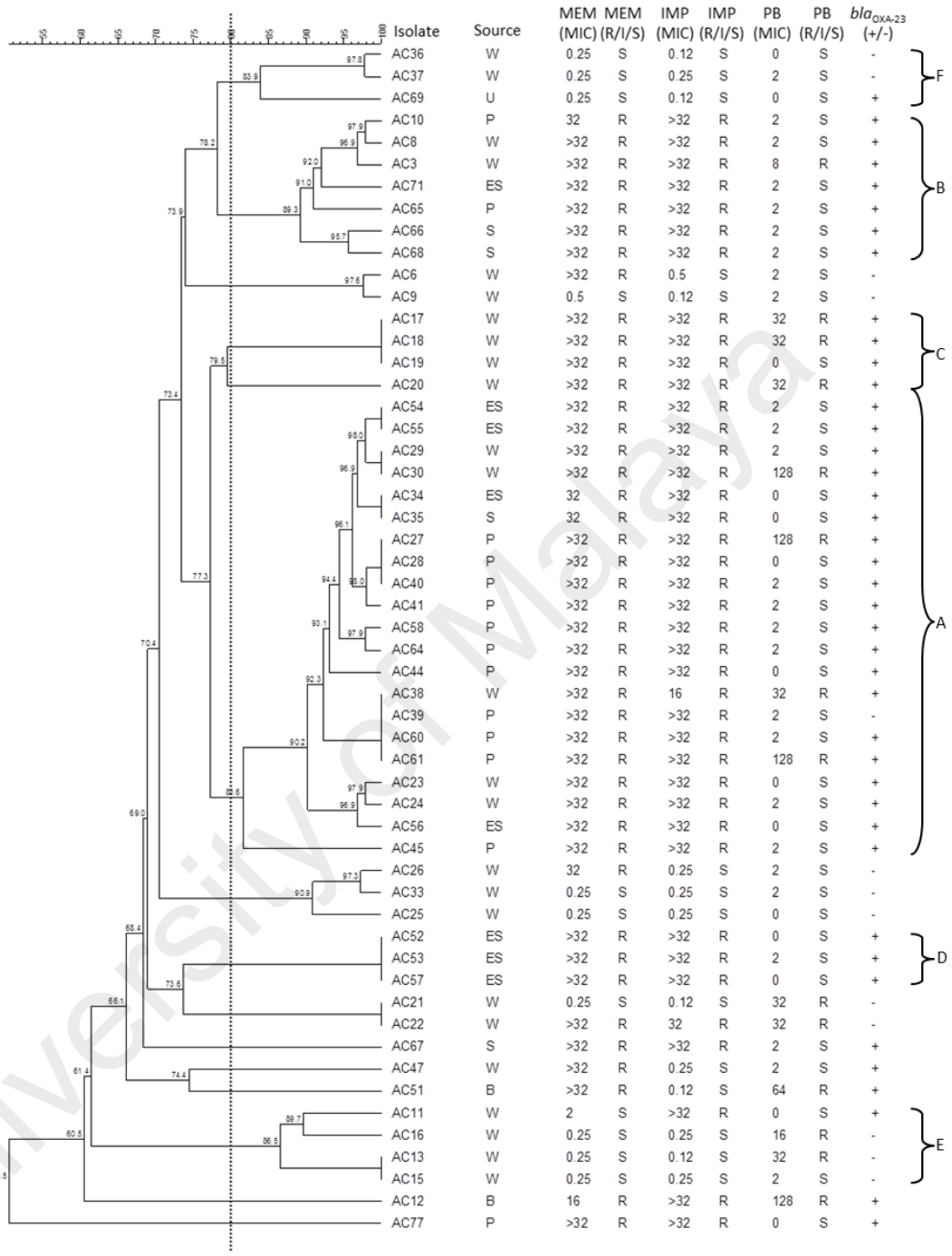


Figure 2: Dendrogram of the 54 *A. baumannii* strains from Hospital Sultanah Nur Zahirah (HSNZ) using unweighted pair group arithmetic means methods (UPGMA) from *ApaI* PFGE profiles. The dotted vertical line indicates the cut-off point of 80% similarity which clustered the strains into six distinct clusters designated A, B, C, D,

E and F, with cluster A being predominant. The sources of isolation for the *A. baumannii* strains are as indicated (wound, W; pus, P; endotracheal secretion, ES; skin, S; urine, U; blood, B). The MIC values for imipenem (IMP), meropenem (MEM) and polymyxin B (PB) for the strains are shown as are their susceptibility states (resistant, R; intermediate, I; susceptible, S) according to CLSI (2013). PCR detection results for $bla_{\text{OXA-23}}$ are also indicated ('+' for present; '-' for absent).

Cluster A is the largest cluster with 21 carbapenem-resistant strains which were closely related (81.6% similarity), with 4 band differences at most (F value = 0.90–1.00). Four of the strains within the A cluster were also polymyxin resistant and XDR. In addition, all the carbapenem-resistant strains in clusters A – D were all positive for the $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23}}$ carbapenemase genes, whereas the susceptible strains in clusters E and F were negative for $bla_{\text{OXA-23}}$. However, there was no clustering among pulsotypes of the polymyxin-resistant, XDR strains. In fact, strains with identical PFGE patterns would differ in their resistance to polymyxin B. For the three strains in cluster C which shared identical PFGE patterns, two of these (AC17 and AC18) were resistant to polymyxin B but the third strain, AC19, was polymyxin-sensitive. Likewise, AC29 and AC30 within cluster A shared an identical PFGE pattern but AC29 was sensitive to polymyxin B whereas AC30 was resistant.

Even though no certain pulsotype was dominant in a specific ward of the hospital, strains with different pulsotypes were found to spread to several wards throughout this study period. There were various antibiotic susceptibility phenotypes under the

same clusters based on the PFGE analysis. However, most of the cases of cabapenem and more polymyxin resistant strains were grouped into cluster A, as compared to the other clusters.

4.3 WHOLE GENOME SEQUENCING

4.3.1 Genome Features

To further characterize in greater detail the genetic makeup of the Terengganu *A. baumannii* strains, three strains were chosen for whole genome sequencing: two XDR strains, AC12 and AC30, and another MDR strain, AC29. AC29 had an identical *Apa*I pulsotype with AC30 although AC29 was MDR and polymyxin-susceptible whereas AC30 was XDR and polymyxin-resistant.Based on their PFGE profiles, AC29 and AC30 belonged to the major cluster A while AC12 was genetically distinct and did not belong to any cluster. AC29 and AC30 were chosen to find out why strains with indistinguishable pulsotype could have different susceptibility profiles. Detailed genomic analysis could elaborate on the differences in antimicrobial susceptibilities of the strains. Whole genome sequence analysis indicated that the estimated genome size of AC12 (accession no. CP007549) was 3.8 Mbp while each of AC29 and AC30 (accession no. CP007535 and CP007577, respectively) was 3.9 Mbp each. All three *A. baumannii* strains had G+C contents of approximately 38% (Table 7).

A. baumannii AC12 consists of a circular chromosome and an 8,731 bp plasmid designated pAC12 whereas *A. baumannii* AC30 consists of a circular chromosome and three plasmids, pAC30a (8,729 bp), pAC30b (16,236 bp) and pAC30c (71,433

bp). On the other hand, *A. baumannii* AC29 consists of a circular chromosome and two plasmids, pAC29a (8,737 bp) and pAC29b (74,749 bp). The general features of the genomes are listed in Table 7.

Table 7: General genomic features of the whole genome sequences of *A. baumannii* strain AC12, AC30 and AC29.

Feature	Strains		
	AC12	**AC30**	**AC29**
Accumulated length	3,848,312 bp	3,925,274 bp	3,935,134 bp
Average GC content	38.93%	38.98%	38.84%
Number of contigs	86	91	102
Number of ORF	3643	3646	3728
Number of tRNA	42	58	66
Number of rRNA	3	3	4

The *A. baumannii* AC12, AC30 and AC29 genomes have a total of 2322, 2290 and 2393 conserved coding regions (CCDs), respectively. These defined core genes encode for amino acids and derivatives that function in metabolic pathways and biosynthesis in the pathogen. 64%, 62.8% and 64.2% of the core genome from AC12, AC30 and AC29, respectively, is orthologous to the non-pathogenic environmental strain, *Acinetobacter* sp. ADP1. Although the organization of ADP1 and *A. baumannii* chromosome varies, metabolic genes in ADP1 were found to be conserved across *A. baumannii* strains (Di Nocera et al., 2011).

4.3.2 Multilocus Sequence Typing (MLST) and Phylogenetic Analysis

The sequence type (ST) of *A. baumannii* AC12, AC30 and AC29 were determined by two existing MLST schemes: the Bartual scheme and the Pasteur Institute

scheme. According to the Bartual scheme, the seven housekeeping genes used in determining the ST were *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* (Bartual et al., 2005). Whereas in the Pasteur Institute scheme, the housekeeping genes used was *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB* (Jolley et al., 2004). Results from these two different schemes showed that all three *A. baumannii* strains (i.e. AC12, AC30 and AC29) were assigned to ST 195 (1-3-3-2-2-96-3) using the Bartual scheme and ST2 (2-2-2-2-2-2-2) using the Pasteur scheme. Both ST2 and ST195 strains belongs to Clonal Complex 92 which are grouped under the Global Clonal 2 lineage (Karah et al., 2011; Kim et al., 2013).

Phylogenetic analysis was carried out using the whole genome sequences of the three strains in comparison with all other available complete genomes of *A. baumannii*. *Acinetobacter* sp. ADP1 was used as an outgroup to root the phylogenetic tree (Zhou et al., 2011; Huang et al., 2012; Tan et al., 2013; Zhu et al., 2013). Based on the phylogenetic analysis (Figure 3), *A. baumannii* AC12, AC29 and AC30 were closely related and the closest neighbours of the three *A. baumannii* strains were *A. baumannii* OIFC032 and BJAB0715, both of which do not belong to either of the major GC1 or GC2 groups but rather, to the GC3 (or sometimes referred to as the International Clone III, or IC-III group).

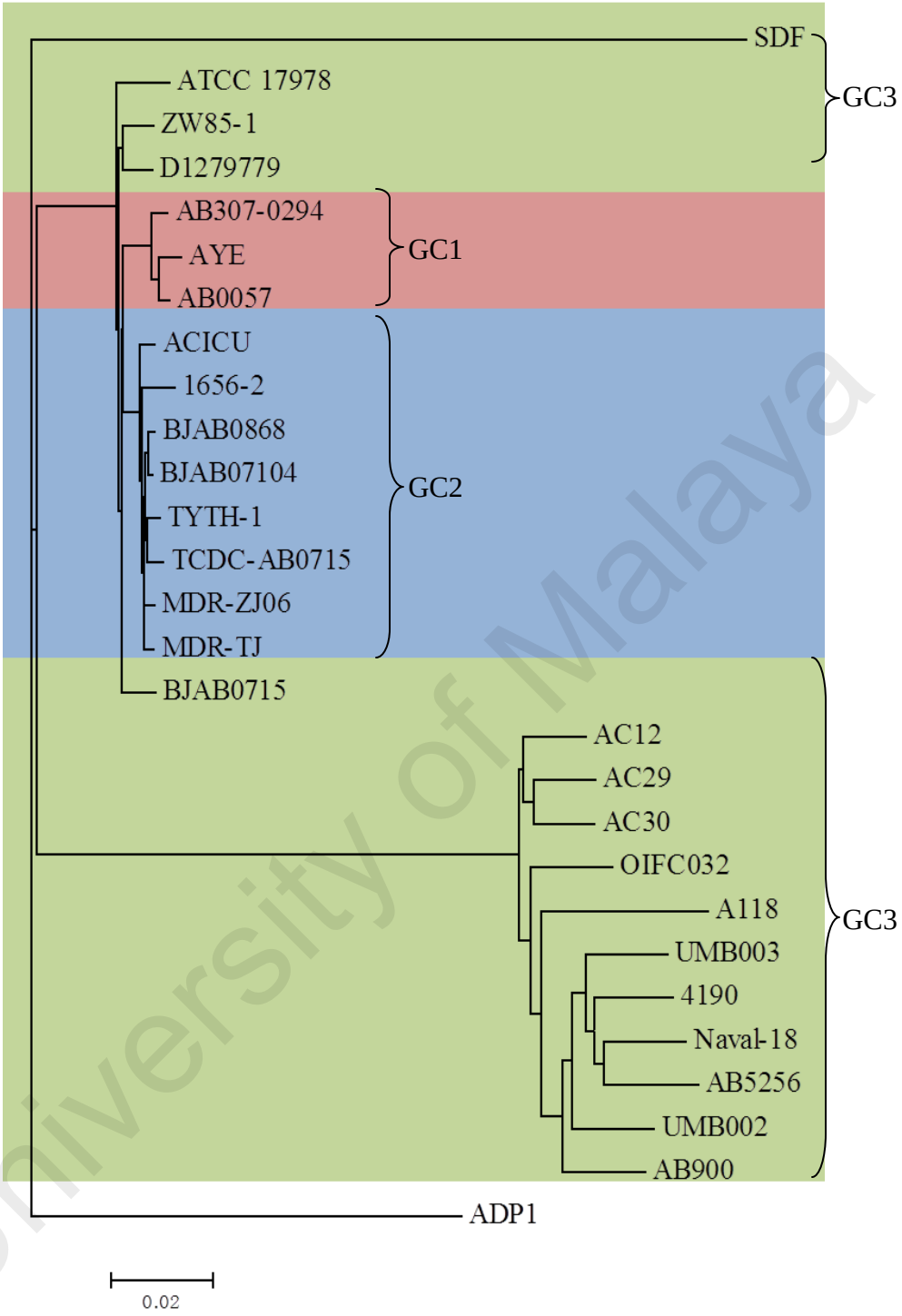


Figure 3: Phylogenetic analysis of *A. baumannii* AC12, AC30, AC29 and completed *A. baumannii* genomes obtained from NCBI. The phylogenetic tree was constructed using CVTree and the neighbor-joining tree was constructed using MEGA5

software. Strains belonging to the global clonal groups GC1, GC2 and GC3 are indicated.

4.3.3 Genome Synteny

Synteny plot produced by r2cat software allows for the comparison, arrangement and synteny visualization of genomes. Orientations of the contigs in the *A. baumannii* AC12, AC30 and AC29 genomes were determined by using the closely related full genomes of *A. baumannii* BJAB0715 and OIFC032 as reference based on the previous phylogenetic analysis. Comparison of *A. baumannii* AC12 to both reference genomes showed that r2cat was able to align 69 out of 79 contigs in AC12 (Figures 4a and 4b), which were previously assembled by CLCBio Genomics Workbench 5.0. A total of 79 out of 92 contigs from *A. baumannii* AC30 was able to be aligned (Figures 4c and 4d). As for *A. baumannii* AC29, 88 out of 102 contigs can be aligned to the *A. baumannii* BJAB0715 and OIFC032 genomes (Figures 4e and 4f). Sequences in *A. baumannii* AC12, AC30 and AC29 which could not be aligned to the reference genomes may represent regions unique to the three genomes itself. Those unaligned and/or unmatched sequences from the r2cat software were then later identified as resistance islands, plasmids and accessory genes that were only present in the *A. baumannii* AC12, AC30 and AC29 genomes.

Arrangements of the *A. baumannii* AC12, AC30 and AC29 genomes were further verified by using Progressive Mauve which generated multiple alignments of the genome sequences with rearrangements. The resulting genome alignments from Mauve showed the same outcome as in r2cat, including those previously unaligned sequences (i.e. also known as unique regions) in r2cat. Details of these unique regions will be discussed in the next section.

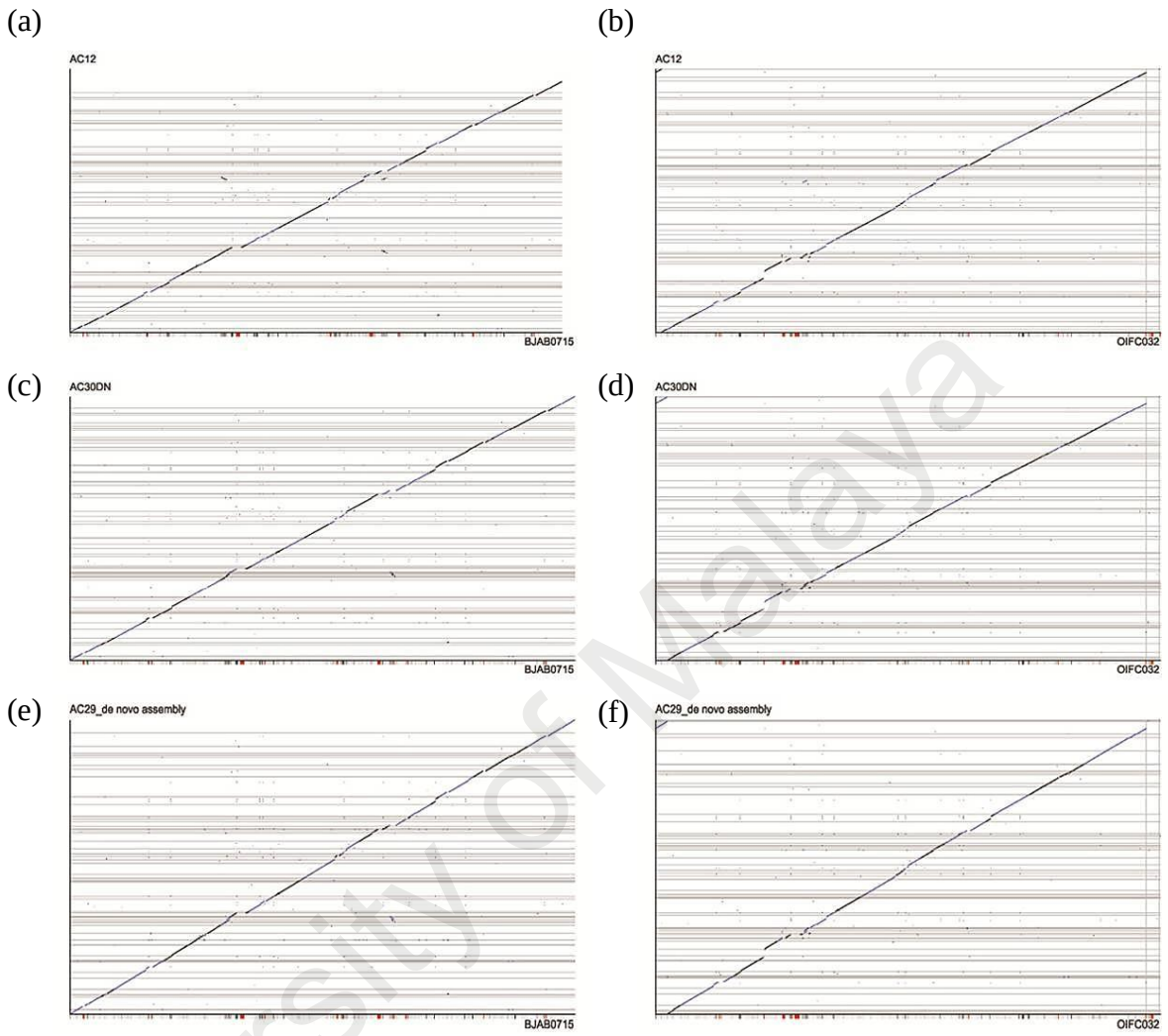


Figure 4: Synteny plots of the *A. baumannii* AC12, AC30 and AC29 genomes against *A. baumannii* BJAB0715 and OIFC032 as the closest reference genome for contigs arrangements. Plot between (a) *A. baumannii* AC12 and BJAB0715, (b) *A. baumannii* AC12 and OIFC032, (c) *A. baumannii* AC30 and BJAB0715, (d) *A. baumannii* AC30 and OIFC0715, (e) *A. baumannii* AC29 and BJAB0715, and (f) *A. baumannii* AC29 and OIFC032.

