MOLECULAR MECHANISMS OF BIOFILM-SPECIFIC ANTIBIOTIC RESISTANCE IN *ACINETOBACTER BAUMANNII*

ABEBE MEKURIA SHENKUTIE

PhD

The Hong Kong Polytechnic University

2021

The Hong Kong Polytechnic University

Department of Health Technology and Informatics

MOLECULAR MECHANISMS OF BIOFILM-SPECIFIC ANTIBIOTIC RESISTANCE IN *ACINETOBACTER BAUMANNII*

ABEBE MEKURIA SHENKUTIE

A thesis submitted in partial fulfilment of the requirements

for the degree of Doctor of Philosophy

April 2021

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgment has been made in the text.

_____ (Signed)

ABEBE MEKURIA SHENKUTIE (Name of student)

ABSTRACT

Acinetobacter baumannii is a priority I antibiotic-resistant microbial pathogen. Although it has a remarkable capacity to acquire external genetic determinants of resistance, this is not the sole reason for failure to treat *A. baumannii* infections with antibiotics. Biofilm formation is an important strategy by which pathogens survive exposure to antibiotics. The emergence and spread of *A. baumannii* depends on both planktonic and biofilm-specific antibiotic resistance mechanisms. Antibiotic resistance mechanisms have been well studied in planktonic cultures of *A. baumannii*. However, limited in-depth studies have been conducted on the antibiotic resistance mechanisms associated with *A. baumannii* biofilms. To understand the connection between biofilm formation, antibiotic resistance and the mechanisms involved in the regulation of biofilm-specific antibiotic resistance, this thesis aims to (i) investigate the relationship between antibiotic susceptibility and the biofilm formation capability of clinical *A. baumannii* strains; (ii) compare the transcriptomes of *A. baumannii* biofilms to those of biofilm cells treated with sub-optimal concentrations of antibiotics; and (iii) investigate the regulatory role of small RNA00203 (sRNA00203) in biofilm formation and the development of biofilm-specific antibiotic resistance in *A. baumannii*.

To understand the connection between biofilm formation capability and antibiotic susceptibility, 104 clinical *A. baumannii* strains were studied. We found that 59.6% of the strains were biofilm formers. We also observed that non-multidrug-resistant *A. baumannii* strains were strong biofilm formers. The antibiotic susceptibility of biofilm cells was evaluated in nine biofilm formers. The antibiotic concentrations required to eradicate the biofilm were 44–364 times higher than those required to kill planktonic

bacteria. Of the nine strains tested, *A. baumannii* ST1894 developed the highest level of resistance to imipenem, ciprofloxacin and colistin. The reversibility test for antibiotic susceptibility showed that biofilm formation induced reversible antibiotic tolerance in the non-multidrug-resistant strains but resulted in a higher level of irreversible resistance in the extensively drug-resistant strain.

To understand the mechanisms involved in the regulation of biofilm-specific antibiotic resistance, the hyper biofilm-forming strain *A. baumannii* ST1894 was subjected to global transcriptome profiling. Planktonic and biofilm cells of this strain were treated with colistin and imipenem at sub-inhibitory concentrations. The transcriptome profiles were obtained by RNA sequencing using an Illumina NovaSeq platform. We found that 1592 (51.8%) of the 3,075 transcribed genes were differentially expressed between the mature biofilm and planktonic cells; 106 and 368 biofilm-specific genes were differentially expressed in biofilm cells treated with sub-inhibitory concentrations of imipenem and colistin, respectively. The differentially expressed genes induced by imipenem and colistin in biofilm cells included genes that encoded outer membrane transport proteins, resistance–nodulation–cell division multidrug efflux systems, fimbrial proteins, homoserine lactone synthases and matrix synthesis proteins. The expression levels of metabolism-related genes, such as those coding for acinetobactin biosynthesis, DNA replication and translation and transport of D- and L-methionine, were significantly reduced in the biofilm cells. The effects of suboptimal concentrations of imipenem and colistin on biofilm-specific genes and their regulatory pathways may account for the reduced susceptibility of biofilm cells to antibiotics.

When we investigated the regulatory role of sRNA00203, we identified 138 sRNAs as regulatory targets based on the transcriptome data of *A. baumannii* ST1894. Among the identified sRNAs, the gene encoding sRNA00203 was more strongly upregulated in the biofilm cells than in the planktonic cells. We found that deletion of this gene using suicide plasmid-mediated allelic exchange substantially impaired biofilm formation in *A. baumannii* ST1894. Deletion of sRNA00203 also increased the susceptibility of the biofilm cells to imipenem and ciprofloxacin. We showed that deletion of sRNA00203 significantly decreased the expression levels of genes that code for efflux pumps (novel00738), matrix synthesis (*pgaB*), preprotein translocase subunit (*secA*) and the CRP transcriptional regulator. These findings demonstrate a clear role for sRNA00203 in the regulation of biofilm formation and development of antibiotic susceptibility in *A. baumannii* ST1894. The inhibition of sRNA00203 presents a new strategy for treating biofilm-specific infections by impairing biofilm production and increasing the antibiotic susceptibility of biofilm cells.

In summary, this thesis demonstrates that non-multidrug-resistant *A. baumannii* strains are strong biofilm formers. The biofilm state of growth promoted reversible antibiotic tolerance in non-multidrug-resistant strains and higher levels of irreversible resistance in extensively drug-resistant strains. The transcriptome analysis demonstrated that the differential expression of genes associated with antibiotic resistance and metabolic quiescence could be responsible for the reduced susceptibility of biofilm cells to antibiotics. Furthermore, this thesis is the first of its kind to demonstrate the effect of sRNA00203 on the expression of genes associated with biofilm formation and the development of antibiotic resistance in clinical *A. baumannii* strains.

PUBLICATION

Scientific Journal Paper

Shenkutie, A.M.; Yao, M.Z.; Siu, G.K.-h.; Wong, B.K.C and Leung, P.H.-m. Biofilm-induced antibiotic resistance in clinical *Acinetobacter baumannii* isolates. *Antibiotics***,** 2020, *9*, 817. https://doi.org/10.3390/antibiotics9110817

Shenkutie, A.M. and Leung, P.H.-m. Comparative transcriptomic profiles of untreated and antibiotic-treated biofilm cells clinical *A. baumannii* isolates. (Submitted to *Biofilm and Microbiomes*).

Shenkutie, A.M. and Leung, P.H.-m. Impact of Small Non-Coding RNA00203 on biofilm-specific antibiotic resistance in *A. baumannii*. (Submitted to *Virulence*).

ACKNOWLEDGMENTS

I want to take this opportunity to thank all of the people who have guided me in my studies and given me their unlimited support and eternal appreciation. Without their assistance, guidance, and inspiration, I would never have been able to complete my PhD.

First of all, I would like to extend my heartfelt gratitude to my chief supervisor, Dr Polly Leung, for her immense support, encouragement and valuable guidance throughout my PhD studies. I was fortunate to have a supervisor who cared very much for my work and replied very quickly to all of my questions and inquiries. I am so thankful for the freedom, commitment and courage she has provided me throughout my study period.

I will be forever grateful to my lab mates, Dr Mou Qianqian and Mr Yao Mian Zhi, for their kind support and teamwork.

I would like to thank Dr Gilman Siu for his encouraging discussion and feedback as he evaluated my project progress over the last three and half years.

Special thanks are due to the Research Grants Council of Hong Kong for granting me the Hong Kong PhD Fellowship to study in Hong Kong.

I sincerely thank the staff and students of the Department of Health Technology and Informatics for their support, cooperation and goodwill.

I owe a debt of gratitude to Saint Paulo's Hospital and Millennium Medical College for their unwavering support throughout my PhD study.

I owe a great deal to my dearest mother (Likie Fekadu), wife (Lemlem Degife), and other family members whose affection, support and understanding have always been with me during my studies and have carried me through all of the hardships of my life.

Above all, I am grateful to **Almighty GOD** for his unending blessings, strength, peace of mind, and good health throughout my life!

TABLE OF CONTENTS

CONTENTS **PAGE**

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CBD	Calgary biofilm device
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
CRP	Cyclic-AMP receptor protein
DEGs	Differentially expressed genes.
DNA	Deoxyribonucleic acid
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
FPKM	Fragments per kilobase of transcript sequence per million base pairs
GO	Gene Ontology
ICU	Intensive care unit
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria-Bertani
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
MLST	Multilocus sequence typing
MDR	Multiple drug-resistant
MIC	Minimum biofilm inhibitory concentration
mRNA	Messenger RNA
ncRNA	Non-coding RNA
NGS	Next-generation sequencing
OD	Optical density
OMP	Outer membrane protein
PDR	Pan-drug-resistant
PFGE	Pulsed-field gel electrophoresis
RBS	Ribosome binding site
RND	Resistance–nodulation–cell division

ROS	Reactive oxygen species
rpm	Revolutions per minute
RRF	Ribosomal release factor
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SPSS	Statistical Package for the Social Sciences
sRNA	Small RNA
ST	Sequence type
TLR	Toll-like receptor
UTR	Untranslated region
XDR	Extensively drug-resistant