4.3.4 Resistance Island Structures

One of the known hotspots for the insertion of resistance islands in *A. baumannii* is the *comM* gene (Post et al., 2010; Zhou et al., 2011; Huang et al., 2012; Kim et al., 2013). Analyses of *A. baumannii* AC12, AC30 and AC29 genome sequences revealed that the *comM* gene in these three strains was interrupted by the insertion of a 23 kb, 26 kb and 23 kb resistance island, respectively. These islands were later designated AC12-RI1, AC30-RI1 and AC29-RI1 and contained clusters of antibiotic resistance determinants. Gaps in the various contigs that cover the island and their order were determined and validated by PCR using appropriately designed primers (Appendix 6) and sequencing the PCR products.

Both AC12-RI1 and AC29-RI1 consisted of the backbone of Tn*6167* with a copy of ΔTn*6022* (Figure 5a and 5b). ΔTn*6022* was a truncated version of Tn*6022*, in which a 2.85 kb fragment consisting of the entire *tniD* and part of the *tniE* gene were deleted from the AC12-RI1. AC29-RI1 also consisted of a variant copy of ΔTn*6022*, whereby only the entire *tniC* was deleted. Similar to AC12-RI1 and AC29-RI1, the AC30-RI1 island shared the same Tn*6167* backbone. This indicates that the Tn*6167* backbone is a common feature across the three resistance islands. In contrast to AC12-RI1 and AC29-RI1, AC30-RI1 carries a complete Tn*6022*, which is similar to that in AbaR22 (Figure 5c and 5d). Structures of AC12-RI, AC30-R1 and AC29-RI1 presented only one copy of either ΔTn*6022* or Tn*6022*, whereas similar types of resistance islands displayed two copies of ΔTn*6022* and/or Tn*6022* (Figure 5d and 5e). Drug resistance genes found in the ACRI12-1, AC29-RI1 and ACRI30-1 islands were *sulI* (conferring sulphonamide resistance), $bla_{\text{OXA-23}}$ (conferring carbapenem

resistance), *tetA* and *tetB* (conferring tetracycline resistance), and *strA* with *strB* (conferring streptomycin resistance). In all three RIs, the $bla_{\text{OXA-23}}$ gene was found flanked by two copies of the IS*Aba1* insertion element in a composite transposon structure similar to Tn*2006* and denoted as ΔTn*2006* (Figure 5). The presence of IS*Aba1* upstream of $bla_{\text{OXA-23}}$ may contribute to carbapenem resistance by increasing the expression level of the gene (Zhu et al., 2013). The Tn*2006*-like structure was inserted immediately downstream from ΔTn*6022* or Tn*6022*, and interestingly in AC12-RI1 and AC29-RI1, the *orf4* gene found at the right-most end of ΔTn*6022* was duplicated, resulting in the ΔTn*2006*-like structure being flanked by *orf4* on either end (Figure 5). Such an arrangement has not been reported in similar resistance islands before.

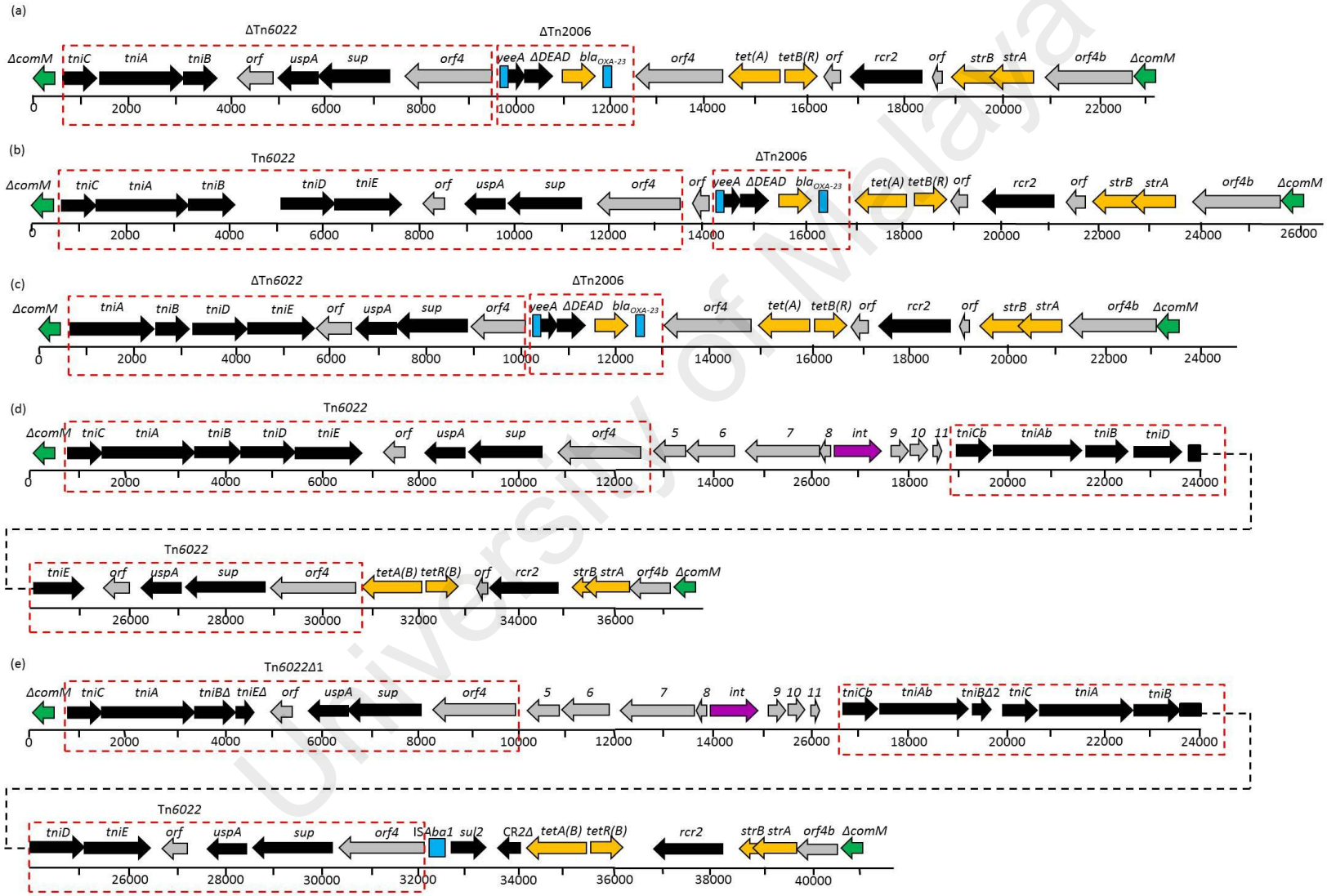


Figure 5: Structures of resistance islands present in *A. baumannii* AC12, AC30 and AC29 as compared to similar islands in *A. baumannii* MDR-ZJ and MDR-TJ. (a) Structure of resistance island ACRI12-1 present in *A. baumannii* AC12. Multiple antibiotic resistance regions are indicated by thick lines, ΔTn*6022* is indicated in the red dotted-line box. Directions of genes and ORFs are indicated by arrows above the central thick line and the names are as given above. Green boxes represent truncated Δ*comM*, turquoise blue boxes represent insertion element, IS*Aba1* and orange boxes represent genes conferring antibiotics resistance. Numbered boxes in grey represent genes encoding for hypothetical proteins. Comparison between structures of (a) AC12-RI1, (b) AC30-RI1, (c) AC29-RI1, (d) AbaR22 and (e) RI_{MDR-TJ}, revealed similarities in the islands, as indicated by the same colors and red dotted-line box.

A second RI was identified in the genome of *A. baumannii* AC12 based on sequence similarities with the AbGRI2-1 island found in the GC2 Australian *A. baumannii* WM99c strain (Nigro et al., 2013). This RI was designated as AC12-RI2 and its structure was shown in Figure 6. AC12-RI2 was found interrupting a D-serine/D-alanine/glycine transporter gene at the 5'-end of the island and a flavin monooxygenase at the 3'-end (Figure 6). AC12-RI2 was relatively shorter (10.3 kb in size) as compared to AbGRI2-1 which was 19.5 kb, but both islands were flanked by two copies of IS*26*. AC12-RI2 lacks the class 1 integron containing the *aacC1-orfP-orfP-orfQ-aadA1* array that was present in AbGRI2-1 along with an incomplete Tn*21* that was absent from its 3'-end (Nigro et al., 2013). The resistance genes found in AC12-RI2 were *aphA1b,* which conferred aminoglycoside resistance and bla_{TEM}, which conferred β-lactam resistance. Both *aphA1b* and bla_{TEM} were flanked by two copies of IS*26* in a composite transposon-like structure. However, this RI is only found in *A. baumannii* AC12 and was absent from AC30 and AC29.

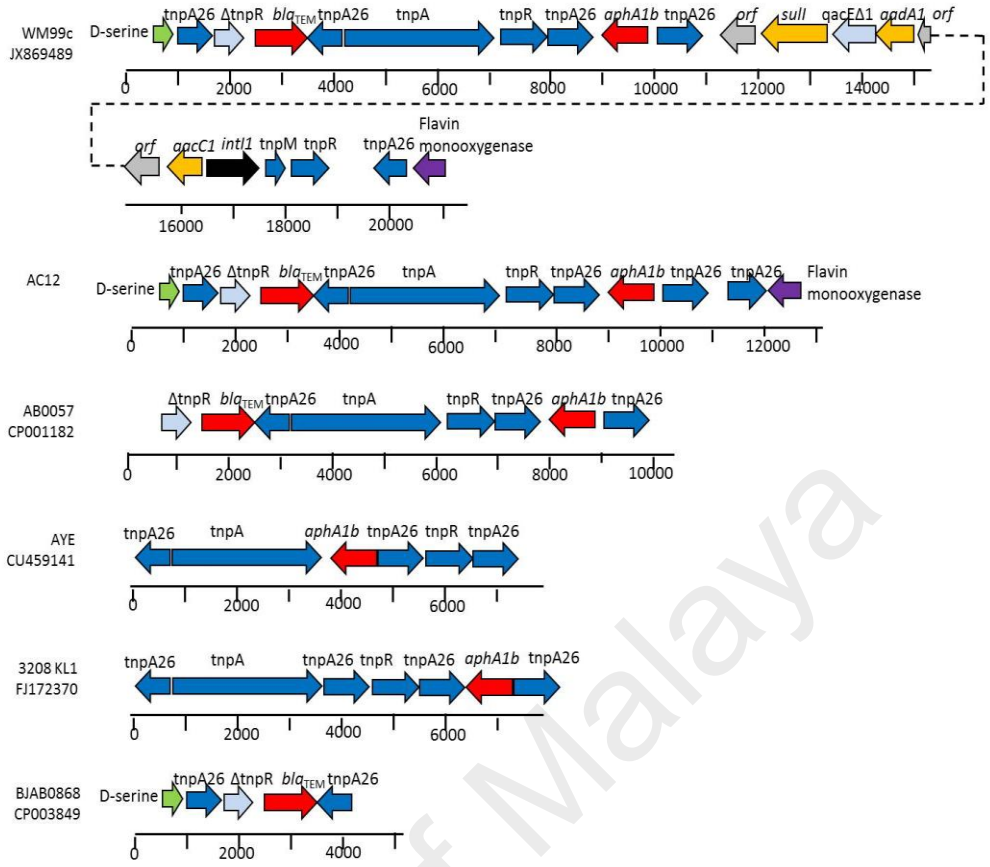


Figure 6: Structures of the AC12-RI2 resistance island present in *A. baumannii* AC12 in comparison with AbGRI2-1 from *A. baumannii* WM99c, AB0057, AYE, 3208 KL1 and BJAB0868. Resistance determinants in the island are presented in red boxes. Genes encoding for transposases are indicated by blue boxes, whereas Δ*tnpR* is indicated by light blue boxes. D-serine and flavin monooxygenase encoding genes are indicated by green and purple boxes, respectively. *sulI*, *aadA1* and *aacC1* genes are presented in orange boxes. Directions of genes and ORFs are indicated by arrows above the central thick line with gene names given above.

Genome alignment in Mauve revealed an approximately 7 kb fragment present in *A. baumannii* AC12, which is unique to the AC12 chromosome and absent in the BJAB0715 and OIFC032 genomes. This region contains the Tn*1548*::*armA* island which was previously reported in the pMDR-ZJ06 plasmid of *A. baumannii* MDR-ZJ06 (Zhou et al., 2011) as well as plasmids from several Enterobacteriaceae including pKT51748 of *Klebsiella pneumoniae* (accession no. FJ715937), pNDM-HK of *Escherichia coli* (accession no. HQ451074), pXD1 of *Salmonella enterica* Paratyphi B (accession no. JN225877) and pNDM-CIT plasmid of *Citrobacter freuendii* (accession no.JX182975). This 7 kb fragment includes genes encoding transposases (*tnp*U and *tnp*D) from Tn*1548*::*armA*, the 16S rRNA methylase (*armA*) which confers resistance to aminoglycosides, a macrolide efflux protein (*mel*) and a macrolide 2'-phosphotransferase (*mph*2) that confers macrolide resistance (Figure 7). The Tn*1548::armA* island was subsequently found on the pAC30b plasmid from strain AC30 as well as the chromosome of AC29 where Tn*1548::armA* was located in the same region and with an identical genetic environment as that in AC12.

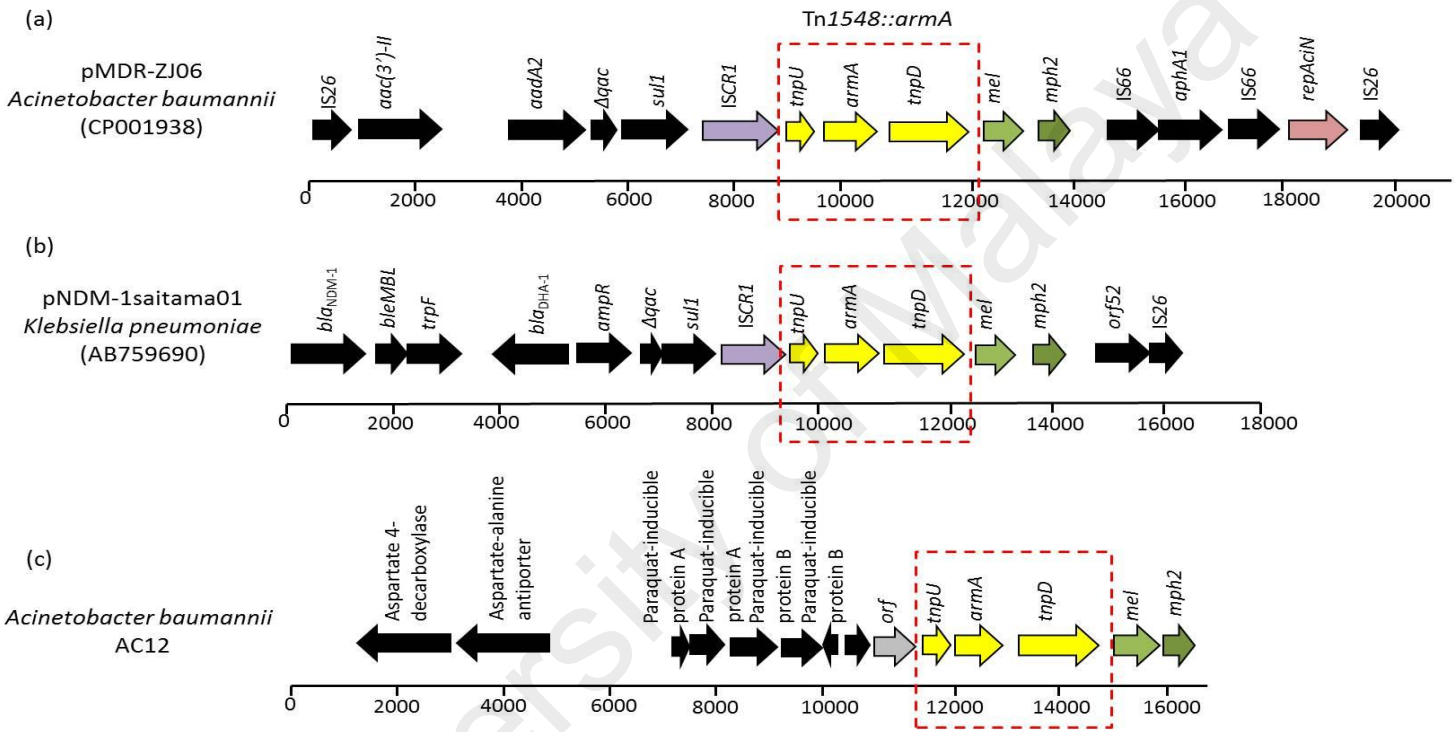


Figure 7: Comparative analysis of the Tn*1548*::*armA* structure as found in (a) plasmid pMDR-ZJ06 of *A. baumannii* MDR-ZJ06, (b) *Klebsiella pneumoniae* plasmid pNDM-1saitama01 and (c) the chromosome of *A. baumannii* AC12. The Tn*1548*::*armA* element is indicated in the red dotted box. Open reading frames are

indicated by coloured boxes above black thick lines. Orientations of genes are indicated by arrows above the central thick line and the names are as given above. Grey boxes represent genes encoding for hypothetical proteins.

4.3.5 Plasmids

In general, three types of plasmids were found within the three sequenced *A. baumannii* strains (i.e. AC12, AC30 and AC29). The three types of plasmids could be categorized as a cryptic plasmid of 8.7 kb in size, a resistance-plasmid of ~16 kb and a conjugative plasmid of ~70 kb in size.

All three *A. baumannii* strains from Terengganu harboured a cryptic 8.7 kb plasmid designated pAC12 (in AC12), pAC30a (in AC30) and pAC29a (in AC29). The actual sizes of pAC12, pAC30a and pAC29a were 8,731 bp, 8,729 bp and 8,737 bp; respectively. This shared plasmid (Figure 8) had an average GC content of 34.4% and is nearly 100% identical to plasmids pAB0057 (8,729 bp) (Adams et al., 2008) and p1ABTCDC0715 (8,731 bp) (C.-C. Chen et al., 2011) found in *A. baumannii* AB0057 and TCDC-AB0715, respectively. Features on this plasmid across strains were the same and all of them contained a total of nine coding sequence (CDS) (Figure 8). No antibiotic resistance gene was found on the plasmid. Two plasmid replication genes, designated *repA* and *repB*, were found on these plasmids along with an iteron sequence made up of four direct repeats of 5'-ATA TGT CCA CGT TTA CCT TGC A-3' located 53 nucleotides upstream of the *repB* gene. Also found on these plasmids are a Sel1 repeat protein-encoding gene (*sel1*) which is flanked by XerC/XerD-like recombination sites in an inverted repeat formation, an outer membrane TonB-dependent receptor gene, a gene encoding for putative septicolysin and two hypothetical protein-encoding genes. A toxin-antitoxin (TA) system designated AbkB/AbkA (Mosqueda et al., 2013) was also encoded on these 8.7 kb plasmids.