1 CHAPTER ONE: INTRODUCTION AND THESIS ORGANISATION

1.1 Introduction

Acinetobacter baumannii is a Gram-negative bacterium that has evolved from an organism of questionable pathogenicity to a high-priority infectious agent (Fournier & Richet, 2006; WHO, 2017). The increased incidence of infections caused by *A. baumannii* has become a significant global concern (Dijkshoorn et al., 2007; Gonzalez-Villoria & Valverde-Garduno, 2016; Ho et al., 2013; Piperaki et al., 2019). The typical clinical manifestations caused by this pathogen include ventilator-associated pneumonia, bloodstream infections, urinary tract infections, surgical site infections and more recently, severe cases of necrotising fasciitis (Cheng et al., 2018; Peleg et al., 2008).

From 2011 to 2016, the detection rates of carbapenem- and β-lactam-resistant *A. baumannii* strains rose by > 30% worldwide (Xie et al., 2018). The carbapenem resistant strains cause 34–61.6% mortality, making it a priority pathogen among those that cause nosocomial infections (Abbo et al., 2007). The rise in carbapenem-resistant strains has led to an increase in the use of colistin as salvage therapy. In turn, the repeated use of colistin has caused the emergence of pan-drug-resistant (PDR) strains (Methé et al., 2012), the increased occurrence of which implies the limited utility of last-resort antibiotic classes, such as polymyxin. The capacity of *A. baumannii* to withstand antibiotics arises from cellular and biofilm-specific antibiotic resistance mechanisms (Flemming et al., 2016). Cellular antibiotic resistance mechanisms enable the pathogen to survive the presence of antibiotics through enzymatic inactivation, target modification and drug efflux from the bacterial cell (Gordon & Wareham, 2010; Nikaido, 1998; Paulsen, 2003).

Antibiotic resistance mechanisms have been studied thoroughly under planktonic conditions (Penesyan et al., 2019). Such studies have been based on transcriptomic and mutational analyses of planktonic cells to identify differentially expressed genes (DEGs) and genetic mutations associated with the development of antibiotic resistance (Esterly et al., 2011; Qin et al., 2018). Another mechanism of antibiotic resistance is through the formation of biofilms, which allow the pathogen to survive in antibiotic-exposed environments by developing either antibiotic resistance or tolerance (Hoyle & Costerton, 1991; Penesyan et al., 2015). Lewis (2008) described resistance as the means by which an antimicrobial agent is prevented from interacting with its intended target. Following this concept, processes involving the sequestration of antibiotics by extracellular DNA (eDNA), antibiotic efflux pumps and matrix β-lactamases have been considered to act as resistance mechanisms (Hall & Mah, 2017).

Antibiotic tolerance has also been defined as a process that prevents the antimicrobial agent from exerting its downstream toxic effects, even if the agent is bound to its target (Lewis, 2008). In biofilm cells, the mechanism of antibiotic tolerance involves slow biofilm growth, activation of the general stress response and the presence of persister cells (Hall & Mah, 2017; Høiby et al., 2010; Mah, 2012). These antibiotic tolerance mechanisms may arise from phenotypic alterations associated with growth arrest, eventually preventing the target of the antibiotic from exerting its downstream effects (Hall & Mah, 2017). During biofilm formation, pathogenic bacteria such as *A. baumannii* can harness both tolerance and resistance mechanisms to survive in antibiotic-exposed environments (Hall & Mah, 2017; Van Acker et al., 2014). Resistance and tolerance result in the reduced susceptibility of biofilms to antibiotics, referred to as recalcitrance (Hall & Mah, 2017). However, the genetic determinants that confer biofilms with reduced susceptibility to antibiotics have not yet

been identified in *A. baumannii* strains. Therefore, this study aimed to investigate the molecular mechanisms underlying the reduced susceptibility of biofilm cells to antibiotics.