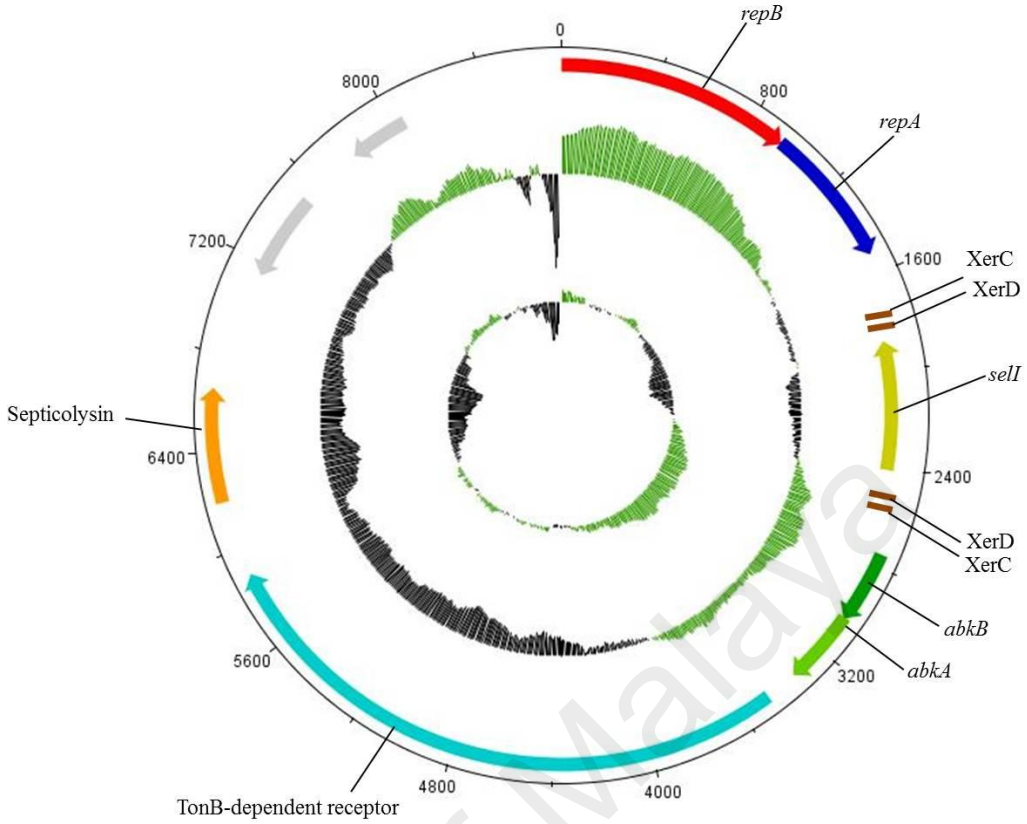


Figure 8: Circular map of the 8.7 kb plasmid pAC12, pAC30a and pAC29a found in *A. baumannii* AC12, AC30 and AC29, respectively. Outer circle of the map represents ORFs found in the plasmid, grey ORFs are genes encoding for hypothetical protein. The two inner circles represent GC plot and GC skew, whereby the green circle stands for above average (38.9%) and black circle stands for below average G+C content. The XerC/XerD-like recombination sites are marked as brown rectangular boxes.

The second type of plasmid that was identified was approximately 16 kb in size. However, this plasmid was only found in *A. baumannii* AC30, and was designated pAC30b. Plasmid pAC30b is 16,236 bp in size, consisted of 11 CDS and is nearly identical in certain sections to plasmid pMDR-ZJ06 which is 20,301 bp in size and found in *A. baumannii* MDR-ZJ06 (Zhou et al., 2011) (Figure 9). Both pAC30b and pMDR-ZJ06 are resistant plasmids, as indicated by the presence of resistance determinants shown in Figure 9 and this includes the 16S rRNA methylase gene (*arm*A) and aminoglycoside 3'-phosphotransferase gene (*aph*A1) which confer resistance to aminoglycosides, along with macrolide 2'-phosphotransferase (*mph2*) and macrolide efflux protein-coding (*mel*) genes which conferred resistance to macrolides (Zhou et al., 2011). The pMDR-ZJ06 plasmid harboured a class 1 integron encoding the aminoglycoside acetyltransferase (*aacC1*) and adenyltransferase (*aadA1*) along with *sulI* that conferred sulphonamide resistance (Zhou et al., 2011) but this integron was absent in pAC30b. The 7 kb Tn*1548::armA* located in the chromosome of *A. baumannii* AC12 was found in pAC30b as well as pMDR-ZJ06. However, the *tnpU* putative transposase gene located at the 3'-end of Tn*1548::armA* was truncated in pAC30b whereby only the last 480 bp of the 836-bp gene could be found and was thus designated *ΔtnpU*. The pAC30b plasmid encodes a number of putative transposases: *ΔtnpU* and *tnpD* from Tn*1548::armA*, *tnpA* and *tnpB* transposases from IS*66*, a 2,888-bp *tnpA* from the Tn*3* family of transposases (or Tn*1000*-like) and immediately adjacent to that, another smaller 704-bp *tnpA* transposase belonging to IS*26*. The aminoglycoside resistance gene *aphA1* is found downstream from IS*26*.

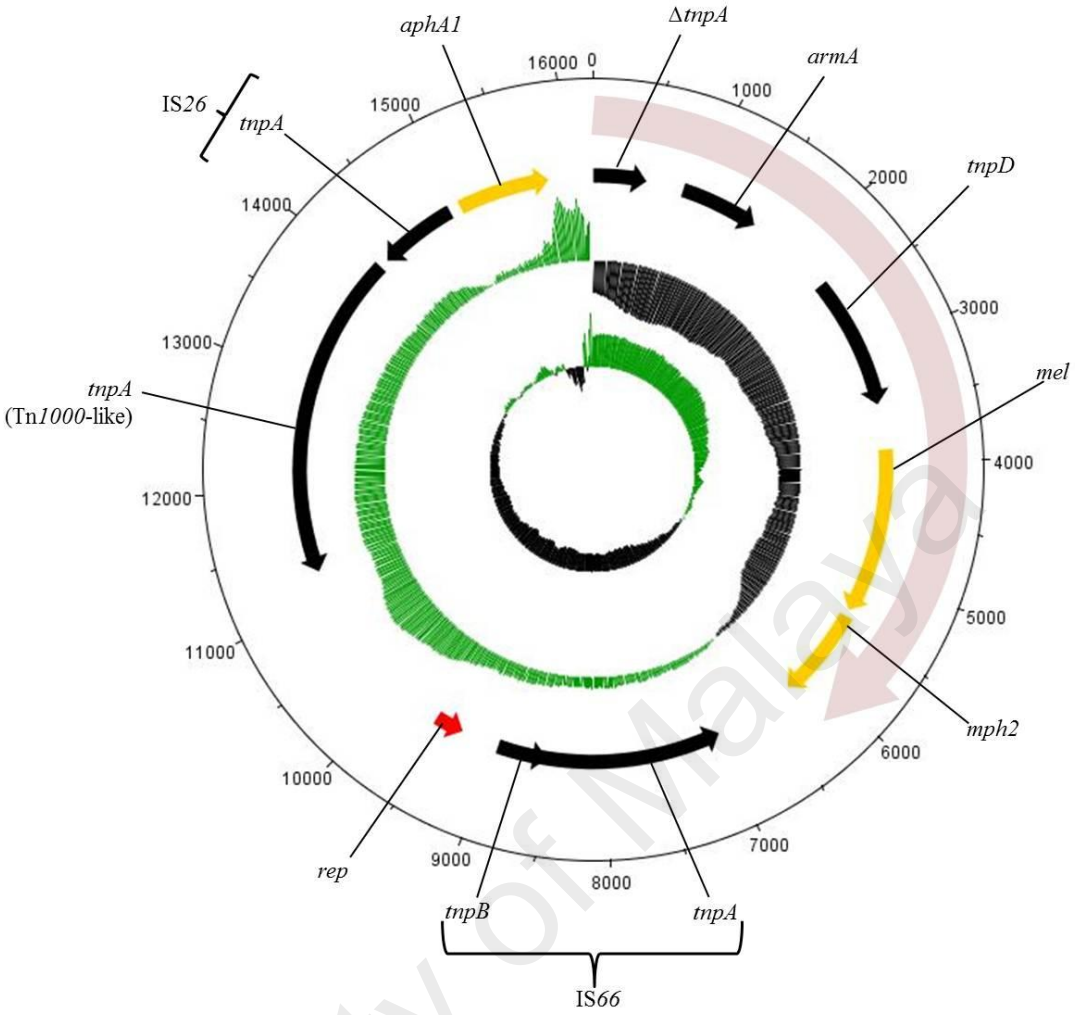
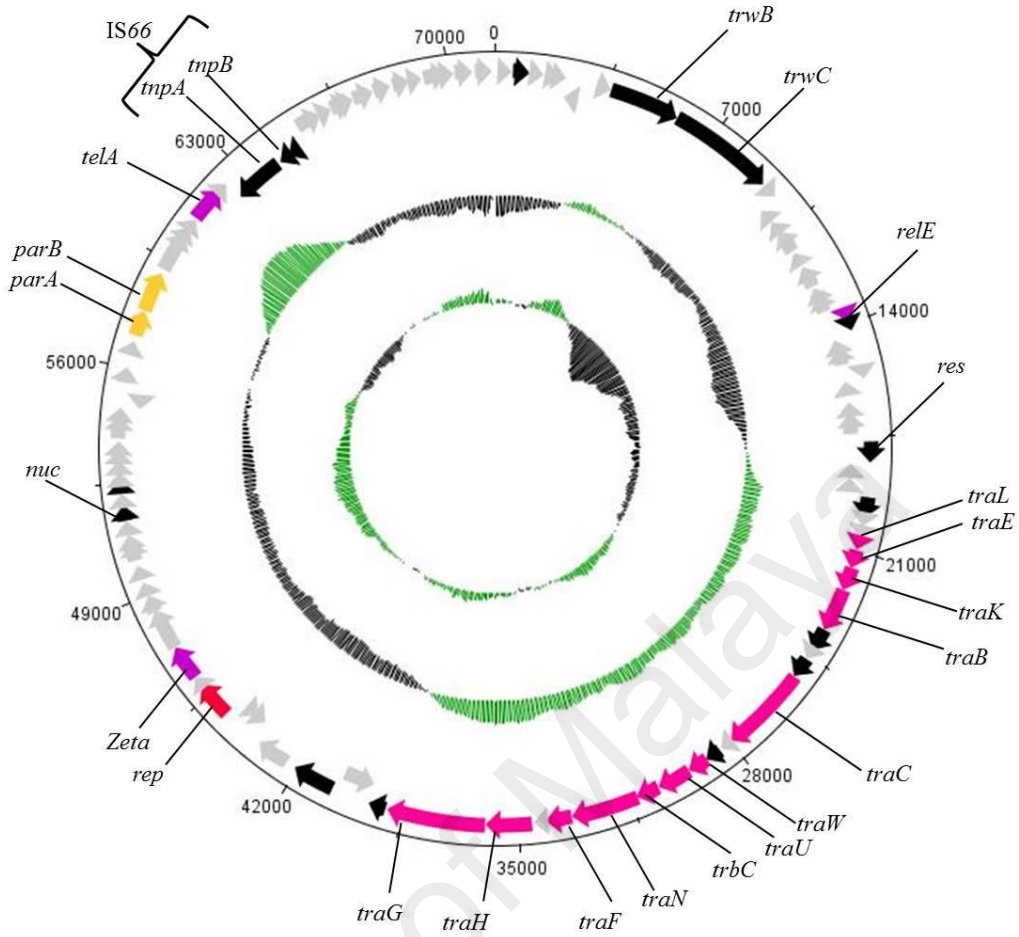


Figure 9: Circular map of 16 kb plasmid pAC30b. Outer circle of the map represents ORFs found in the plasmid. Area shaded with light pink arrow represents Tn*1548*::*armA* region, and both IS*66* and IS*26* were indicated by brackets. The two inner circles represent GC plot and GC skew, whereby the green circle stands for above average and black circle stands for below average G+C content.

The largest plasmid that could be found within the sequenced *A. baumannii* genomes was a conjugative plasmid of approximately 70 kb in size, harboured in *A. baumannii* AC30 and AC29. Designated as pAC30c and pAC29b in *A. baumannii* AC30 and AC29, these two plasmids were 71,433 bp and 74,749 bp in size. There were a total of 96 CDS in pAC30c and 101 CDS in pAC29b. Both plasmids were categorized as conjugative plasmids due to the presence of a complete *tra* locus (Figure 10a and 10b), similar to the *tra* locus found in pACICU2 (Iacono et al., 2008) and p2ABTCDC0715 (C.-C. Chen et al., 2011) (Chen et al. 2011) pAb-G7-2 (Hamidian & Hall, 2014), and pA85-3 (Hamidian et al., 2014). The complete *tra* locus in pAC30c and pAC29b encode for a type IV secretion system (T4SS) which are related to the bacterial conjugation machinery and mediate horizontal gene transfer (Juhas et al., 2008). Genes for the T4SS in pAC30c and pAC29b are clustered in two separate regions: the main *tra* region responsible for mating pair formation spans approximately 20 kb from *traL* to *traG*, and a second smaller region containing *traD* and *traI* (or *trwB* and *trwC*) responsible for plasmid mobilization. Both pAC30c and pAC29b carry the *rep* gene designated *rep*Aci6, similar to pACICU2, pAb-G7-2 and pA85-3. Both plasmids also appeared to harbour solitary toxin genes without their corresponding antitoxins – one encoding for a Zeta family toxin and another for a RelE family toxin. The Zeta toxin found here is one of the solitary Zeta toxins that have been found in other plasmids (Chan et al., 2012) without a corresponding Epsilon antitoxin. In the absence of typical canonical toxin-antitoxin systems, the only other identifiable genes that could contribute to plasmid stability in pAC30c and pAC29b are the *parAB* genes which likely encode proteins that are involved in plasmid partitioning.

Plasmid pAC29b is larger by 3,316 bp when compared to pAC30c. However, there is a 14,374 bp fragment in pAC30c which has no homology with pAC29b. The additional fragment found in pAC29b included the β-lactamase gene bla_{OXA-23} and two transcriptional regulators, *tetR* and *asnC*. Other identifiable genes within this pAC29b-unique fragment include *yeeA* (encoding DNA methyltransferase), *yeeB* (encoding ATP-dependent helicase), *yhbS* (encoding N-acetyltransfease), *mscM* (encoding mini-conductance mechanosensitive channel), *iutA* (encoding ferric aerobactin receptor), *lrp* (encoding leucine-responsive regulatory protein) and *aroQ* (encoding monofunctional chorismate mutase) were also found within the pAC29b plasmid. The actual functions of these protein encoding genes are yet to be determined.

(a)



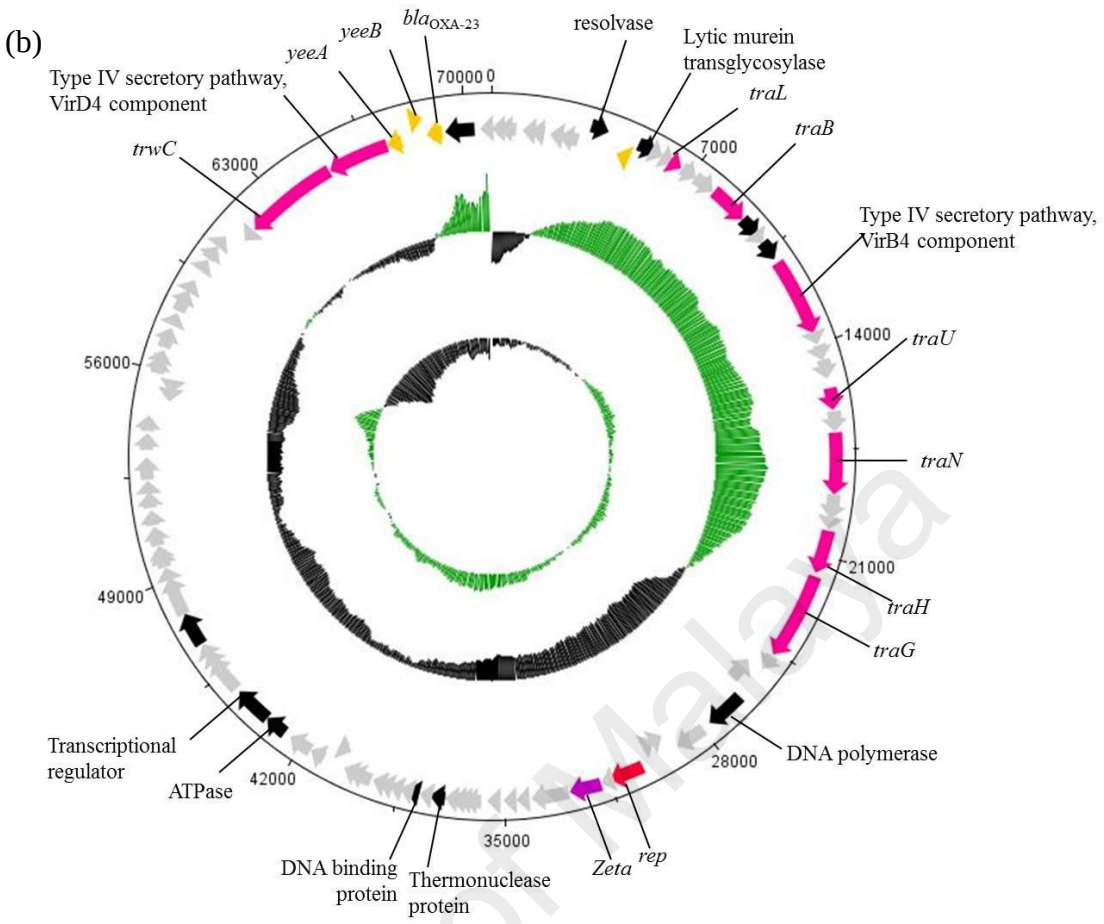


Figure 10: Circular map of the ~70 kb plasmids pAC30c (Figure 10a) and pAC29b (Figure 10b). Outer circle of the map represents ORFs found in the plasmid, grey ORFs are genes encoding for hypothetical proteins. The two inner circles represent GC plot and GC skew, whereby the green circle stands for above average and black circle stands for below average G+C content. Red colored arrow represents plasmid replication gene, *rep*; orange colored arrow represents resistant determinants; pink colored arrow represents *tra* genes; turquoise colored arrow represents genes encoding proteins with known functional homologues; purple colored arrow represents putative toxin genes; and grey colored arrow represents genes encoding hypothetical proteins.

4.3.6 Efflux Pumps

A large number of multidrug transporters were identified in the genome of *A. baumannii* AC12, AC30 and AC29 (Table 8) and these include putative efflux pumps from the resistance-modulation-cell division (RND) family (12 ORFs), the major facilitator superfamily (MFS) (8 ORFs), the amino acids, polyamines and organic cations transporter (APC) family (4 ORFs), ATP-binding cassette (ABC) superfamily (1 ORF), multidrug and toxic efflux (MATE) family (1 ORF), drug/metabolite transporter (DMT) family (2 ORFs) and the small multidrug resistance (SMR) family (1 ORF). Within the RND family, the *adeABC* genes along with its two-component regulatory genes *adeRS* were found in all three *A. baumannii* AC12, AC29 and AC30 genomes, as were the *adeIJK* genes. However, a third RND efflux pump encoded by the *adeFGH* operon (Poole, 2004) was not found in AC12, AC30 and AC29.

Table 8: Drug transporter and efflux pumps found in *A. baumannii* AC12, AC30 and AC29.

Drug Transporters	Genes	Contig Location			Gene Products
		AC29	AC30	AC12	
APC family transporter	*cyc*A	16_60	3_157	6_60	D-serine/D-alanine/glycine transporter
	*cyc*A2	3_70	10_70	7_82	amino acid APC transporter
		3_71	10_71	7_83	amino acid APC transporter
	*mmu*P	67_1	22_32	30_94	S-methylmethionine APC transporter
	*pro*Y	17_59	1_59	13_96	proline-specific permease ProY
RND family transporter	*ade*A	31_11	16_59	29_13	membrane-fusion protein
	*ade*B	31_10	16_60	29_12	cation/multidrug efflux pump
	*ade*C	31_9	16_61	29_11	multidrug efflux protein AdeC
	*ade*I	15_38	34_39	20_39	multidrug efflux protein AdeI
	*ade*J	15_39	34_40	20_40	multidrug efflux protein AdeJ
	*ade*K	15_40	34_41	20_41	multidrug efflux protein AdeK
	*ade*R	31_21	16_58	29_14	AdeR
	*ade*S	31_13	16_57	29_15	AdeS
	*fus*E	9_83	19_57	60_14	Putative FusE-MFP/HlyD membrane fusion protein
	*mdt*A	20_59	14_55	11_53	multidrug ABC transporter
	*nol*F	35_29	4_13	36_13	NolF secretion protein
	RND efflux pumps	35_29	4_13	36_13	NolF secretion protein
MFS family transporter	*emr*B	26_33	27_212	17_33	major facilitator superfamily multidrug resistance protein
	fsr	25_59	27_36	17_210	major facilitator superfamily permease
	bcr	35_12	47_5	16_5	MFS superfamily bicyclomycin/multidrug transport protein
	MFS superfamily protein	12_101	13_186	56_30	transporter, major facilitator family protein
	MFS transporter	19_98	8_90	3_90	major facilitator superfamily permease
	*yga*Y	8_35	18_33	9_41	transporter, major facilitator family protein

	*nor*M	9_13	19_5	31_57	multidrug ABC transporter
	cmr	22_155	22_42	30_104	major facilitator superfamily multidrug/chloramphenicol efflux transporter
ABC family transporter	ABC efflux pump	9_13	19_5	31_57	multidrug ABC transporter
Na+ driven transporters	Na+ driven efflux pump	17_88	1_88	13_67	Na+-driven multidrug efflux pump
Efflux pumps	*abe*M	9_13	19_5	31_57	multidrug efflux pump AbeM
	dmt	16_81	3_136	6_81	EamA-like transporter family protein
	*ywf*M	56_27	12_128	52_40	DMT family permease
	MATE efflux pump	17_88	1_88	13_67	MATE efflux family protein
SMR	*smr*	22_155	14_62	11_47	multidrug resistance protein, SMR family
		20_66	14_1	45_14	Smr protein/MutS2

4.3.7 Virulence Genes

Various virulence determinants could be identified from the genome sequences of *A. baumannii* AC12, AC30 and AC29 (Table 9), by using local database of virulence genes. In the current study, a cluster of virulence genes including *barA*, *barB*, *basA*, *basB*, *basC*, *basD*, *basE*, *basF*, *basG*, *basH*, *basI*, *bauA*, *bauB*, *bauC*, *bauD*, *bauE* and *bauF* (Table 9) were found existing within the same contig. All the genes in this cluster were neighboring with each other and functions as multidrug transporters, acinetobactin and enterobactin production, siderophore interacting protein, and important catalytic enzymes (Figure 14). This 15 kb cluster is flanked by IS4 insertion element at one end and a tRNA (tRNA-Val-GAC) at another end. To date, there has been very little data and reports on pathogenicity islands (PAI) identified from clinical strains of *A. baumannii*. This cluster is a plausible candidate PAI (cPAI) as it fulfills the following criteria for a PAI (Yoon et al., 2005; Yoon et al., 2007):

i. Genes within the island encode for virulence determinants such as toxins, adhesins and invasins
ii. This structure is only present in pathogenic strains but absent in non-pathogenic strains such as environmental strain *A. baumannii* SDF and *A. baylyi* ADP1.
iii. G+C content of this island is different from its chromosome (38.25% in AC12 and AC30, 38.0% in AC29)
iv. The size of the candidate 'pathogenicity island' is large (>10 kb), comparable to that of resistance islands

Although the three *A. baumannii* AC12, AC30 and AC29 genomes shared a similar set of virulence genes (Table 9), there were still differences between the

strains, especially between the two polymyxin-resistant (AC12 and AC30) and the polymyxin-susceptible strain AC29. The MDR but polymyxin-susceptible strain AC29 showed differences from the other two strains through the absence of several virulence factors such as *araJ, degT, entA, entE, iucAC, lpsE, menG* and *mviM* (Table 9). These determinants were responsible for transport and metabolism, which could be essential in *A. baumannii* to strive as a successful pathogen in the hospital settings. For instance, the presence of *entA* in the *A. baumannii* genomes was associated with acinetobactin biosynthesis (Peleg et al., 2012).

Table 9: List of virulence genes found in *A. baumannii* AC12, AC30 and AC29.

Gene	Contig location			Product	Function
	AC12	AC30	AC29		
araJ	13_134	1_21	N/A	arabinose efflux permease family protein	Carbohydrate transport and metabolism
	1_38	10_12	17_21	arabinose efflux permease	Carbohydrate transport and metabolism
	14_94	6_94	3_12	Arabinose efflux permease	Carbohydrate transport and metabolism
barA	25_39	24_85	34_94	GacS-like sensor kinase protein	covalent modification, demodification, maturation
	26_11	37_11	49_39	multidrug ABC transporter ATPase and permease	ABC-type transport system involved in cytochrome bd biosynthesis, ATPase and permease components [Energy production and conversion / Posttranslational modification, protein turnover, chaperones
barB	26_10	37_10	14_11	multidrug ABC transporter ATPase and permease	multidrug ABC transporter ATPase and permease
basA	26_26	37_26		acinetobactin biosynthesis protein	adenylation domain of nonribosomal peptide synthetases (NRPS)
basB	26_25	37_25	14_26	non-ribosomal peptide synthetase module	Phosphopantetheine attachment site; Condensation domain
	26_10	37_10	14_25	multidrug ABC transporter ATPase and permease	putative bacteriocin export ABC transporter
basC	26_17	37_17	14_10	lysine/ornithine N-monooxygenase	Secondary metabolites biosynthesis, transport, and catabolism
basD	26_16	37_16	14_17	BasD	
basE	26_15	37_15	14_16	enterobactin synthase subunit E	bifunctional 2,3-dihydroxybenzoate-AMP ligase/S-dihydroxybenzoyltransferase; activates the carboxylate group of 2,3-dihydroxy-benzoate forming (2,3-dihydroxybenzoyl)adenylate then catalyzes the acylation of holo-entB with 2,3-dihydroxy-benzoate adenylate

basF	26_14	37_14	14_15	isochorismate hydrolase	Isochorismatase, also known as 2,3 dihydro-2,3 dihydroxybenzoate synthase, catalyses the conversion of isochorismate
basG	26_13	37_13	14_14	histidine decarboxylase	catalyzes the formation of histamine from L-histidine
basH	26_9	37_9	14_13	acinetobactin biosynthesis protein	Thioesterase domain
basI	26_8	37_8	14_9	4'-phosphopantetheinyl transferase family protein	Putative phosphopantetheinyl transferase component of acinetobactin biosynthesis (BasI)
basJ	26_7	37_7	14_8	acinetobactin biosynthesis protein	chorismate binding enzyme
bauA	26_18	37_18	14_7	bauA	
bauB	26_19	37_19	14_18	enterochelin ABC transporter periplasmic protein	ABC-type enterochelin transport system, periplasmic component [Inorganic ion transport and metabolism]
bauC	26_21	37_21	14_19	enterochelin ABC transporter periplasmic protein	ABC-type enterochelin transport system, periplasmic component [Inorganic ion transport and metabolism]
bauD	26_22	37_22	14_21	bauD	Transmembrane subunit (TM), of Periplasmic Binding Protein (PBP)-dependent ATP-Binding Cassette (ABC) transporters
bauE	26_20	37_20	14_22	enterochelin ABC transporter ATPase	ABC-type enterochelin transport system, ATPase component [Inorganic ion transport and metabolism]
bauF	26_27	37_27	14_20	siderophore-interacting protein	Siderophore-interacting protein [Inorganic ion transport and metabolism]
csuA	18_5	2_22	14_27	protein CsuA	Spore Coat Protein U domain
csuAB	18_4	2_21		protein CsuA/B; secreted protein related to type I pili	Biofilm production
	18_6	2_23	62_22	putative type 1 pili subunit CsuA/B protein	Spore Coat Protein U domain
csuB	18_6	2_23	62_23	putative type 1 pili subunit CsuA/B protein	Spore Coat Protein U domain
csuC	18_7	2_24	62_21	P pilus assembly protein, chaperone PapD	P pilus assembly protein, chaperone PapD [Cell motility and secretion / Intracellular trafficking and secretion

csuD	18_8	2_25	62_21	P pilus assembly protein, porin PapC	P pilus assembly protein, chaperone PapD [Cell motility and secretion / Intracellular trafficking and secretion
csuE	18_9	2_26	62_20	protein CsuE	Spore Coat Protein U domain
degT	7_40	10_113	N/A	glutamine--scyllo-inositol transaminase	DegT/DnrJ/EryC1/StrS aminotransferase family
entA	20_56	39_1	N/A	lipid A phosphoethanolamine transferase	lipid A phosphoethanolamine transferase, associated with polymyxin resistance
	55_5	43_1	N/A	lipid A phosphoethanolamine transferase	lipid A phosphoethanolamine transferase, associated with polymyxin resistance
	N/A	52_5	N/A	lipid A phosphoethanolamine transferase	lipid A phosphoethanolamine transferase, associated with polymyxin resistance
entE	26_15	37_15	N/A	enterobactin synthase subunit E	activates the carboxylate group of 2,3-dihydroxy-benzoate forming (2,3-dihydroxybenzoyl)adenylate then catalyzes the acylation of holo-entB with 2,3-dihydroxy-benzoate adenylate
iucAC	3_92	8_91	N/A	siderophore synthetase component	Siderophore synthetase component [Secondary metabolites biosynthesis, transport, and catabolism]
	3_91	8_92	3_113	siderophore synthetase component	Siderophore synthetase component [Secondary metabolites biosynthesis, transport, and catabolism]
	3_88	8_88	29_1	siderophore synthetase component	Siderophore synthetase component [Secondary metabolites biosynthesis, transport, and catabolism]
lpsB	2_54	5_54	15_55	glycosyltransferase	Cell envelope biogenesis, outer membrane
lpsC	23_31	26_31	38_1	glycosyltransferase	Cell envelope biogenesis, outer membrane
lpsE	23_32	26_32	N/A	glycosyltransferase	Cell envelope biogenesis, outer membrane
menG	3_63	8_63	N/A	ribonuclease activity regulator protein RraA	regulator of RNase E; increases half-life and abundance of RNAs; interacts with RNase E possibly inhibiting catalytic activity
mviM	7_38	10_115	N/A	oxidoreductase, NAD-binding domain protein	oxidoreductase, NAD-binding domain protein
ompA	63_12	55_12	19_96	outer membrane protein	Outer membrane protein and related peptidoglycan-associated

					(lipo)proteins [Cell envelope biogenesis, outer membrane]
	17_7	27_238	19_97	OmpA family protein	Outer membrane protein and related peptidoglycan-associated (lipo)proteins [Cell envelope biogenesis, outer membrane]
	23_92	26_92	19_100	outer membrane protein	Outer membrane protein beta-barrel domain
	6_93	3_124	33_54	gammaproteobacterial enzyme transmembrane domain protein	
pbpG	24_29	38_28	23_74	D-alanyl-D-alanine endopeptidase	
pepN	48_3	35_104	23_73	aminopeptidase N	aminopeptidase N; Provisional
pilA	30_138	22_76	19_125	Tfp pilus assembly protein, major pilin PilA	Cell motility and secretion / Intracellular trafficking and secretion
plD	23_41	26_41	3_115	phospholipase D endonuclease domain-containing protein	Putative catalytic domain
ptk	7_34	10_119	34_139	protein tyrosine kinase	
tonB	4_13	41_13	26_7	periplasmic protein TonB	Cell envelope biogenesis, outer membrane
	3_48	8_48	23_13	TonB-dependent receptor	Cell envelope biogenesis, outer membrane
vipA	7_37	10_116	16_93	WecC protein	Vi polysaccharide biosynthesis protein TviB; Provisional
wbbJ	7_39	10_114	54_28	WbbJ protein	putative trimer interface, polypeptide binding

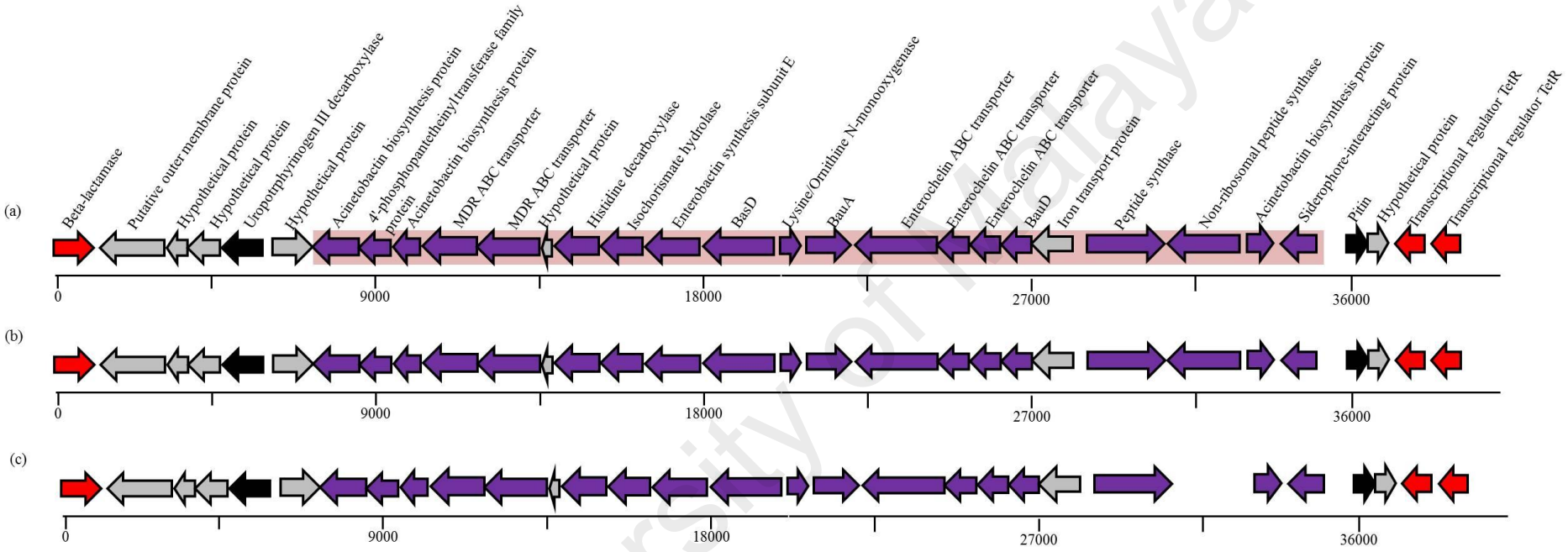


Figure 11: Schematic diagram representing the candidate pathogenicity island indicated in light pink colored area. Red arrow represents antibiotic resistant determinants, grey arrow represents gene encoding hypothetical protein, black arrow represents known homologues of functional genes, and purple arrow represents virulence determinants. Structures indicated are as found in the genome of *A. baumannii* strain (a) AC12, (b) AC30, and (c) AC29.

4.4 RESISTANCE MECHANISMS

4.4.1 Various Resistance Determinants in *A. baumannii* AC12, AC29 and AC30

Genome analyses of *A. baumnnii* AC12, AC30 and AC29 have identified genes associated with resistance to various antimicrobials (Table 10). The three genomes shared the same set of resistance determinants responsible for β-lactams and non β-lactams resistance. Resistance determinants that encode for β-lactamases from four different classes (i.e. Ambler class A, B, C and D) were identified. For instance, bla_{TEM}, which encodes for class A extended spectrum β-lactamases (ESBL), was found in all three *A. baumannii* strains. In AC12, bla_{TEM} was located in the AC12-RI2 island, whereas for AC30 and AC29, the same bla_{TEM} was not located in any detectable RIs in the chromosome. A putative Class B carbapenemase-encoding *mbl* gene (encoding a protein of the MBL superfamily), was also shared across *A. baumannii* AC12, AC30 and AC29. The class C β-lactamase which includes the bla_{AmpC}-encoded extended spectrum cephasporinase (ESC) was detected in all three genomes as well (see next section). The last class of β-lactamases, the class D carbapenemase, was represented by OXA-23 and the $bla_{\text{OXA-23}}$ gene was found in all three strains within Tn*2006* in the AbaR4-like island which interrupted the *comM* gene. In AC29, another copy of $bla_{\text{OXA-23}}$ was located on the pAC29b plasmid.

One of the main mechanism of fluoroquinolone resistance is mutations that alter the drug targets. The targets of fluoroquinolone action are the bacterial enzymes DNA gyrase (encoded by *gyrA* and *gyrB*) and DNA topoisomerase IV (encoded by *parC* and *parE*), both of which work together in replication, transcription, recombination and repair of DNA. Fluoroquinolones inhibit these two enzymes

leading to formation of lethal double-stranded DNA breaks (Jacoby, 2009). Fluoroquinolone resistance involves amino acid substitutions in a region of GyrA or ParC called the quinolone-resistance-determining region, which for GyrA, includes amino acids between positions 51 – 106 with mutational "hot spots" particularly at positions 83 and 87 (Jacoby, 2009). The AC12, AC30 and AC29-encoded *gyrA* and *parC* showed the Ser → Leu amino acid substitutions at positions 83 and 80, respectively, which have been implicated in fluoroquinolone resistance (Fournier et al., 2006b; Maragakis & Perl, 2008; Wisplinghoff, 2002). However, four additional novel point mutations (G145D, S118G, L644P and T872A) were observed in the AC12, AC30 and AC29-encoded *gyrA* and whether these mutations contribute to fluoroquinolone resistance would require further investigations.

Table 10: Genes conferring antibiotic resistance found in *A. baumannii* AC12, AC30 and AC29.

Antibiotics	Genes	Contig Location			Products
		AC29	AC30	AC12	
Aminoglycosides	*aad*A	3_40	10_40	1_10	Adenyltransferase
	*aad*A1a	3_40	10_40	1_10	Aminoglycoside adenylyltransferase
	*aph*A1b	88_1	91_1	67_1	Aminoglycoside 3'-phosphotransferase
	*str*A	54_7	38_7	24_8	Phosphotransferase
	*str*B	54_6	38_6	24_7	Phosphotransferase
Beta-lactams	*amp*C	14_1	37_1	26_1	Beta-lactamase Class C
	bla_{TEM}	85_1	87_1	76_2	Beta-lactamase TEM
	Class A beta-lactamase	34_58	6_58	14_58	Beta-lactamase Class A
	MBL	16_33	3_184	6_33	Metallo-beta-lactamase family protein
	bla_{OXA-23}	60_16	40_16	42_64	Beta-lactamase OXA-23
	bla_{OXA-51}	53_6	60_1	71_1	Beta-lactamase OXA-51
Carbapenems	*car*O	16_123	6_123	3_94	Putative porin protein associated with imipenem resistance
Chloramphenicol	*cml*A	35_12	47_5	16_5	Major facilitator superfamily permease
	*cml*A	35_12	47_5	16_5	cmlA transporter
Fluoroquinolones	*par*C	45_19	49_19	58_20	DNA topoisomerase IV subunit A
	parE	17_100	1_100	13_55	DNA topoisomerase IV subunit B
	*gyr*A	16_68	3_149	6_68	DNA gyrase subunit A
	*gyr*B	40_38	23_38	47_38	DNA gyrase subunit B
Tetracyclines	*tet*A, Class A	54_1	38_1	24_2	Tetracycline resistance protein
	*tet*R	54_2	38_2	24_3	Tetracycline repressor protein
Trimethoprim	*dhfr*I	33_27	5_27	2_27	Dihydrofolate reductase
Sulfonamides	*sul*I	16_16	57_14	6_16	Dihydropteroate synthase

Polymyxins	*pmr*A	15_53	34_55	20_54	Polymyxin resistance component, PmrA
	*pmr*B	15_54	34_54	20_55	Polymyxin resistance component, PmrB
	*pmr*C	15_55	43_1/39_1	20_56	Polymyxin resistance component, PmrC
	*lpx*A	29_1	13_136	12_21	UDP-N-acetylglucosamine acyltransferase
	*lpx*B	38_1	8_113	3_113	Lipid-A-disaccharide synthase
	*lpx*C	12_51	1_129	13_26	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase
	*lpx*D	19_75	13_138	12_19	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
	*lpx*H	17_129	35_87	48_20	UDP-2,3-diacylglucosamine hydrolase
	*lpx*K	12_53	8_19	3_19	Tetraacyldisaccharide 4'-kinase

4.4.2 Extended-spectrum cephalosporin resistance

The class C cephalosporinase AmpC, are a class of enzymes that target extended-spectrum cephalosporins and most β-lactams (Poole, 2004) with novel AmpC-type enzymes from *Acinetobacter* designated ADC (for *Acinetobacter*-derived cephalosporinases) (Rodríguez-Martínez et al., 2010). AmpC enzymes displaying extended-spectrum cephalosporin resistance activities have been reported to display mutations in the vicinity of several active sites, namely the Ω loop, the H-10 helix, the H-2 helix and the protein's C terminal (José-Manuel Rodríguez-Martínez, Patrice Nordmann, 2010). Multiple sequence alignment of AmpC from *A. baumannii* AC12, AC30 and AC29 (Figure 11) showed identical mutations as in the active sites of ADC-7 (Rodríguez-Martínez et al., 2010; Hujer et al., 2005), with two additional novel mutations G246S and R80S. The G246S mutation is located within the Ω loop whereas the R80S mutation is located at a non-active site.