1.2 Thesis organisation

Chapter 2 consists of a literature review that covers the characteristics, clinical significance and treatment of *A. baumannii* infections. The general process of biofilm formation, biofilm-specific antibiotic resistance and the regulatory role of small RNAs (sRNAs) are also reviewed. Chapter 3 explains the phenotypic characterisation of 104 clinical *A. baumannii* strains, with a focus on biofilm formation and the development of antibiotic resistance. In this part of the study, we found that non-multidrug-resistant (MDR) strains were strong biofilm formers and were resistant to several antibiotic classes when grown in the biofilm state. *A. baumannii* ST1894 is a hyper biofilm-forming strain that has demonstrated the highest-recorded degree of multidrug-tolerance to antibiotics during biofilm growth. This strain was therefore selected to elucidate the molecular mechanisms responsible for biofilm formation and the development of reduced antibiotic resistance. Chapter 4 describes the global transcriptional responses of biofilm cells left untreated or treated with suboptimal concentrations of imipenem and colistin. We observed 1592 differentially expressed genes (DEGs) between mature biofilm and planktonic *A. baumannii* ST1894 cells. We identified 106 and 368 biofilm-specific DEGs in biofilm cells treated with suboptimal concentrations of imipenem and colistin, respectively. We also determined that among all differentially expressed sRNAs, the expression of sRNA00203 was the most highly upregulated in the biofilm cells. Chapter 5 describes the role of sRNA00203 in biofilm formation and the emergence of biofilm-specific antibiotic resistance. Chapter 6 concludes the main findings and discusses future work.

2 CHAPTER TWO: LITERATURE REVIEW

2.1 Characteristics of *A. baumannii*

Acinetobacter is a Gram-negative coccobacilli characterised by strict aerobic, catalase-positive, oxidase-negative and non-motile cells (Peleg et al., 2008). These organisms are ubiquitous and can be isolated from environmental sources in clinical settings (Jamal et al., 2018). Based on 16S ribosomal RNA (rRNA)-based sequencing, *Acinetobacter* has been shown to include 73 species, of which 59 have been accurately identified (Parte, 2018). The most frequently isolated species of *Acinetobacter* belongs to the *A. calcoaceticus-A. baumannii* (ACB) complex, which comprises *A. calcoaceticus* (genomic species 1), *A. baumannii* (genomic species 2), *A. pittii* (genomic species 3) and *A. nosocomialis* (genomic species 13TU). All ACB members except *A. calcoaceticus* are clinically relevant and cause frequent outbreaks of nosocomial infection (Kamolvit et al., 2014).

2.2 Identification of *A. baumannii*

The high phenotypic similarity between species presents the main challenge to classifying the ACB complex into species using phenotypic-based approaches such as API 20NE and Vitek 2 systems. Genotypic methods such as amplified rRNA gene restriction analysis and DNA-DNA hybridisation, however, can classify ACB complex members into species. These methods are time-consuming, so it is difficult to apply them to routine diagnostic procedures (Bosshard et al., 2006; La Scola et al., 2006). Sequencing methods that involve the *rpoB* and 16S–23S rRNA gene spacer regions can be used reliably to identify *Acinetobacter* species (Hsein et al., 2005; La Scola et al.,

2006). In addition, a *gyrB*-based PCR method has been used to identify members of the ACB complex (Higgins et al., 2010; Hsein et al., 2005; Kamolvit et al., 2014).

2.3 Virulence and pathogenicity

Over the last few years, *A. baumannii* has emerged as one of the most important nosocomial pathogens associated with infections of various anatomical sites. This pathogen owes its success due to the possession of several virulence factors, which are molecular characteristics that enable pathogens to interact successfully with and colonise the host cell (Harding et al., 2018). Studies based on genomic, phenotypic and infection models have identified several potential virulence factors in *A. baumannii*. These virulence determinants are associated with the ability of the pathogen to adhere to epithelial cells, produce enzymes and possess anti-phagocytic surface components (Tomaras et al., 2003).

The virulence determinants that have been implicated in these functions in *A. baumannii* include glycoconjugates, outer membrane proteins (OMPs), phospholipases and protein secretion systems (Antunes et al., 2014; Harding et al., 2018). More recent studies have demonstrated the ability of *A. baumannii* to spread widely owing to its capacity to form biofilms and acquire antibiotic resistance determinants, which will be reviewed in subsequent sessions.

2.3.1 Glycoconjugates

Bacterial carbohydrates, also referred to as glycans or glycoconjugates, form an interface between the pathogen and the environment. Glycoconjugates play a significant structural function in *A. baumannii* and mediate the first line of protection against various stresses and immune evasion and regulation (Harding et al., 2018). The

most common bacterial glycoconjugates are lipopolysaccharides (LPSs), capsular polysaccharides, glycosylated proteins and peptidoglycans.