To investigate if the bla_{AmpC} gene harboured by *A. baumannii* AC12, AC30 and AC29 contribute to extended-spectrum cephalosporin resistance, these genes were cloned and expressed in *E. coli* BL21(DE3)(pLysS) through the IPTG-inducible T7 promoter of the pET30a vector as outlined in the Materials and Methods. PCR-amplified bla_{AmpC} genes from AC12, AC30 and AC29 were initially cloned into pGEM-T Easy and the recombinant plasmids validated by Sanger sequencing prior to subcloning into pET30a through the *Bam*HI-*Hin*dIII sites introduced into the PCR primers. Once the recombinant pET30a plasmids containing the bla_{AmpC} genes from AC12, AC30 and AC29 were obtained, they were transformed into *E. coli* BL21(DE3)(pLysS). Heterologous expression was carried out by growing the transformed *E. coli* BL21 cells in the presence of 100 mM IPTG and MIC values against several β-lactam antibiotics were determined.

The results (Table 11) indicated that expression of the *ampC*$_{AC12}$, *ampC*$_{AC30}$ and *ampC*$_{AC29}$ genes conferred the *E. coli* BL21 cells with resistance towards the extended spectrum cephalosporins ceftazdime and cefepime, the monobactam aztreonam and interestingly, the carpabenem imipenem. This indicates that the *ampC* gene in these three strains likely contributes towards extended spectrum cephalosporin resistance and may also contribute towards carbapenem resistance besides the *bla*$_{OXA-23}$ gene.

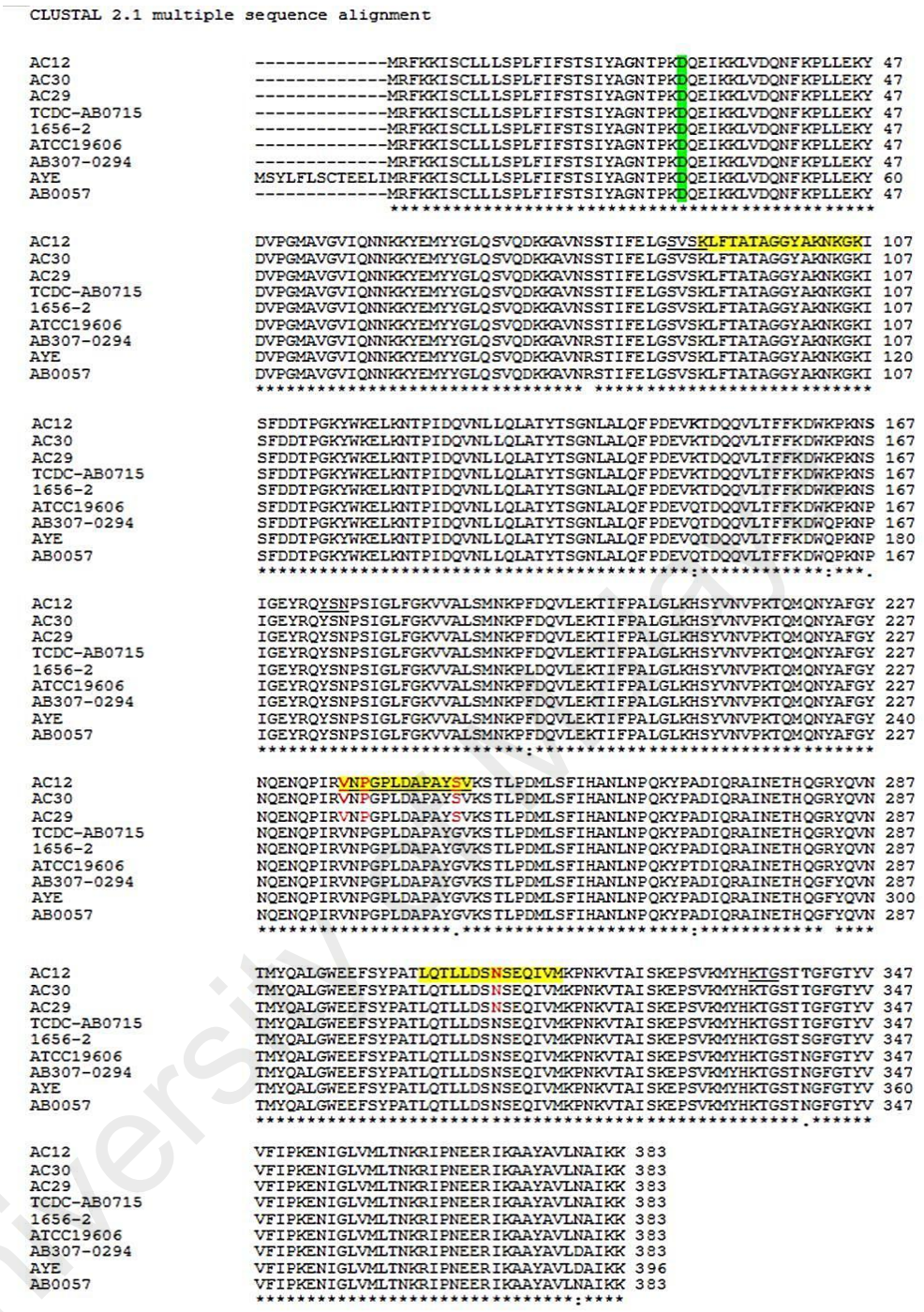


Figure 12: Multiple sequence alignment of AmpC showing alignment comparisons between different strains of *A. baumannii*. The typical domains including β-lactamase active site (SVSK), conserved triad (KTG) and class C typical motif (YXN) were underlined. H-2 and H-10 helicases were indicated in yellow highlight. The Ω loop of AmpC was highlighted in yellow and

underlined. Differences in the H-10 helicase and Ω loop were presented in red letters. Region highlighted in green indicates cleavage site for signal peptide.

Table 11: MIC values of the *ampC* clones in IPTG-induced *E. coli* BL21 towards selected antibiotics.

Clones	MIC value (μg/mL)			
	Aztreonam	Ceftazidime	Imipenem	Cefepime
BL21_ampC-AC12	16	16	32	16
BL21_ampC-AC30	16	16	32	16
BL21_ampC-AC29	16	16	32	16
Control (BL21_pET30a)	2	2	2	2

4.4.3 Polymyxin resistance

Several genetic loci have been implicated in the resistance towards polymyxins in *Acinetobacter*, namely the *pmrCAB* operon in which *pmrAB* encodes a two-component signal transduction system and *pmrC* mediates phosphoethanolamine modification of lipid A of LPS (Arroyo et al., 2011; Beceiro et al., 2011; Park et al., 2011) and the *lpxA, lpxC, lpxD* (Henry et al., 2012; Moffatt et al., 2010, 2011) and *lpsB* genes that are involved in LPS biosynthesis (Hood et al., 2013). Sequences for these genes were extracted from the whole genome sequences of polymyxin-resistant strains AC12 and AC30 as well as the polymyxin-susceptible strain AC29. The remaining 12 polymyxin-resistant strains of the 54 Terengganu *A. baumannii* strains were subjected to PCR amplification of *pmrCAB, lpxA, lpxC, lpxD* and *lpsB* and the resulting amplicons were sequenced. Three strains (AC6, AC11 and AC19) that were susceptible to polymyxin B were used as controls along with the reference strains *A. baumannii* ATCC 17978 and ATCC 19606.

As many as 11 different point mutations were found within the sensor kinase *pmrB* gene of the polymyxin-resistant strains when compared to the reference strains ATCC 17978 and ATCC 19606 but these mutations were similarly found in the polymyxin-susceptible strains. Thus, when the *pmrB* sequences of the 14 polymyxin-resistant strains were compared with the 4 polymyxin-susceptible strains, no mutations within the *pmrB* gene could be found. Similarly, when comparing the sequences of the response regulator gene *pmrA*, three different point mutations (R149K, T153I and Q165P) were observed but these mutations were commonly found in both susceptible and resistant strains. However, all 14

resistant strains had a P102H mutation within *pmrA* that was absent in the susceptible strains (Table 12). When comparing the sequences for the *pmrC* gene that encodes lipid A phophoethanolamine transferase, up to 8 point mutations were found in comparison with the reference strains. However no difference was found amongst the polymyxin-susceptible and polymyxin-resistant strains.

Overexpression of the *pmrCAB* operon is usually observed in polymyxin resistant *A. baumannii* strains (Arroyo et al., 2011; Beceiro et al., 2011; Park et al., 2011). To investigate if overexpression of *pmrCAB* was similarly observed in the resistant strains in this study, quantitative real-time reverse transcriptase PCR (qRT-PCR) was carried out on two of the polymyxin resistant strains in which the whole genome sequences were determined, i.e., AC12 and AC30, in comparison with two polymyxin-susceptible strains, *A. baumannii* AC29 and ATCC19606. The results of the qRT-PCR (Figure 12) indicated that expression of *pmrA* in AC12 was eight-folds higher when compared to the susceptible strain AC29, but *pmrA* expression in AC30 was lower (at only 0.25 folds as compared to susceptible strains). On the other hand, for *pmrB* expression, the levels were two-folds and five-folds higher for AC12 and AC30, respectively. Thus, higher levels of expression of *pmrB* and *pmrA* (in the case of AC12) were observed in the polymyxin-resistant strains, as had been reported for other polymyxin-resistant strains elsewhere (Arroyo et al., 2011; Beceiro et al., 2011; Park et al., 2011).

The *lpxA*, *lpxC* and *lpxD* genes encode the first three enzymes in the lipid A biosynthesis pathway (Moffatt et al., 2010, 2011). No mutation was found in *lpxA*. In contrast, *lpxD* showed up to 4 amino acid mutations in each resistant strain while *lpxC* had 1 – 2 amino acid mutations (Table 12). The 14 resistant

strains had the K141R, S158R or both mutations in *lpxC* that encodes the enzyme involved in the second step of the lipid A biosynthesis pathway. The *lpxD* gene showed the most number of mutations among the resistant strains with a total of 13 different amino acid substitutions. Each resistant strain had between 2 – 4 amino acid substitutions in *lpxD* and most of the mutations occurred between amino acid residues 150 – 190 of LpxD. Further comparison of the *lpxD* sequence was carried out with known polymyxin-sensitive strains in the database and results of the multiple sequence alignment indicated that the amino acid substitutions found in the Terengganu polymyxin-resistant strains were unique.

The *lpsB* gene encodes a glycosyltransferase involved in the biosynthesis of the LPS core and was recently implicated in *A. baumannii* colistin resistance (Hood et al., 2013). Comparison of *lpsB* sequences among the 14 resistant strains, the 3 susceptible strains and the 2 ATCC reference strains initially showed that each resistant strain contained as many as 4 amino acid substitutions in *lpsB*. However, when the *lpsB* sequence from other polymyxin-sensitive strains in the database was taken into account, majority of these amino acid substitutions were commonly shared among the other polymyxin-sensitive strains. The only unique mutation was H181Y and this substitution was found in 8 of the 14 resistant strains, including AC12 and AC30 (Table 12). The remaining 6 resistant strains did not harbour any unique mutation within *lpsB*.

To investigate if the mutations observed in the *lpxD*, *lpxC* and *lpsB* would result in deficiencies of the lipopolysaccharide (LPS) layer as had been previously reported (Hood et al., 2013; Moffatt et al., 2010, 2011), the LPS from two polymyxin-resistant strains, AC12 and AC30, along with two polymxyin-susceptible strains AC29 and ATCC19606 were extracted and analyzed on 15%

SDS-polyacrylamide gels (Figure 13). SDS-PAGE of the extracted LPS yielded a band of ~10 kD, which was within the expected molecular weight (between 6 and 10 kD) for LPS. Results indicated that the LPS in the polymyxin resistant strains AC12 and AC30 were not totally absent as had been previously reported in other polymyxin-resistant strains (Hood et al., 2013; Moffatt et al., 2010, 2011) but the intensity of the LPS band was considerably less when compared with the LPS band of the polymyxin-susceptible strains. This suggested that the mutations found in the *lpxD*, *lpxC* and *lpsB* genes in the two polymyxin-resistant strains may have led to impairment but not a total loss of the LPS. This, along with the overexpression of the *pmrAB* genes would possibly contribute to the relatively high polymyxin resistance observed in AC12 and AC30.

Table 12: Mutational points found in the polymyxin-resistant determinants among the 14 polymyxin-resistant *A. baumannii* strains as compared to susceptible strains.

Isolate	Mutations			
	pmrA	*lpxD*	*lpxC*	*lpsB*
AC12	P102H	S102T, V141I, R173G	K141R	H181Y
AC30	P102H	S102T, V141I, R173G	K141R	H181Y
AC3	P102H	T104K, I178V	S158R	-
AC13	P102H	T121A, N151D, G169S	S158R	-
AC16	P102H	E50D, V141I	K141R	H181Y
AC17	P102H	E50D, V141I	K141R	H181Y
AC18	P102H	E50D, V141I	K141R	H181Y
AC20	P102H	T15A, T121A, N151D, G186S	K141R, S158R	H181Y
AC21	P102H	T15A, T121A, N151D, G186S	K141R, S158R	-
AC22	P102H	E50D,S102T, I178V	K141R, S158R	H181Y
AC27	P102H	S102T, G169S, R173G	S158R	H181Y
AC38	P102H	S102T, G169S, I178V, G186S,	K141R	-
AC51	P102H	E50D, R173G	K141R, S158R	-
AC61	P102H	I178V, S181N	K141R, S158R	-

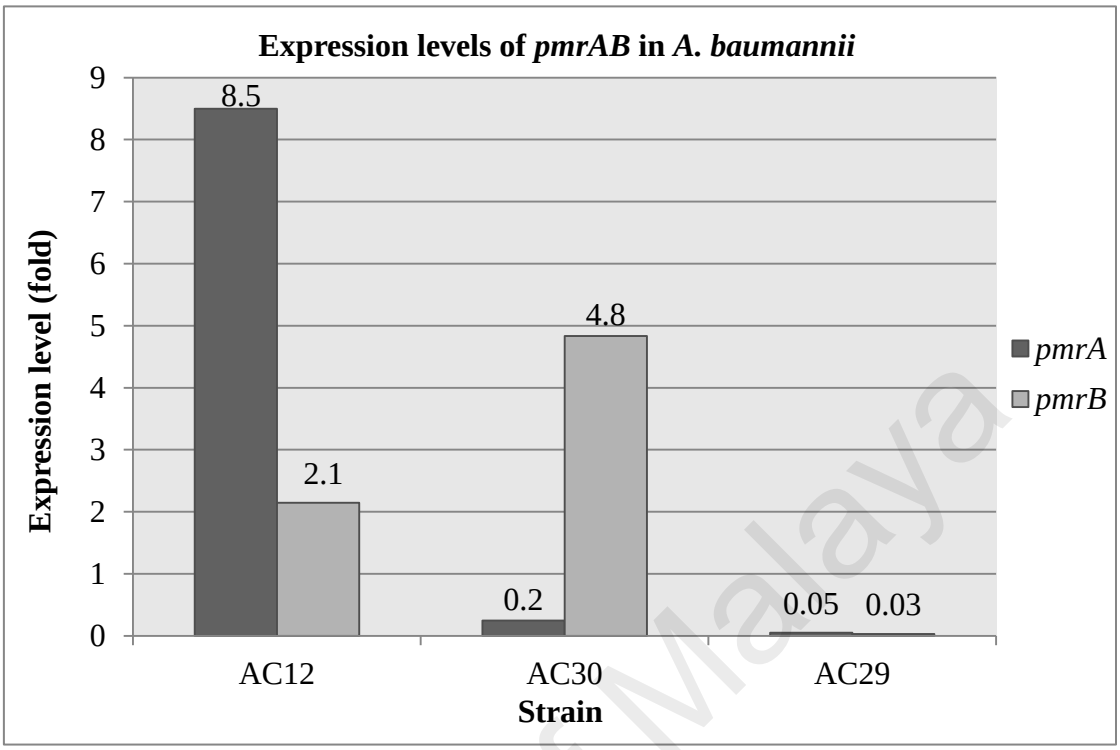


Figure 13: Relative expression levels of *pmrAB* of the polymyxin-resistant *A. baumannii* AC12 and AC30 as compared to the polymyxin-susceptible *A. baumannii* AC29 as determined by Quantitative Real-Time reverse transcriptase-PCR (qRT-PCR).

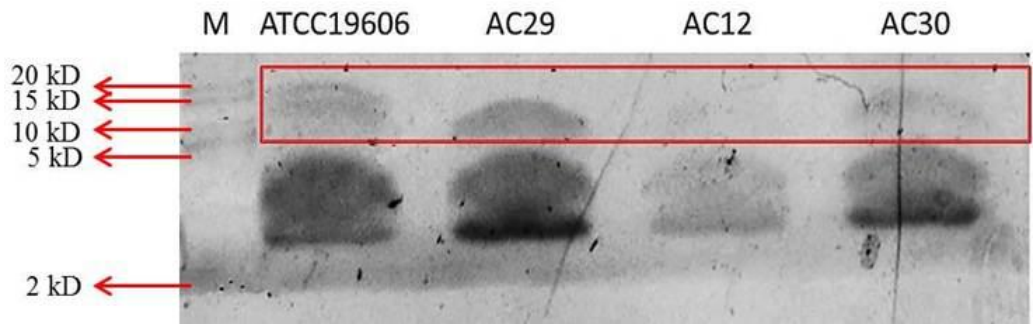


Figure 14: SDS-PAGE analysis of the extracted lipopolysaccharide (LPS) layer of the *A. baumannii* ATCC19606 (control; polymyxin susceptible strain), AC12 (polymyxin resistant strain), AC29 (polymyxin susceptible strain) and AC30 (polymyxin resistant strain). M stands for the protein marker Precision Plus Protein Dual Xtra Standards (BioRad) with the sizes as indicated in kD.

CHAPTER 5

5.0 DISCUSSIONS

5.1 MOLECULAR SUBTYPING

Clinically isolated *Acinetobacter baumannii* nowadays are generally multidrug resistant (MDR) due to the increasing rates of resistance against most, if not all current antibiotics (Feizabadi et al., 2008 ; Nigro et al., 2011). This has developed into a serious therapeutic problem because *A. baumannii* is one of the most important nosocomial pathogens which causes deadly infections such as pneumonia in immunocompromised patients (Feizabadi et al., 2008). The few publications on clinical *A. baumannii* strains from Malaysia were indicative of high resistance rates against most antibiotics (Kong et al., 2011; Dhabaan et al., 2012) but these studies were carried out on strains obtained from hospital around Kuala Lumpur on the west coast of Peninsular Malaysia. No such data is, to our knowledge, available for strains isolated from other parts of Malaysia. Therefore in this study, *A. baumannii* strains isolated from Hospital Sultanah Nur Zahirah (HSNZ), the main tertiary hospital in Kuala Terengganu which is located on the east coast of Peninsular Malaysia, were characterized.