2.3.1.1 Lipopolysaccharide (LPS)

LPS is a significant component of the outer membrane of Gram-negative bacteria and is also the ligand for Toll-like receptor 4 (TLR4) (Harding et al., 2018). As *A. baumannii* lacks an O antigen, its LPS is appropriately referred to as lipooligosaccharide (LOS), which can act as an endotoxin. The endotoxin portion is a potent inflammatory signalling stimulator of human monocyte cells that is dependent on Toll-like receptor 2 (TLR2) and TLR4 (Erridge et al., 2007). The pathology of *Acinetobacter* infections has been linked to the LOS-mediated innate immune reaction (Kim et al., 2013; Knapp et al., 2006). LOS is also toxic to neutrophils and causes impaired migration and phagocytosis (Kamoshida et al., 2020). A previous study has shown that LOS-deficient *A. baumannii* is weakly activated by neutrophils, reducing the production of reactive oxygen species (ROS) and secretion of inflammatory cytokines. However, due to the increased susceptibility to antibacterial lysozyme and lactoferrin, neutrophils can more easily kill LOS-deficient *A. baumannii* than wild-type strains (Kamoshida et al., 2020).

The loss of LOS necessitates the use of compensatory mechanisms, such as the overexpression of lipoproteins to stabilise the outer membrane of *A. baumannii* (Harding et al., 2018). Furthermore, an increase in the production of the capsular polysaccharide poly-beta-1,6-N-acetylglucosamine has been observed to function as an adaptative compensatory mechanism in LOS-deficient strains, although it has not been commonly observed (Boll et al., 2016).

2.3.1.2 Capsular polysaccharides

Capsular polysaccharides are another kind of determinant of the virulence of *A. baumannii*. A thick capsular polysaccharide (Russo et al., 2010) and an O-linked protein glycosylation system have been identified in most *A. baumannii* strains (Iwashkiw et al., 2012). Capsular polysaccharides protect bacteria from external threats such as host defence and desiccation (Harding et al., 2018; Merino & Tomás, 2015), and are the primary agents responsible for the extraordinary resistance of *A. baumannii* strains to complement-mediated killing (Russo et al., 2010). For instance, the K1 capsule isolated from the clinical strain *A. baumannii* AB307-0294 plays the essential role of inhibiting the bactericidal activity of the complement cascade (Russo et al., 2010; Wong et al., 2017). *A. baumannii* capsular polysaccharides are essential virulence factors, as the capsule-free strains are avirulent and easily killed by the complement system (Lees-Miller et al., 2013).

The glycans attached to proteins located in the periplasm or the outer membrane are not involved in complement resistance. Glycans are attenuated in many infection models, indicating that protein glycosylation plays a role in the effective adaptation of pathogens to the host environment. However, cells deficient in glycosylation also have impaired biofilm formation capabilities (Iwashkiw et al., 2012).

Capsular polysaccharides are also involved in desiccation resistance, which is the capacity of *A. baumannii* to maintain viability under dry conditions (Ophir & Gutnick, 1994). The ability of the *A. baumannii* capsule to retain water during biofilm formation makes the pathogen resistant to desiccation and enables it to survive longer in hospital environments (Espinal et al., 2012; Harding et al., 2018).

2.3.2 Outer membrane proteins (OMPs)

The OMP (OmpA) is one of the most abundant and essential virulence factors secreted by *A. baumannii*. OmpA is involved in epithelial cell invasion and apoptosis and mediates the adherence, invasion and penetration of *A. baumannii* into epithelial cells during infection (Guardabassi et al., 2000; Gaddy et al., 2009). OmpA has been shown to enable *A. baumannii* to bind to and invade eukaryotic cells (Cos-7, HEp-2 and macrophages) (Cerqueira & Peleg, 2011). This protein also destroys human respiratory epithelial cells by initiating apoptosis through the release of the pro-apoptotic cytochrome c and apoptosis-inducing factor during the early stages of *A. baumannii* infection (Choi et al., 2005). In epithelial cells (HeLa), *A. baumannii* induces apoptosis via a caspase 3-dependent mechanism (Choi et al., 2005). OmpA from *A. baumannii* ATCC19606 was shown to play a significant role in the formation of stable biofilms on plastic surfaces. Strains with a mutant *ompA* gene do not form biofilms, whereas reconstituting the *ompA* gene fully restores the capacity of *A. baumannii* strains to form biofilms (Gaddy et al., 2009).

2.3.3 Type IV pili

In *A. baumannii*, type IV pili are responsible for the twitching motility that results from the repeated rounds of extension and retraction meant to propel bacterial cells forward (Harding et al., 2013; Wilharm et al., 2013). There is no direct link between type IV pili, twitching motility and *A. baumannii* virulence, but the genes predicted to encode the proteins necessary for type IV pili biogenesis have been found to be upregulated during bacterial growth in human serum. The upregulation of these genes suggests that type IV pili may be a significant virulence factor during bacteraemia (Jacobs et al., 2012). Most *A. baumannii* strains harbour homologous genes encoding the proteins necessary for type IV pili biogenesis. However, variations in the sequence of the main

pilin subunit gene *pilA* have been observed across *A. baumannii* strains. Structural analysis of the *A. baumannii pilA* sequence has revealed that these divergences cause a degree of structural variation that is much higher than expected (Piepenbrink et al., 2016). Thus, any *A. baumannii* type IV pilin-specific vaccines would undoubtedly fail due to sequence and structural variations.

2.3.4 Type VI secretion system (T6SS)

The T6SS is a potent weapon used by many Gram-negative bacteria to kill their competitors. Similarly, *A. baumannii* strains use a T6SS to kill other bacteria (Carruthers et al., 2013). This structure is especially relevant in the context of polymicrobial infections, as the *Acinetobacter* T6SS has not been shown to mediate eukaryotic cytotoxicity. Nevertheless, it offers an *in vivo* fitness gain due to its potent antibacterial activity. Some strains express their T6SSs and release effectors constitutively, whereas others have developed exquisite forms of control. Several MDR *A. baumannii* strains regulate the expression of their T6SSs by carrying a conjugative plasmid that contains the T6SS regulatory and antibiotic resistance genes (Weber et al., 2015). Spontaneous plasmid loss in *A. baumannii* triggers T6SS expression, enabling these bacteria to outcompete other microbes; the T6SS-mediated killing of competing bacteria aids overall survival but renders *A. baumannii* susceptible to antibiotics (Weber et al., 2015). This observation has revealed an apparent survival strategy used by *A. baumannii* when certain strains lose their resistance to antibiotics.

2.3.5 Phospholipases

Phospholipases comprise another group of virulence factors of *A. baumannii*. These enzymes are involved in host cell phospholipid cleavage, thereby facilitating bacterial invasion (McConnell et al., 2013). In one study, the inactivation of phospholipases in

A. baumannii diminished the viability of bacteria in serum, impaired epithelial cell invasion and decreased pathogenesis (Jacobs et al., 2010).

2.4 Clinical relevance of *A. baumannii*

Given the increase in the number of infection cases over the last three decades, MDR *A. baumannii* strains have become clinically significant pathogens (Almasaudi, 2018; McConnell et al., 2013). *A. baumannii* is emerging as a frequent cause of difficult-to-treat infections, with some isolates being resistant to all clinically relevant antibiotics. This pathogen is widely known to cause both hospital- and community-acquired infections (Anstey et al., 1992; Leung et al., 2006; Scott et al., 2007; Sengstock et al., 2010).

2.4.1 Hospital-acquired infections

A. baumannii has emerged as an important nosocomial pathogen that causes skin and soft tissue infections, wound infections, urinary tract infections and fatal secondary meningitis in intensive care unit (ICU) patients (Weiner et al., 2016). Of the several types of infections, ventilator-associated pneumonia and bloodstream infections are the most frequent and have the highest mortality rates (Dijkshoorn et al., 2007). Ventilator-associated pneumonia is more common in patients who require mechanical ventilation in the ICU. Mechanical ventilation introduces microorganisms from the surrounding environment to the respiratory tracts of patients, thus causing pneumonia (Dijkshoorn et al., 2007). A previous report indicated that ventilator-associated pneumonia has a crude mortality rate of 40–70% (Garnacho et al., 2003).

Bloodstream infection is the second most frequently reported type of infection caused by *A. baumannii* in the ICU (Wisplinghoff et al., 2004). The crude mortality rate of nosocomial *A. baumannii* bacteraemia has been reported to be 21.2%, based on a 5-

year study in Japan (Fujikura et al., 2016). However, higher rates of 29–63.5% have also been reported by studies conducted in China (Ballouz et al., 2017; Liu et al., 2015). Bloodstream infections can arise from contaminated intravascular devices, from the lower respiratory tract or by dissemination from other infected sites (Jung et al., 2010).

A. baumannii can also cause infection outbreaks when hospital emergency units are under extreme pressure (Antunes et al., 2014). Between 2000 and 2015, 150 *A. baumannii* outbreaks were recorded worldwide, of which 113 were caused by MDR strains. These outbreaks resulted in severe infections and several deaths (Cerceo et al., 2016; Wieland et al., 2018). They were primarily recorded as occurring in ICUs after antibiotic use and mechanical ventilation, with a mortality rate of up to 47% (Wieland et al., 2018).