Like their counterparts from the west coast of Peninsular Malaysia, *A. baumannii* strains that were isolated from HSNZ in 2011 were found to be highly resistant to most antibiotics. The highest resistance rate shown among 54 *A. baumannii* strains studied was against tetracycline (of which 87% were resistant). Of concern, resistance rates for carbapenems were also high with 77.8% for meropenem and

74.1% for imipenem. Although the carbapenem resistance rates were lower when compared to the rates reported from another medical facility in Kuala Lumpur (96.5% for imipenem and 98.2% for meropenem; (Kong et al., 2011), the finding that more than 50% of the strains from HSNZ were carbapenem-resistant is certainly a cause for concern as carbapenems have been the drug of choice for *Acinetobacter* infections for over a decade (Nordmann & Poirel, 2008; Poirel et al., 2010). This suggests that carbapenems were increasingly compromised in local *A. baumannii* strains and were no longer the effective treatment for *Acinetobacter* infections in Malaysia. The over-use of carbapenems in hospitals to treat *Acinetobacter* infections has led to outbreaks of carbapenem-resistant *A. baumannii* (Devereux & Wilkinson, 2004), which was likely the case at HSNZ. Resistance rates of *A. baumannii* differ across continents and healthcare centers, however, antibiotic resistance rates of HSNZ isolated *A. baumannii* are considered as high with MDR prevalence at 72.2% and with the resistance rates of most antibiotics tested at >50%.

The spread of carbapenem-resistant MDR *A. baumannii*, has led to the reintroduction of polymyxins as a therapeutic option for *Acinetoabcter* infections. This renewed interest in the polymyxins was due to its antibacterial activity against a wide spectrum of Gram-negative pathogens, including *A. baumannii*, despite its previously reported nephrotoxicity (Landman et al., 2008). Since most Gram-negative nosocomial pathogens including *A. baumannii* were susceptible to polymyxins, polymyxin B or colistin (polymyxin E) served as a replacement for carbapenems. However, with the re-introduction of polymyxin B and colistin as therapeutic option and a drug of "last resort" for *A. baumannii* infections,

polymyxin resistant and heteroresistant *A. baumannii* has recently been reported (Cai et al., 2012).

Prior to this study, there has yet to be any published reports of polymyxin-resistant *A. baumannii* in Malaysia. However, in this study, it was found that out of 54 *A. baumannii* strains from HSNZ, 14 (or 25.9%) were resistant to polymyxin B, of which, 4 had MIC values for polymyxin B at >128 μg/mL. This resistance rate towards polymyxin B was higher than that reported in Ko et al (2007) from Korea, which was 18.1% and is indeed a cause of concern. However, Ko et al. (2007) did report a 27.9% resistance rate for colistin and a later report from Spain (Arroyo et al., 2009) indicated an even higher resistance rate of 40.7%. Even though polymyxin resistance in *A. baumannii* could still be categorized as rare worldwide (Cai et al., 2012; Ko et al., 2007), the discovery of polymyxin-resistant XDR *A. baumannii* in HSNZ would certainly pose a serious threat within the hospital settings. It is feared that there will be no effective antimicrobial agents for *Acinetobacter* infections, except tigecycline (Ko et al., 2007) but tigecycline resistance has already been reported (Dhabaan et al., 2012). The seemingly high resistance could be attributed to the use of the drugs in the hospital concern although this is a controlled drug (Unpublished reports). Therefore, careful and accurate selection of antibiotics is crucial in hospitals along with rigorous screening to prevent the dissemination of XDR and especially PDR *A. baumannii* where there will indeed be no treatment options available.

PFGE results indicated that the polymyxin-resistant strains were genetically diverse. This is in contrast to the carbapenem-resistant strains which were clonally related. The carbapenem-resistant cluster A was the largest cluster inferring that this is the

major clonal group of *A. baumannii* existing within the hospital in 2011. Four of the 21 strains within the A cluster were polymyxin resistant and XDR with three of these strains having MIC values for polymyxin B > 128 μg/mL. Similar results were obtained whereby polymyxin-resistant strains were found in clusters B, C and E although the MIC values for these strains were ≤ 32 μg/mL. The clustering of carbapenem resistant strains has indeed been previously reported (Chaulagain et al., 2012; Kouyama et al., 2012). Carbapenem resistance is due mainly to the acquisition of carbapenemases such as OXA-23 which are encoded on plasmids and other mobile elements. Strains that acquire such genes will then undergo clonal expansion (D'Arezzo et al., 2011; Karah et al., 2012). In contrast, polymyxin resistance has been shown to develop through mutations in certain intrinsic determinants in response to selection pressure (Arroyo et al., 2011; Adams et al., 2009; Henry et al., 2012; Moffatt et al., 2010; Moffatt et al., 2011). Thus these strains were likely to develop randomly and in response to the presence of polymyxins. So far, polymyxin resistance has been found to be due to alterations or the complete absence of the outer membrane LPS (Arroyo et al., 2011; Adams et al., 2009; Henry et al., 2012; Moffatt et al., 2010; Moffatt et al., 2011) and this may incur a fitness cost to the bacterium. Thus, these strains may not persist in the absence of polymyxin which could explain the random, non-clustering nature of the polymyxin-resistant strains. This was indeed reported in a recent paper (Snitkin et al., 2011) where colistin-susceptible strains out-competed resistant strains upon withdrawal of colistin in three out of four cases. Colistin-resistant *A. baumannii* has also been reported to have reduced virulence in mice (López-Rojas et al., 2011) although a more recent report indicated contrasting results whereby colistin

resistance may not necessarily intrinsically affect virulence (Lin et al., 2012). Since polymyxin resistant strains appeared to be less fit and the trait is not due to the acquisition of certain genes such as *bla*$_{\text{OXA-23}}$ for carbapenem resistance, the possibility of polymyxin-resistant *A. baumannii* to spread would be less when compared to carbapenem-reisstant strains. Thus, proper and regulated use of polymyxins as a therapeutic option only when it is absolutely necessary should be able to contain the spread of polymyxin-resistant strains in hospitals and healthcare facilities.

5.2 WHOLE GENOME SEQUENCING of *A. baumannii*

Having the whole genome sequences of clinically important pathogens serves to provide a more thorough understanding on the pathogens' basic features and helps in the process of developing effective treatments for the ultimate control of infections (Zhu et al., 2013). The advent of next generation sequencing (NGS) technology enabled the whole genome sequences of bacterial pathogens to be determined at a much faster rate and lower cost. Decoding the *A. baumannii* genome enabled the evaluation of its resistance mechanisms, pathogenicity and genomic organization (Zhu et al., 2013). Even though many clinically important and prevalent *A. baumannii* have been sequenced, there is still a lack of *A. baumannii* genome data from Southeast Asia. In this study, three of the 54 clinical *A. baumannii* strains isolated from the main tertiary hospital in Terengganu, Malaysia, were subjected to whole genome sequencing: these were the XDR strains AC12 and AC30 and the MDR strain AC29, which shared a similar PFGE profile with AC30. All three strains belonged to the ST195 lineage and were closely related to each other. In a recent survey of 108 carbapenem-resistant *A.baumannii* obtained throughout Asia, ST195 strains were only found in two locales, Malaysia and Thailand (Kim et al., 2013) and were part of the clonal complex 92 (CC92) lineage that was predominant in Asia. It is thus intriguing that phylogenetic clustering of the assembled genomes placed the three *A. baumannii* strains AC12, AC29 and AC30 clearly within the GC3 group instead of GC2. MLST only examines the sequences of seven housekeeping loci in the genome as compared to the sequences of the entire genome for whole genome sequencing. Furthermore, *A. baumannii* is well known for undergoing frequent horizontal gene transfer among strains (Nordmann

and Poirel, 2008). In a recent paper comparing PFGE with whole genome sequencing for vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* and *A. baumannii*, it was found that *A. baumannii* showed the largest amount of discrepancies between the PFGE results and that of whole genome sequencing due likely to rapid horizontal gene transfer among strains (Salipante et al., 2015). AC12, AC29 and AC30 are currently the only three ST195 *A. baumannii* strains that have been fully sequenced and available in the database. Whether the discordance in the ST195 grouping is limited to the Terengganu strains would await analysis of the whole genome sequences of other ST195 strains when they are available.

5.2.1 Resistance Islands

One of the interesting features of the *A. baumannii* genome is the presence of genomic islands containing clusters of several antibiotic resistance determinants termed resistance islands (RIs) (Fournier et al., 2006; Post et al., 2010b; Nigro et al., 2011). RIs have been previously reported from a range of different pathogens such as *Shigella flexineri*, *Salmonella enterica*, *Vibrio cholerae* and *Staphylococcus aureus* (Fournier et al., 2006). Therefore, it is not surprising that RIs could be found within the genomes of clinical strains of *A. baumannii*. This mobile element functions as a vehicle for the transport and acquisition of antibiotic resistant determinants within *A. baumannii* and across different pathogens (Nigro & Hall, 2012). RIs in *A. baumannii* generally ranged in size between 20 kb and 60 kb, with the largest RI, AbaR1, reported at 86 kb and harboured up to 45 different resistance determinants in the genome of *A. baumannii* AYE (Fournier et al., 2006).

AbaR-type resistance islands can be identified in most global clones of *A. baumannii* (Nigro & Hall, 2012; Kim et al., 2013) with the majority of these RIs categorized under the AbaR-type in *A. baumannii* and usually interrupt the *comM* gene (Kim et al., 2013; Post et al., 2010). Genome analyses of *A. baumannii* AC12, AC30 and AC29 revealed that the *comM* gene in all three genomes was interrupted by the insertion of a novel ~23 kb AbaR4-type RI which were designated AC12-RI1, AC30-RI1 and AC29-RI1, respectively. These islands shared some similarities with Tn*6167* from *A. baumannii* A91 (Nigro & Hall, 2012), RI_{MDR-TJ} from *A. baumannii* MDR-TJ (Huang et al., 2012) and AbaR22 from *A. baumannii* MDR-ZJ06 (Zhou et al., 2011). AC12-RI1 and AC29-RI1 contained only one copy of ΔTn*6022*, a truncated version of Tn*6022* with the loss of a ~3 kb fragment whereas AC30-RI1 contained a full version of Tn*6022*. In AC12-RI1, the entire *tniD* and *tniE* genes were missing from Tn*6022* whereas in AC29-RI1, *tniD* and *tniE* were present but the entire *tniC* and *tniE* were missing.

When compared to the other similar RIs, both RI_{MDR-TJ} (Huang et al., 2012) and Tn*6167* (Nigro & Hall, 2012) contained Tn*6022*Δ1 in the same position as AC12-RI1 and AC29-RI1. It was located immediately downstream of the interrupted *comM* gene and on the left end of the RI (Figure 5) whereas for AC30-RI1 and AbaR22, the full length Tn*6022* was present at the same location. Comparison of the ΔTn*6022* structure between RI_{MDR-TJ}, AC12-RI1 and AC29-RI1 indicated that one of the main differences is in the deletion derivatives of the *tni* genes within Tn*6022* (Figure 5). RI_{MDR-TJ} consisted of incomplete *tniB*, *tniD* and *tniE* (denoted as Δ*tniB*, Δ*tniD* and Δ*tniE*, respectively) genes within Tn*6022*Δ1. In the case of AC12-RI1, *tniD* and *tniE* were completely lost from the same region which was denoted as

ΔTn*6022*. However, in AC29-RI1, the ΔTn*6022* was missing the *tniC* gene on its far left end, but full-length *tniD* and *tniE* genes were present in the transposon. AC30-RI1 contained the full length Tn*6022*. Thus, in all three AC12-RI1, AC30-RI1 and AC29-RI1 islands, only the full length *tniA* and *tniB* genes were commonly shared on the left (or 5'-end) of ΔTn*6022*/Tn*6022*; however, on the right (or 3'-end) of the transposon, all three islands contained the same hypothetical *orf*, *uspA*, *sup* and *orf4* CDS. In RI_{MDR-TJ} and AbaR22, another copy of the complete Tn*6022* was located next to the *tetA*(B) gene but in the three islands presented here, the second copy of Tn*6022* was absent.

All three AC12-RI1, AC30-RI1 and AC29-RI1 islands contained the composite transposon Tn*2006* which was inserted immediately following *orf4* of Tn*6022*/ΔTn*6022*. Interestingly, Tn*2006* was found flanked by *orf4* of Tn*6022*/ΔTn*6022* and a second copy of *orf4* (Figure 5), an arrangement which has not been reported before. Tn*2006* comprises of the bla_{OXA-23} carbapenase-encoding gene, a DEAD/DEAH box helicase-like gene and an ATPase gene (*yeeA*) flanked by two copies of IS*Aba1*. Besides that, comparison between the Tn*2006* present in the three RIs and Tn*6167* showed some differences. Tn*6167* harbours Tn*2006*, but at a different location whereas both RI_{MDR-TJ} and AbaR22 do not contain Tn*2006*. However, the right end of the islands were identical with Tn*6167*, RI_{MDR-TJ} and AbaR22 and comprises of *tetA*(B)-*tetR*(B) (conferring tetracycline resistance), the small mobile element CR2, *strA*-*strB* (conferring streptomycin resistance) and *orf4b*, a hypothetical ORF related to *orf4* of Tn*6022* and Tn*6022*Δ1 (Figure 5). The structure of AC12-RI1, AC30-RI1 and AC29-RI1 indicated that they are novel variants of the AbaR4-type RIs that were recently reported in a survey of RIs found

in *A. baumannii* strains throughout Asia (Kim et al., 2013). Kim et al. (2013) also reported that AbaR4-type RIs were commonly found among carbapenem-resistant CC92 strains which included the ST195 lineage.

A second RI which was only identified in the genome of *A. baumannii* AC12 was based on sequence similarities with the AbGRI2-1 island found in the GC2 Australian *A. baumannii* WM99c strain (Nigro et al., 2013a). This RI is designated AC12-RI2 and is located at the same chromosomal region as the AbGRI2-1 island, i.e., interrupting a D-serine/D-alanine/glycine transporter gene at the 5'-end of the island and a flavin monooxygenase at the 3'-end. Both AC12-RI2 and AbGRI2-1 are flanked by two copies of IS*26*. However, AC12-RI2 is 10.3 kb whereas AbGRI2-1 is 19.5 kb, with AC12-RI2 missing the class 1 integron containing the *aacC1-orfP-orfP-orfQ-aadA1* array as well as an incomplete Tn*21* found at the 3'-end (right-most end) of the AbGRI2-1 structure (Figure 6). The resistance genes found in AC12-RI2, *aphA1b* (conferring kanamycin and neomycin resistance) and bla_{TEM} (β-lactam resistance), are each flanked by two copies of IS*26*, an arrangement that is identical to that in AbGRI2-1 (Nigro et al., 2013a). All 5 copies of IS*26* in AbGRI2-1 are present in AC12-RI2. Different variants of AbGRI2-1 have been reported in other GC2 strains such as MDR-TJ which lacks the *aphA1b* and bla_{TEM} segments and contains only 3 copies of IS*26* (Nigro et al., 2013a).

With the mosaic nature of these RIs, it is thus not surprising that segments of the RI are found in the genomes of other *A. baumannii* strains. As pointed out by Nigro et al. (2013a), the AbGRI2-1 segment spanning the portion of Tn*1* (containing bla_{TEM}) to the end of Tn*6020* (containing *aphA1b*) is identical to a part of AbaR islands found in GC1 strains. Indeed smaller versions of AC12-RI2 are found in the

genomes of *A. baumannii* AB0057 (accession no. CP001182) (spanning part of *tnpR* of Tn*1* and *bla*$_{TEM}$ until the IS*26* adjacent to *aphA1b*) and 3208 KL1 (accession no. FJ172370), which contained only *aphA1b* and the Tn*1000*-like portion but without the *bla*$_{TEM}$ segment (Figure 6). In BJAB0868 (accession no. CP003849), only *bla*$_{TEM}$ and flanking IS*26* (i.e., the Tn*1* portion) were found in the same location as AC12-RI2. Since this AbGRI2-1-type island does not seem to be a very common feature across completed *A. baumannii* genome available in the NCBI database, it is interesting that the island is found in *A. baumannii* AC12 but not in AC30 and AC29 as these three strains are closely related to each other. This suggests that the AC12-RI2 may be a recent acquisition to the *A. baumannii* strains isolated from Terengganu.

A. baumannii AC12 was also found to harbour another unique RI through genome alignment of its genome sequence to its closest neighbors using Mauve. One of the regions that appeared unique to AC12 was an approximately 7 kb fragment containing the Tn*1548*::*armA* island which was previously found in the pMDR-ZJ06 plasmid of *A. baumannii* MDR-ZJ06 (Zhou et al., 2011) as well as plasmids from several *Enterobacteriaceae* including pKT51748 of *Klebsiella pneumoniae* (accession no. FJ715937), pNDM-HK of *Escherichia coli* (accession no. HQ451074), pXD1 of *Salmonella enterica* Paratyphi B (accession no. JN225877) and pNDM-CIT plasmid of *Citrobacter freuendii* (Dolejska, Villa, Poirel, Nordmann, & Carattoli, 2013). This 7 kb fragment includes genes encoding transposases (*tnpU* and *tnpD*) from Tn*1548::armA*, the 16S rRNA methylase (*armA*) which confers resistance to aminoglycosides, a macrolide efflux protein (*mel*) and a macrolide 2'-phosphotransferase (*mph2*) that confers macrolide resistance and was

also subsequently found on plasmid pAC30b in *A. baumannii* AC30 (see next section) and the same region in the AC29 chromosome.

It is noteworthy that thus far, the Tn*1548::armA-mel-mph2* fragment was located on plasmids and a recent analysis showed that a *repAciN* replicase gene was always located immediately downstream of *mph2* and this entire region was bounded by two copies of IS*26* (Dolejska et al., 2013). Such an arrangement was not observed in the AC12 genome where only the Tn*1548::armA-mel-mph2* genes were found without the presence of *repAciN*. Although Tn*1548*::*armA* was discovered in pMDR-ZJ06 from *A. baumannii* MDR-ZJ06, the *armA* gene is still absent from most of the plasmids recovered from *A. baumannii* (Dolejska et al., 2013). A recent report indicated the presence of a Tn*1548*-like RI in the chromosome of several *A. baumannii* strains from the United States (Wright et al., 2014). However, the location and the resistance gene content of this RI [*armA*, *aadA1*, *aac*(6')-*Ib*, *aphA1* for aminoglycoside resistance, and *catB8* for chloramphenicol resistance] differs to that of the Tn*1548*::*armA-mel-mph2* island in AC12. Comparative analysis of this Tn*1548*::*armA* region strongly suggested mobilization of this transposon across species, in which the transposition was IS*26*-mediated and originally targeted plasmids (Dolejska et al., 2013), thereby promoting the spread of the *armA* gene.

5.2.2 Plasmids

Plasmids are extra-chromosomal elements which exist naturally within bacteria (Camp & Tatum, 2010). Categorized as mobile genetic elements, plasmids are transferrable and capable of integrating into bacteria of the same and/or other species (Camp & Tatum, 2010; Sahl et al., 2011). Therefore, it became one of the vehicles of transmission for antibiotic resistance genes, facilitating antimicrobial

resistance when resistance determinants happened to be located in a plasmid (Vila et al., 2007; Camp & Tatum, 2010; Sahl et al., 2011; Tan et al., 2013). Among mobile genetic elements such as transposons and integrons, plasmids have been well recognized for its influence in the acquisition of multidrug resistance and new resistance mechanisms (Vila et al., 2007; Sahl et al., 2011). This was shown by a plasmid recovered by Goldstein et al. in the 1980s, whereby the plasmid carried three different antibiotic resistance determinants encoding β-lactamase TEM-1, aminoglycoside-modifying enzyme APH(3')(5') and ADD(3")(9) (Vila et al., 2007). Acquisition of such antibiotic resistance plasmids in *A. baumannii* plays an important role in enhancing its multidrug resistant phenotypes, as it makes the establishment of new resistance determinants possible (Vila et al., 2007; Camp & Tatum, 2010; Sahl et al., 2011; Tan et al., 2013).

One of the interesting features of the three sequenced *A. baumannii* genomes is the discovery that *A. baumannii* AC12, AC30 and AC29 shared the same small cryptic plasmid. The ~8 kb plasmid, designated pAC12, pAC30a and pAC29a in their respective strains, however contained no antibiotic resistance gene. The plasmids are almost identical to pAB0057 and p1ABTCDC0715 reported in Adams et al (2008) and Chen et al (2011), respectively. A similar *A. baumannii* plasmid, pABVA01 (8,963 bp), was found to harbor the $bla_{\text{OXA-24}}$ carbapenese gene flanked by XerC/XerD-like recombination sites (D'Andrea et al., 2009) and this arrangement was also subsequently reported in the 8,771 bp plasmid pMMCU1 (Merino et al., 2010). The XerC/XerD-like recombination sites were present in pAC12/pAC30a/pAC29a but with the *selI* gene instead of $bla_{\text{OXA-24}}$ within the potential recombination region. The pAB0057 plasmid from *A. baumannii* AB0057

(Adams et al., 2008) also contained the *sel1* gene in between the XerC/XerD sites. The function of the *sel1* gene is currently unknown. Other similar *Acinetobacter* plasmids harboured different sized fragments between the XerC/XerD recombination sites with p2ABAYE from *A. baumannii* AYE (Fournier et al., 2006) for example, harbouring a putative alcohol dehydrogenase gene (Figure 15). The $bla_{\text{OXA-24}}$ gene is so far reported only in strains from Italy and Spain (D'Andrea et al., 2009; Merino et al., 2010). This was corroborated in a recent multicenter study which showed that the $bla_{\text{OXA-24}}$/$bla_{\text{OXA40-like}}$ genes in *A. baumannii* strains isolated from Spain was predominantly carried by small 8-12 kb plasmids with two of the sequenced plasmids, pAbATCC223 and pAbATCC329, harbouring the $bla_{\text{OXA-24}}$ gene in between the XerC/XerD sites (Mosqueda et al., 2013). Xer recombination is a site-specific recombination mechanism involved in events such as the integration of phage CTX-Φ at the *dif1* site in the *Vibrio cholera* chromosome (D'Andrea et al., 2009). Moreover, the proteins required for Xer recombination, such as the XerC and XerD recombinases and PepA are encoded in the chromosome of several *A. baumannii* strains (Merino et al., 2010). Thus, the XerC/XerD-like sites on these 8 kb plasmids could act as site-specific recombination targets responsible for mobilization of discrete gene modules such as $bla_{\text{OXA-24}}$ and *sel1* within *Acinetobacter* plasmids (D'Andrea et al., 2009; Merino et al., 2010).

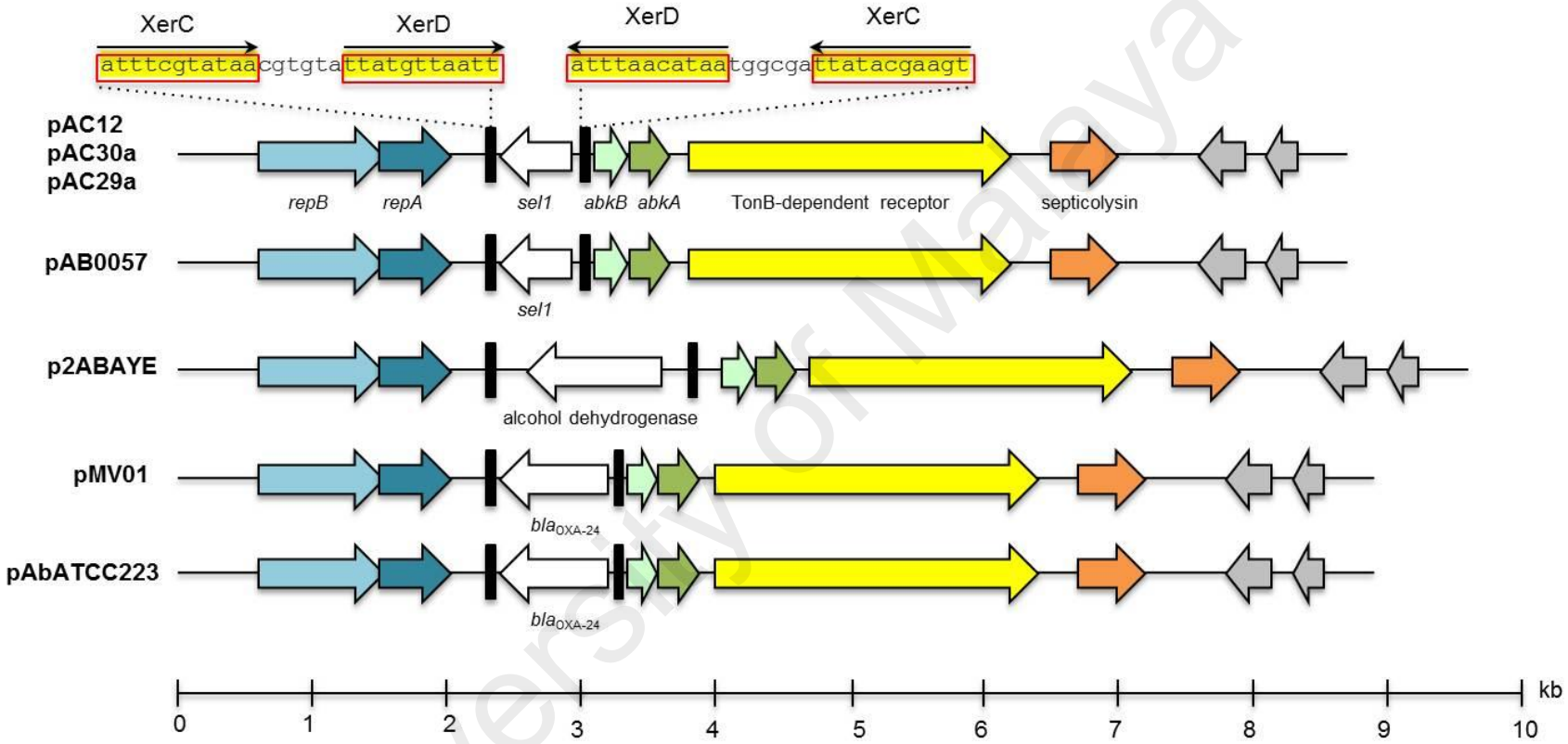


Figure 15: Genetic organization of plasmids pAC12, pAC29a and pAC30a in comparison with other similar *A. baumanii* plasmids. The XerC/XerD-like recombination sites are indicated in black rectangular boxes with the nucleotide sequences as indicated above the boxes. The two plasmid replication genes, *repB* and *repA*, are indicated in blue whereas the putative *abkA/abkB* toxin-antitoxin genes are indicated in green arrows. Hypothetical ORFs are shaded grey. The TonB-dependent receptor gene is indicated in yellow whereas the putative septicolysin gene is in orange.

Another interesting feature of these small plasmids is the presence of a toxin-antitoxin (TA) system designated AbkB/AbkA (Mosqueda et al., 2014). TA systems are usually characterized by two co-transcribed genes with the antitoxin gene preceding the toxin gene; the antitoxin gene encoding either a labile antisense RNA which prevents translation of the toxin mRNA (type I TAs) or an unstable protein which binds tightly to the toxin protein preventing it from exerting its lethal effect (type II TAs) (Chan et al., 2012; Hayes & Kędzierska, 2014). AbkB/AbkA seems to differ from canonical TA systems as the *abkB* toxin gene precedes the *abkA* antitoxin gene. To date, only three characterized TA loci have been reported to display this unusual genetic arrangement: the *mqsRA* (Brown et al., 2009), the *higBA* (Tian et al., 1996) and *hicAB* (Jørgensen et al., 2009) modules. AbkB/AbkA was previously identified as one of the four functional TA systems in *A. baumannii* (it was designated SplT/SplA or DUF497/COG3914) whereby overexpression of the toxin was shown to inhibit growth in *E. coli* and this was overcame by co-expression of the cognate antitoxin (Jurenaite et al., 2013). The AbkB (or SplT) toxin was shown to inhibit translation when overexpressed in *E. coli* with cleavage of *lpp* mRNA and transfer-messenger RNA (tmRNA) demonstrated, thus indicating that the AbkB toxin likely functions as an endoribonuclease or RNA interferase. The AbkB/AbkA locus was found to be highly prevalent in small plasmids of *A. baumannii* clinical strains (88.6% prevalence among 476 clinical isolates from Lithuania) (Jurenaite et al., 2013). The presence of a TA system on these plasmids would explain their stability in the absence of any apparent selection pressure,

particularly for the small plasmids without the $bla_{\text{OXA-24}}$/$bla_{\text{OXA40-like}}$ gene such as pAC12, pAC30a and pAC29a.

One of the intriguing genes found in all the ~ 8 kb cryptic plasmids encode a possible TonB-dependent receptor protein. Some TonB-dependent receptors, in particular BauA, play important roles in the acquisition of iron in *A. baumannii* (Dorsey et al., 2004; Mihara et al., 2004) with recent transcriptomic and proteomic analyses indicating approximately 20 TonB-dependent receptors in *A. baumannii*, some of which are regulated by iron (Nwugo et al., 2011; Antunes et al., 2011; Eijkelkamp et al., 2011). TonB-dependent receptors are found in the outer membrane where they interact with TonB and associated inner membrane proteins (ExbB and ExbD) that provide energy needed to transport host iron-carrier and iron-siderophore complexes into the periplasm once these complexes are bound to the TonB-dependent receptors (Zimbler et al., 2013). Thus, along with TonB, the TonB-depended recpetors play an important role in the virulence of *A. baumannii*. Whether the pAC12/pAC30a/pAC29a-encoded TonB-dependent receptor protein plays a similar role in iron acquisition and hence, virulence, awaits further experimentation. Another possible virulence-associated gene encoded in these small plasmids is found downstream of the gene encoding the TonB-dependent receptor. This gene is predicted to encode a 152-amino acids-residue protein homologous to septicolysin, a putative virulence factor (Mosqueda et al., 2014). Septicolysin is a member of thiol-activated cytolysins which have been implicated in the pathogenesis of infections by several Gram-positive pathogens such as *Clostridium perfringens*, *Listeria monocytogenes* and *Streptococcus pneumoniae* and are characterized by their cytolytic activity for eukaryotic cells (Billington et al., 2000).

With two putative virulence factors encoded on these small plasmids, it would therefore be of interest to investigate if these plasmids play a role in the virulence and pathogenesis of *A. baumannii* infections, especially in view of their prevalence among clinical *A. baumannii* isolates.

Another plasmid that was discovered in this study was designated pAC30b and was found only in *A. baumannii* AC30 and not the other two strains. Plasmid pAC30b is a ~16 kb drug resistant plasmid encoding determinants for aminoglycoside (*aphA1* and *armA*) and macrolide resistance (*mel* and *mph2*), harbouring three of these determinants (i.e., *armA*, *mel* and *mph2*) in a Tn*1548*::*armA* island structure that was also located in the chromosome of strains AC12 and AC29. Plasmid pAC30b shared the most identity with pMDR-ZJ06 from *A. baumannii* MDR-ZJ06 (Zhou et al., 2011) as well as p3BJAB0868 from *A. baumannii* BJAB0868 and p2BJAB07104 from *A. baumannii* BJAB07104 (Zhu et al., 2013) and this was mainly an approximately 10 kb fragment that spanned Tn*1548*::*armA*, IS*66* and the *rep* gene. Two other parts of pAC30b that were identical with pMDR-ZJ06, p3BJAB0868 and p2BJAB07104 are IS*26* and the *aphA1* gene, both of which were in different locations in pAC30b when compared with pMDR-ZJ06 (Figure 16), p3BJAB0868 and p2BJAB07104. The *aphA1* gene is flanked by two copies of IS*26* in a composite transposon-like structure designated Tn*6210* in p3BJAB0868 and p2BJAB07104 (Zhu et al., 2013) as well as pMDR-ZJ06 but in pAC30b, only one copy of IS*26* is found adjacent to *aphA1*. It is possible that a deletion had occurred in pAC30b that took out the other copy of IS*26* as well as part of the *tnpU* gene of Tn*1548*::*armA* which is located adjacent to the IS*26*-*aphA1* structure. All three plasmids pMDR-ZJ06, p3BJAB0868 and p2BJAB07104 which were isolated from

A. baumannii strains in China, harboured a class 1 integron but this was absent in pAC30b.

It is noteworthy that in contrast to pAC12, pAC30a and pAC29a, pAC30b has very few similar plasmids in *A. baumannii* besides pMDR-ZJ06, p3BJAB0868 and p2BJAB07104, suggesting that these plasmids are not so prevalent. Tn*1548*::*armA* is indeed found in many other plasmids particularly in the Enterobacteriaceae, as had been discussed in the previous section. What the four *A. baumannii* plasmids (namely, pAC30b, pMDR-ZJ06, p3BJAB0868 and p2BJAB07104) have in common are (1) a number of transposases encoded by IS elements and transposons, and (2) a lack of any plasmid stability genes such as toxin-antitoxin systems. Thus, there is a likelihood that these plasmids are much less stable as compared to the smaller pAC12 which is endowed with the AbkB/AbkA toxin-antitoxin system. Hence, in *A. baumannii* AC12 and AC29, portions of pAC30b are chromosomally-located and there is no widespread occurrence of pAC30b-like plasmids in other *A. baumannii* strains.

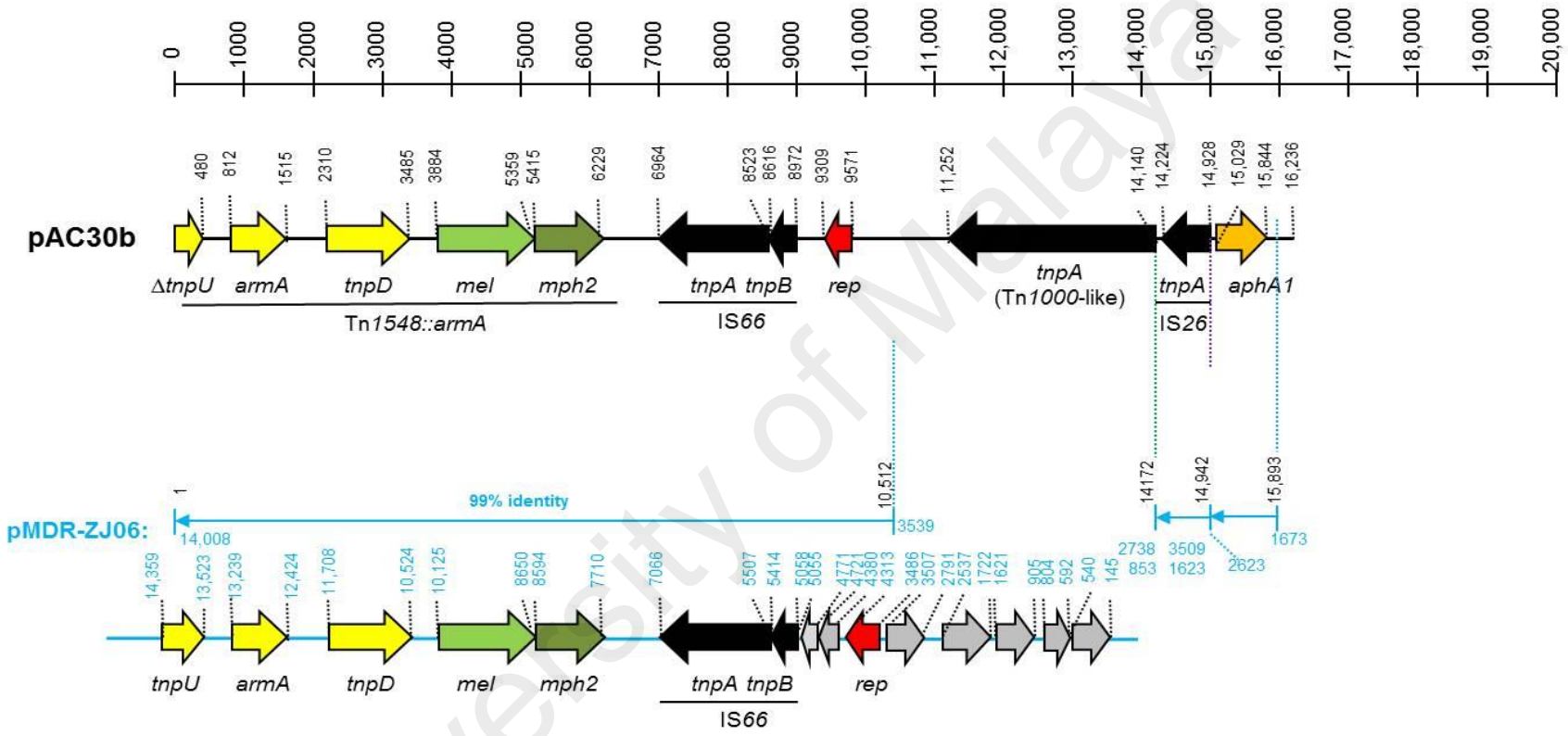


Figure 16: Linear map comparison of the Tn*1548*::*armA* region in plasmid pAC30b from *A. baumannii* AC30 and pMDR-ZJ06 from *A. baumannii* MDR-ZJ06 (NC_017172). The Tn*1548*::*armA* region and insertion elements (IS) were underlined and indicated in the diagram. Percentage nucleotide identities between the structures and gene names were stated in the scaled diagram.

The largest plasmid found in this study is an approximately 70 kb plasmid harboured in the genomes of *A. baumannii* AC30 and AC29 designated pAC30c and pAC29b, respectively. These plasmids are conjugative plasmids as they encode the complete *tra* locus (type IV secretion system or T4SS) and are similar to plasmids pACICU2 (Iacono et al., 2008), p2ABTCDC0715 (C.-C. Chen et al., 2011), pAB CC (Chang et al., 2014), pAb-G7-2 (Hamidian & Hall, 2014) and pAB85-3 (Hamidian et al., 2014). Presence of the complete *tra* locus in these plasmids indicates that they can be transferred freely, as well as integrated into chromosomes and different plasmids (Zhu et al., 2013). Transmissibility of the pAb-G7-2, pAB85-3 and pACICU2 plasmids has been demonstrated recently (Hamidian & Hall, 2014; Hamidian et al., 2014), thus it is likely that pAC30c and pAC29b are similarly transmissible by conjugation.

It is interesting to note that the T4SS genes in pAC30c and pAC29b are separated into two regions, an approximately 20 kb fragment containing 13 genes from *traL* to *traG*, and another containing just two genes, *traD* and *traI*. In pAb-G7-2, the space between these two regions contained the aminoglycoside resistance transposon, Tn*aphA6* (Hamidian & Hall, 2014) whereas in pA85-3, the AbaR4 resistance island is located in the same area (Hamidian et al., 2014). In pAC30c, this region contained several hypothetical ORFs and a solitary *relE* toxin gene without the corresponding *relB* antitoxin gene. However, the two genes flanking the *relE* could perhaps function as the antitoxin as they encode for hypothetical proteins of about the same size as the putative RelE toxin. Toxin-antitoxin pairs are usually about the same size with a few exceptions such as the Zeta toxin which is much larger (~270 amino acids) as compared to their cognate Epsilon antitoxin (~90

amino acids) (Chan et al., 2012) (Jurenaite et al., 2013). Although toxins usually interact with their cognate antitoxin pair, sometimes mixing and matching between different toxin and antitoxin families do occur (Chan et al., 2012) (Hayes & Kędzierska, 2014). Nevertheless, the functionality of the plasmid pAC30c and pAC29b-encoded *relE* toxin gene needs to be ascertained. In the case of the solitary Zeta toxin that was located downstream from the *rep*Aci6 gene, experimental evidence had suggested that its overexpression is non-toxic to *E. coli* and thus, may not function as a typical toxin (Jurenaite et al., 2013). Its putative antitoxin partner, located upstream of its reading frame, does not bear any homology to the Epsilon antitoxin. These solitary Zeta-like toxins have been observed in several plasmids and their function is currently unknown(Chan et al., 2012) (Jurenaite et al., 2013).