Colonised index patients (50%) and inanimate dry surfaces (25.6%), such as curtains, mattresses and bed rails, are the most common sources of *Acinetobacter* outbreaks. If index patients are proven to be carriers of the outbreak strain upon admission and the strain has not been seen previously in other patients or environmental locations, the index patients are regarded to be the primary sources of the outbreak. Index patients transferred from other hospitals (33.3%) or other countries (20%) have accounted for a significant fraction of *A. baumannii* outbreaks (Wieland et al., 2018).

Compared with *Pseudomonas aeruginosa* outbreaks, *Acinetobacter* outbreaks are more frequently reported from ICUs, have a propensity to last longer and are associated with doubled mortality rates. These features may be due to the high tenacity of *A. baumannii* and its ability to survive for up to 5 months on dry surfaces (Kramer et al., 2006); these traits enable the pathogen to spread quickly in ICUs, where there is a high risk of contracting the infection from contaminated surfaces (Huslage et al., 2010; Whiteley et

al., 2015). The pathogens spread from person to person either through direct contact via the hands of patients, staff and visitors or through indirect contact via contaminated surfaces (Wieland et al., 2018). The pathogens get access to the body through open wounds, mechanical ventilators and intravascular catheters, thus causing infections. Infections are most common in patients who have undergone extended hospitalisation for conditions such as diabetes mellitus, chronic obstructive pulmonary disease, renal disease and age-related complications (Peleg et al., 2008; Wisplinghoff et al., 1999).

2.4.2 Community-acquired infections

A. baumannii has caused severe community-acquired infections in the Asian-Pacific region and some other countries with hot and humid climates (Anstey et al., 1992; Chen et al., 2001; Leung et al., 2006). Community-acquired *A. baumannii* (CA-Ab) infections are characterised by acute sepsis and severe pneumonia and can occur either with or without an accompanying bloodstream infection (Leung et al., 2006). The sudden onset of respiratory symptoms is often followed by septic shock and multi-organ failure. Most patients require admission to the ICU due to the severity of their illness, resulting in a mortality rate of 11–64% and death within 48–72 h (Anstey et al., 1992; Chen et al., 2001; Davis et al., 2014; Ong et al., 2009). The risk factors for CA-Ab infection include alcoholism, smoking, diabetes, renal failure and chronic lung disease (Anstey et al., 1992; M. Z. Chen et al., 2001; Falagas et al., 2007). Alcohol intoxication can exacerbate *A. baumannii* pathogenesis by affecting the expression of bacterial genes and impairing phagocytosis by immune cells (Asplund et al., 2013; Gandhi et al., 2014).

Although MDR strains rarely cause CA-Ab pneumonia, evidence indicates the development of resistance against antibiotics that are widely used in the empirical

treatment of community-acquired pneumonia. Because of the virulent nature of CA-Ab infections, effective initial antibiotic therapy is critical to ensure better clinical outcomes (Davis et al., 2014). In addition, CA-Ab can cause meningitis, soft tissue infections, chronic pneumonia, native valve endocarditis and urinary tract infections (Carter et al., 1999; Chang et al., 2000; Ryu et al., 2012).

2.5 Treatment of *A. baumannii* infections

The antibiotic regimen for treating an infection caused by an antibiotic-susceptible *A. baumannii* isolate consists of broad-spectrum cephalosporins, a combination of β-lactam/β-lactamase inhibitors or carbapenem (Table 2-1). Carbapenems are highly bactericidal and have been used successfully to treat infections caused by antibiotic-susceptible *Acinetobacter* strains (Fishbain & Peleg, 2010). Of the β-lactamase inhibitors, sulbactam has shown excellent bactericidal activity against *Acinetobacter* isolates (Fishbain & Peleg, 2010). Observational studies have shown that the efficacy of ampicillin-sulbactam is comparable to that of imipenem (Cisneros et al., 1996; Smolyakov et al., 2003). A combination of ampicillin and sulbactam appeared to be effective in treating patients with ventilator-associated pneumonia caused by imipenem-resistant *A. baumannii* (Wood et al., 2002).

Monotherapies based on β-lactams/β-lactamase inhibitors, cephalosporins and carbapenems have been individually associated with antibiotic resistance (Núñez et al., 1998; Tatman-Otkun et al., 2004). As a result, antimicrobials from these three classes have been used in combination with an anti-pseudomonal fluoroquinolone or aminoglycoside (Bergogne-Bérézin & Towner, 1996; Lesho et al., 2005). Such combinations of antimicrobials are widely used to treat *Acinetobacter* infections to increase the probability of adequate empiric antibiotic coverage before obtaining the

drug susceptibility testing results. Combination treatment also reduces the risk of resistance development and improves the treatment outcomes of MDR infections (Kanafani, 2014). The use of a combination therapy consisting of carbapenem and sulbactam against MDR *A. baumannii* bacteraemia has demonstrated better clinical outcomes compared with carbapenem monotherapy (Kuo et al., 2007; Lee et al., 2007).