No antibiotic resistance determinants could be found in pAC30c but pAC29b harboured $bla_{\text{OXA-23}}$, an extended spectrum β-lactamase-encoding gene. The pA85-3 plasmid from *A. baumannii* A85 harboured the $bla_{\text{OXA-23}}$ gene within the AbaR4 island (Hamidian et al., 2014) whereas both pAb-G7-2 and pACICU2 harboured the aminoglycoside resistance gene *aphA6* within a composite transposon designated Tn*aphA6* (Hamidian & Hall, 2014; Hamidian et al., 2014). The location of these resistance determinants on a transmissible plasmid enables their direct spread which could account for the widespread dissemination of the $bla_{\text{OXA-23}}$ and *aphA6* genes among clinical strains of *A. baumannii* (Hamidian & Hall, 2014; Hamidian et al., 2014).

5.2.3 Efflux Pumps

Multidrug efflux pumps and porins play important roles in *A. baumannii* antimicrobial resistance (Chen et al., 2011). Efflux pumps function to remove particles harmful to the bacterial cytoplasmic membrane. Therefore, efflux pumps are also capable of expelling antibiotics such as β-lacams, quinolones and even aminoglycosides (Bonomo & Szabo, 2006; Wieczorek et al., 2008). Among the five major families of bacterial efflux pumps (i.e. RND, MFS, APC, ABC and MATE), the RND family was widely disseminated in Gram-negative bacteria (Poole, 2004; Bonomo & Szabo, 2006; Nordmann & Poirel, 2008; Wieczorek et al., 2008). Members of the RND family were also often associated with the development of multidrug-resistance in clinically important pathogens (Poole, 2004; Bonomo & Szabo, 2006; Wieczorek et al., 2008). Genome analysis of *A. baumannii* AC12, AC30 and AC29 showed the presence of the complete *adeABC* operon and also its derivatives across the strains. The AdeABC efflux pump, which was categorized under the RND family, has been recognized as one of the main efflux systems in *A. baumannii* (Wieczorek et al., 2008). The AdeABC efflux system works as a multidrug transporter in *A. baumannii*, and has been implicated in the resistance to aminoglycosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones, β-lactams and even the recently used tigecycline (Bonomo & Szabo, 2006; Wieczorek et al., 2008). Overexpression of *adeABC* and to a certain extent, *adeIJK*, has also been associated with the multidrug resistance phenotype in *A. baumannii* (Chen et al., 2011) and it would be of interest to investigate if this is likewise in *A. baumannii* AC12, AC30 and AC29.

5.2.4 Virulence Genes

A large number of known and potential virulence factors were found in the genome sequence of *A. baumannii* AC12, AC30 and AC29. Their presence in the genome could explain the success of *A. baumannii* as a human pathogen (Peleg et al., 2012). It has been hypothesized that *A. baumannii* persists in the hospital environment and causes disease because of its capacity to form biofilms on solid surfaces (McConnell et al., 2013). The CsuA/BABCDE usher-chaperone assembly system is needed for attachment and biofilm formation on abiotic surfaces and is widespread among clinical strains (McConnell et al., 2013), including the three sequenced genomes. The OmpA outer membrane protein is essential for the interaction of *A. baumannii* with human cells and plays a role in adherence and invasion of epithelial cells (Human et al., 2012). Indeed 4 copies of the *ompA* gene were found distributed in the genome of AC12, AC30 and AC29.

A. baumannii produces a siderophore called acinetobactin that is used to scavenge and acquire ferric ions under iron-limited conditions within mammalian hosts. Iron acquisition plays a critical role in *A. baumannii* virulence as a recent report had demonstrated (Human et al., 2012). The full complement of the genes required for the biosynthesis and transport of acinetobactin (Ab) – *bauF*, *basA*, *basB*, *bauDCEBA*, *basDC*, *basEFG-barAB-basHI* and *basJ* – is found in the genome of AC12 and AC30, except AC29 which has a missing *barB*. The *barB* gene functions to encode an ATP-binding protein of a putative ABC transporter, hence, it is likely that the BarB in *A. baumannii* works as an efflux pump for the secretion of siderophore Ab to the extracellular milieu (Mihara et al., 2004). When *barB* was absent in AC29, this may affect the release of Ab, which is a high ferric-ion-specific

chelator involved in the pathogenesis of *A. baumannii*. BarB has been identified as one of the important components for the transport of acinetobactin across the membrane under iron-stress conditions (Mihara et al., 2004). It would therefore be of interest to investigate if the absence of *barB* in AC29 would somehow lead to the strain being less virulent when compared to AC12 and AC30. The acinetobactin biosynthesis and transport genes are clustered and arranged in seven transcriptional units (Mihara et al., 2004) and in AC12, AC30 and AC29, are flanked by IS*4* on one end and a tRNA gene on the other end, indicative of a putative pathogenicity island. Besides, this cluster is not present in the non-pathogenic environmental strain *A. baumannii* SDF. However, this cluster was not in the list of *A. baumannii* genomic islands presented recently (Zarrilli et al., 2011) and Imperi et al. (2011) classified the acinetobactin cluster as part of the *A. baumannii* core genome. Whether this cluster is truly a *bona fide* pathogenicity island would require further in depth investigations.

5.3 RESISTANCE MECHANISMS

Occurrences of *A. baumannii* which were resistant to virtually all available drugs had gained much attention in public health. Despite its own intrinsic resistance to commonly used antibiotics such as aminopenicillins, first- and second- generation cephalosporins and chloramphenicol, this pathogen also possesses a remarkable ability in acquiring resistant mechanisms (Dijkshoorn et al., 2007). Among the acquired mechanisms in *A. baumannii*, includes mechanisms which confer resistance to broad-spectrum β-lactams, aminoglycosides, fluoroquinolones and

tetracyclines (Dijkshoorn et al., 2007). In depth analyses of the resistance mechanisms in *A. baumannii* revealed that the pathogen's multidrug resistance phenotype was mediated by all of the major resistance mechanisms that are known to occur in bacteria, including modification of target sites, enzymatic inactivation, active efflux and decreased influx of drugs (Dijkshoorn et al., 2007).

Besides the resistance genes found in the two RIs and Tn*1548*::*armA*, the genome of *A. baumannii* AC12, AC30 and AC29 also encode other genes associated with resistance to various antimicrobials. Previous reports showed that specific point mutations in the *gyrA* and *parC* genes were responsible for the fluoroquinolones resistance feature, since both genes encode for DNA gyrase and topoisomerase IV which are the targets for fluoroquinolones (Wisplinghoff, 2002; Fournier et al., 2006; Dijkshoorn et al., 2007). The *gyrA* and *parC* genes encoded by *A. baumannii* AC12, AC30 and AC29 showed Ser → Leu amino acid substitutions at positions 83 and 80, respectively, that were previously implicated in fluoroquinolone resistance (Wisplinghoff, 2002; Fournier et al., 2006). However, four additional novel point mutations (G145D, S118G, L644P and T872A) were observed in the *gyrA* gene found in all three genomes and whether these mutations contribute to fluoroquinolone resistance would require further investigations.

Resistance to β-lactam antibiotics via synthesis of β-lactamase encoded by the chromosome and/or plasmids is the most common resistance mechanism observed in *A. baumannii* (Bou & Martínez-Beltrán, 2000). With more β-lactamases involved in resistance to broad spectrum cephalosporins identified, resistance to oxyminocephalosporins was studied. Broad spectrum cephalosporins in *A. baumannii* are usually related to the over production of extended spectrum β-

lactamase (ESBL) in *A. baumannii*, especially AmpC-type β-lactamase designated ADCs (*Acinetobacter*-derived cephalosporinases) (Rodríguez-Martínez et al., 2010). ADCs typically hydrolyze penicillins and narrow- and extended-spectrum cephalosporins but not zwitterionic cephalosporins such as cefepime or carbapenems (Rodríguez-Martínez et al., 2010). However, extended-spectrum AmpCs (ESACs) have been reported in *A. baumannii* that confer reduced susceptibility to all cephalosporins and this includes ADC-33 (Rodríguez-Martínez et al., 2010) and ADC56 (G.-B. Tian et al., 2011). The AmpC encoded by *A. baumannii* AC12, AC30 and AC29 displayed novel mutations (R80S and G246S) that have not been reported before.

To investigate if the mutation in the bla_{AmpC} gene harbored by *A. baumannii* AC12, AC30 and AC29 has any effect on resistance against β-lactams especially cephalosporins, these genes were cloned and expressed in *E. coli* BL21 through the IPTG-inducible T7 promoter of the pET30a vector. Recombinant *E. coli* BL21 carrying the bla_{AmpC} from AC12, AC30 and AC29 displayed resistance to ceftazidime, cefepime, aztreonam and even imipenem. This strongly suggests that the AmpC from AC12, AC30 and AC29 is an ESAC cephalosphorinase. ADC-33 possessed a P210R substitution together with a duplication of the Ala residue at position 215 within the Ω loop, both of which are required for extended spectrum activity (Rodríguez-Martínez et al., 2010). ADC-56 possesed an R148Q mutation also within the Ω loop, that enabled the enzyme to hydrolyze cefepime (G.-B. Tian et al., 2011). Thus, the G246S mutation within the Ω loop of the AmpC from AC12, AC30 and AC29 could be responsible for the extended spectrum activity. This could be examined and verified by site-directed mutagenesis of the pET30a

recombinant clones. Likewise, the contribution of the R80S mutation towards extended spectrum activity should be investigated even though it is located in a non-active site.

Normally, *A. baumannii* strains that showed resistance towards third generation cephalosporins would have an insertion of the IS*Aba1* upstream of the *ampC* gene. IS*Aba1* provides a strong outward-directing promoter that drives the expression of the *ampC* gene (Bou & Martínez-Beltrán, 2000; Rodríguez-Martínez et al., 2010). However, no IS*Aba1* could be found either upstream or in the vicinity of the *ampC* gene in *A. baumannii* AC12, AC30 and AC29. Nevertheless, there have been reported cases where resistance towards extended spectrum cephalosporins in *A. baumannii* was displayed in the absence of an upstream IS*Aba1* (Martínez and Mattar, 2012).

The mechanism for polymyxin resistance in *A. baumannii* has only recently been investigated and the main mechanism appeared to be either covalent modification of the lipid A portion of LPS (Arroyo et al., 2011; Beceiro et al., 2011) or disruption of LPS biosynthesis (Moffatt et al., 2010; Park et al., 2011). By modification and/or mutations in the amino acid sequences, negative charges on the outer membrane can be reduced, leading to reduction in the affinity of the positively-charged polymyxin component, hence giving rise to polymyxin resistance (Adams et al., 2009; Arroyo et al., 2011; Beceiro et al., 2011). It is interesting that all 14 polymyxin-resistant strains (including AC12 and AC30) displayed an identical P102H mutation within the *pmrA* gene and this mutation was identical to that previously reported by Adams et al. (2009) in a colistin-resistant derivative of *A. baumannii* AB0057.

Overexpression of the two-component signal transduction system *pmrAB* and mutations within these genes, especially *pmrB*, were reported to contribute to polymyxin resistance (Adams et al., 2009; Arroyo et al., 2011; Park et al., 2011). Relative quantification of the *pmrAB* transcript levels by qRT-PCR of the polymyxin-resistant strains AC12 and AC30 in comparison with the polymyxin-susceptible strain AC29 indicated upregulation of *pmrA* by about 8-folds in AC12 and about 2-folds in AC30. The expression of *pmrB* is about 4-folds higher in AC30 but in AC12, the expression level of *pmrB* is around 0.25-folds when compared to that of AC29. These expression levels of *pmrAB* were lower than that reported by Beceiro et al (2011), but it should be noted that in this case, no isogenic polymyxin-susceptible strains for the polymyxin-resistant strains were available for comparison. Thus, the expression levels for AC12 and AC30 were compared with the non-isogenic polymyxin-susceptible strains AC29 and the reference strain ATCC19606. Nevertheless, the results do give an indication of some upregulation in the *pmrAB* expression for the polymyxin-resistant strains AC12 and AC30.

Further analysis of the LPS biosynthesis genes identified novel mutations especially in the *lpxD*, *lpxC* and *lpsB* genes. All 14 XDR strains had mutations in both *lpxD* and *lpxC* genes whereas 8 out of the 14 strains (including AC12 and AC30) showed an identical H181Y substitution in *lpsB*. It does appear that disruption in the LPS biosynthesis genes could also be associated with polymyxin-resistance in the *A. baumannii* strains studied. The effects of these mutations on the LPS of these strains were further investigated as they differed from the mutations previously reported (Moffatt et al., 2010; 2011; Hood et al., 2013). Furthermore the crystal structure of the *A. baumannii* LpxD protein has been elucidated (Smani et al., 2012) and one of

the mutations (G186S) lies within the proposed active site of the enzyme. Polyacrylamide gel separation of extracted LPS from AC12, AC30 and AC29 indicated that the mutations in *lpxD, lpxC* and *lpsB* might have led to an impairment of the LPS produced and not a total loss as had been previously reported for other polymyxin-resistant strains (Moffatt et al., 2010; 2011; Hood et al., 2013). It is likely that in the case of AC12 and AC30, polymyxin resistance could be the result of a combination of increased *pmrAB* expression leading to covalent modification of the lipid A moiety of LPS and possibly impaired LPS synthesis as well. Nevertheless, it should be noted that in a recent study (Hood et al., 2013), screening of transposon mutant libraries led to the identification of more than 20 genes that may be involved in inducible colistin resistance in *A. baumannii*. Most of these genes converged on pathways involved in osmotolerance, cell envelope biosynthesis along with protein folding (Hood et al., 2013). The role that these factors may play in the development of polymyxin resistance among the 14 XDR *A. baumannii* strains would also need to be investigated.

CHAPTER 6

6.0 CONCLUSIONS

In this study, 54 strains of *Acinetobacter baumannii* isolated from sporadic infections throughout 2011 in Hospital Sultanah Nur Zahirah, Terengganu was characterized by antimicrobial susceptibility testing (AST) and pulsed-field gel electrophoresis (PFGE). Relatively high percentages of resistance (>50%) were observed for all antibiotics tested and multidrug resistance (MDR) was detected in 39 of the 54 strains (72.2%). High resistance rates of approximately 75% for carbapenems coupled with the relatively high occurrence (25.9%) of polymyxin-resistant and extensive-drug resistant (XDR) *A. baumannii* strains in the hospital strains from Terengganu is indeed a cause for concern as the therapeutic options become severely limited. Hence, a higher level of vigilance is needed and indiscriminate usage of antibiotics curbed to prevent the spread of XDR *A. baumannii* and the development of pandrug resistant (PDR) *A. baumannii* in the hospital environment. PFGE results indicated genetic variability among the 54 *A. baumannii* strains with clustering observed among some carbapenem-resistant strains but not among the XDR polymyxin-resistant strains. This is not surprising as carbapenem resistance is usually mediated by genes such as $bla_{\text{OXA-23}}$ that can be transmitted by mobile genetic elements whereas polymyxin resistance is mediated by mutations in genes that lead to modifications or defects in the lipopolysaccharide layer of the cell membrane. These changes incur a fitness cost to the bacterium and thus, once the selection pressure is off, these polymyxin-resistant clones would likely not be disseminated.

Novel resistance island (RI) variants and plasmids were discovered from the whole genome sequences of two XDR *A. baumannii* strains, AC12 and AC30, and one MDR strain, AC29. All three strains shared a similar AbaR4-type RI of approximately 22 kb interrupting the *comM* gene designated AC12-RI1, AC30-RI1 and AC29-RI1. The carbapenem resistance gene, $bla_{\text{OXA-23}}$ is found within a composite transposon, Tn*2006* that is located within the island that also encodes genes conferring resistance to tetracyclines, sulphonomides and streptomycin. *A. baumannii* AC12 harboured a RI that was absent from the genomes of AC30 and AC29. The 10.3 kb AC12-RI2 island is a derivative of AbGRI2-1 and harbours the aminoglycoside resistance gene *aphA1b* and the β-lactamase gene bla_{TEM}. The 7 kb Tn*1548*::*armA* island encodes resistance determinants for aminoglycosides and macrolides. Tn*1548*::*armA* is usually plasmid-borne but is chromosomally located in *A. baumannii* AC12 and AC29. In *A. baumannii* AC30, some components of this island were located on a 16 kb plasmid, pAC30b. Plasmid pAC30b is found only in AC30 but not AC12 or AC29. Both AC30 and AC29 harboured a ~70 kb conjugative plasmid designated pAC30c and pAC29b with pAC29b containing another copy of $bla_{\text{OXA-23}}$. All three *A. baumannii* strains harboured a small ~8 kb cryptic plasmid which encode two putative virulence determinants (TonB-dependent receptor and septicolysin) and a XerC/XerD recombination site. Thus, genomic islands and, to a lesser extent, conjugative plasmids, appeared to play an important role in the dissemination and acquisition of antibiotic resistance determinants in the Terengganu *A. baumannii* strains. Whole genome sequencing also enabled the identification of a number of potential virulence factors in the *A. baumannii* strains. Genes for iron acquisition appeared to cluster together in a

genomic island-like arrangement. Whole genome sequencing of the three Terengganu *A. baumannii* clinical strains enabled detailed characterization of their genetic repertoire of resistance and virulence determinants, thereby giving us a first look at the genetic blueprint of local strains of this increasingly important and deadly nosocomial pathogen.

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List of Publications and Papers Presented

Proceedings and Poster Presentations

1. Soo-Sum Lean, Zarizal Suhaili, Chew Chieng Yeo, Kwai-Lin Thong. "Genomic Analyses of a Polymyxin-Resistant Strain of Clinical *Acinetobacter baumannii*". Book of Abstracts. 31st Symposium of the Malaysian Society for Microbiology: Microbiology Research in the Omics Era. 13-15th December 2012.

2. Soo-Sum Lean, Zarizal Suhaili, Chew Chieng Yeo, Kwai-Lin Thong. "Characterization of Genetic Determinants for Polymyxin Resistance in *Acinetobacter baumannii* strains Isolated from a Tertiary Hospital in Malaysia". Book of Abstracts. 9th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2013). Containing Antimicrobial Resistance: A Global Mission to be achieved. 13-15th March 2013.

3. Soo-Sum Lean, Zarizal Suhaili, Chew Chieng Yeo, Kwai-Lin Thong. "Genome Sequencing Reveals the Structure of a 32 kb ACRI12-1 Resistance Island in an Extensive-drug Resistant Clinical Strain of *Acinetobacter baumannii"*. Book of Abstracts. 20th MSMBB Annual Scientific Meeting. 26-27th June 2013.

4. Soo-Sum Lean, Zarizal Suhaili, Chew Chieng Yeo, Kwai-Lin Thong. "Characterization of multidrug- and extensive drug-resistant *Acinetobacter baumannii* isolated from a local tertiary hospital*"*. Book of Abstracts. Monash Science Symposium. 18-19th June 2014.

5. Soo-Sum Lean, Zarizal Suhaili, Chew Chieng Yeo, Kwai-Lin Thong. "Resistance islands: what is new in an extensive-drug resistant clinical strain of *Acinetobacter baumannii*?". Book of Abstracts. 21st Malaysian Society of Molecular Biology and Biotechnology (MSMBB) Annual Scientific Conference. 1st-3rd October 2014.

Publications

1. Gan Han Ming, Lean Soo-Sum, Zarizal Suhaili, Chew Chieng Yeo, and Kwai-Lin Thong. (2012). Genome sequence of *Acinetobacter baumannii* AC12, a polymyxin-resistant strain isolated from Terengganu, Malaysia. Journal of Bacteriology. **194**(21): 5979-80.

2. Lean Soo-Sum, Zarizal Suhaili, Salwani Ismail, Nor Iza A. Rahman, Norlela Othman, Fatimah Haslina Abdullah, Zakaria Jusoh, Chew Chieng Yeo, and Kwai-Lin Thong. (2014). Prevalence and genetic characterization of polymyxin-resistant *Acinetobacter baumannii* isolated from a tertiary hospital in Terengganu, Malaysia . *ISRN Microbiology*, 1–28.

3. Lean Soo-Sum, Zarizal Suhaili, Chew Chieng Yeo, and Kwai-Lin Thong. (2014). Whole genome analyses of an extensive drug-resistant clinical isolate of *Acinetobacter baumannii* AC12: insights into the mechanisms of resistance of an ST195 clone from Malaysia. *International Journal of Antimicrobial Agents,* **45**: 178-182.