Currently, polymyxins and tetracyclines (minocycline and tigecycline) are the only viable therapeutic choices for MDR and extensive drug-resistant (XDR) *A. baumannii* isolates. The most commonly used antimicrobial agents used to treat infections caused by *Acinetobacter* isolates resistant to first-line drugs are polymyxins (polymyxin B) and colistin (polymyxin E). Colistin has been used to treat XDR isolates that cause serious infections, such as meningitis, pneumonia and bacteraemia (Falagas & Kasiakou, 2005; Michalopoulos et al., 2005). However, renal dysfunction has been observed as an adverse effect in 19–27% of patients treated with colistin (Jiménez-Mejías et al., 2002; Michalopoulos et al., 2005). More recently, however, the use of colistin for the treatment of serious *A. baumannii* infection was shown to be associated with low renal toxicity, suggesting that the frequency of colistin-related systemic toxicity might have been overestimated (Garnacho-Montero et al., 2003; Pintado et al., 2008). When used to treat pneumonia caused by MDR *A. baumannii*, parenteral colistin yielded a response rate of 25–71% (Linden & Paterson, 2006; Pintado et al., 2008).

Tigecycline has been successfully used for the treatment of infections caused by imipenem-resistant *A. baumannii* isolates. Although carbapenem-resistant isolates of *A. baumannii* do not exhibit altered tigecycline susceptibility, tigecycline-resistant isolates of *A. baumannii* have begun to emerge from clinical settings in recent years (Pachón-Ibáněz et al., 2004; Navon-Venezia et al., 2007; Reid et al., 2007). The

emergence of tigecycline-resistant isolates suggests that *A. baumannii* has evolved into a PDR strain with no effective treatment options. Several other studies have also shown an increase in the number of drug-resistant strains with few or no therapeutic options (Fishbain & Peleg, 2010).

Table 2-1. Antimicrobial agents used to treat *A. baumannii* infections.

Class	**Drugs**	**Dosage**	**Route**	**Toxicity**
Cephalosporin (first-line drug)	Ceftazidime	2 g every 8 h	IV	Stomach discomfort
	Cefepime			
β-lactamase inhibitor (first-line drug)	Sulbactam	6 g per day	IV	Dermatological, nephritis
Carbapenem (First-line drug)	Imipenem	500 mg every 6 h	IV	Phlebitis, anaphylaxis
	Meropenem	500 mg every 8 h	IV	Headache, seizure
	Doripenem	500 mg every 8 h	IV	Anaemia, anaphylaxis
Aminoglycosides	Amikacin	15 mg/kg per day	IV	Nephrotoxicity, ototoxicity
	Tobramycin	4–7 mg/kg per day	IV	Nephrotoxicity, ototoxicity
Polymyxin	Colistin	5 mg/kg per day	IV	Nephrotoxicity, neurotoxicity
Glycylcycline	Tigecycline	100 mg once or 50 mg every 12 h	IV	Shock, pancreatitis

Source: (Fishbain & Peleg, 2010). IV: intravenous.

2.6 Antibiotic resistance mechanisms in *A. baumannii*

A. baumannii was once considered an opportunistic pathogen due to its low abundance of virulence factors. However, it has quickly elicited public health concerns due to its ability to develop antibiotic resistance through different mechanisms (Figure 2-1) and prolonged environmental survival capabilities (Fournier & Richet, 2006; WHO, 2017). *A. baumannii* is resistant to various antibiotic groups, as it can produce β-lactamases that hydrolyse and confer resistance to various drugs such as penicillins, synthetic cephalosporins and carbapenems (Singh et al., 2013). Structural and functional alterations and the overexpression of genes encoding efflux pumps also facilitate reduced antibiotic uptake by bacterial cells (Abbott et al., 2013). *A. baumannii* is naturally resistant to many antibiotics, such as aminopenicillins, chloramphenicol, ertapenem, aztreonam, fosfomycin and trimethoprim, which are commonly used to treat infections caused by Gram-negative bacteria. In addition to its natural antibiotic resistance, *A. baumannii* has acquired genes associated with antimicrobial resistance, thereby contributing to the emergence and dissemination of MDR strains across the globe (Howard et al., 2012; Lee et al., 2017). The fundamental mechanisms of antibiotic resistance observed in planktonic *A. baumannii* cells are summarised in the following section.

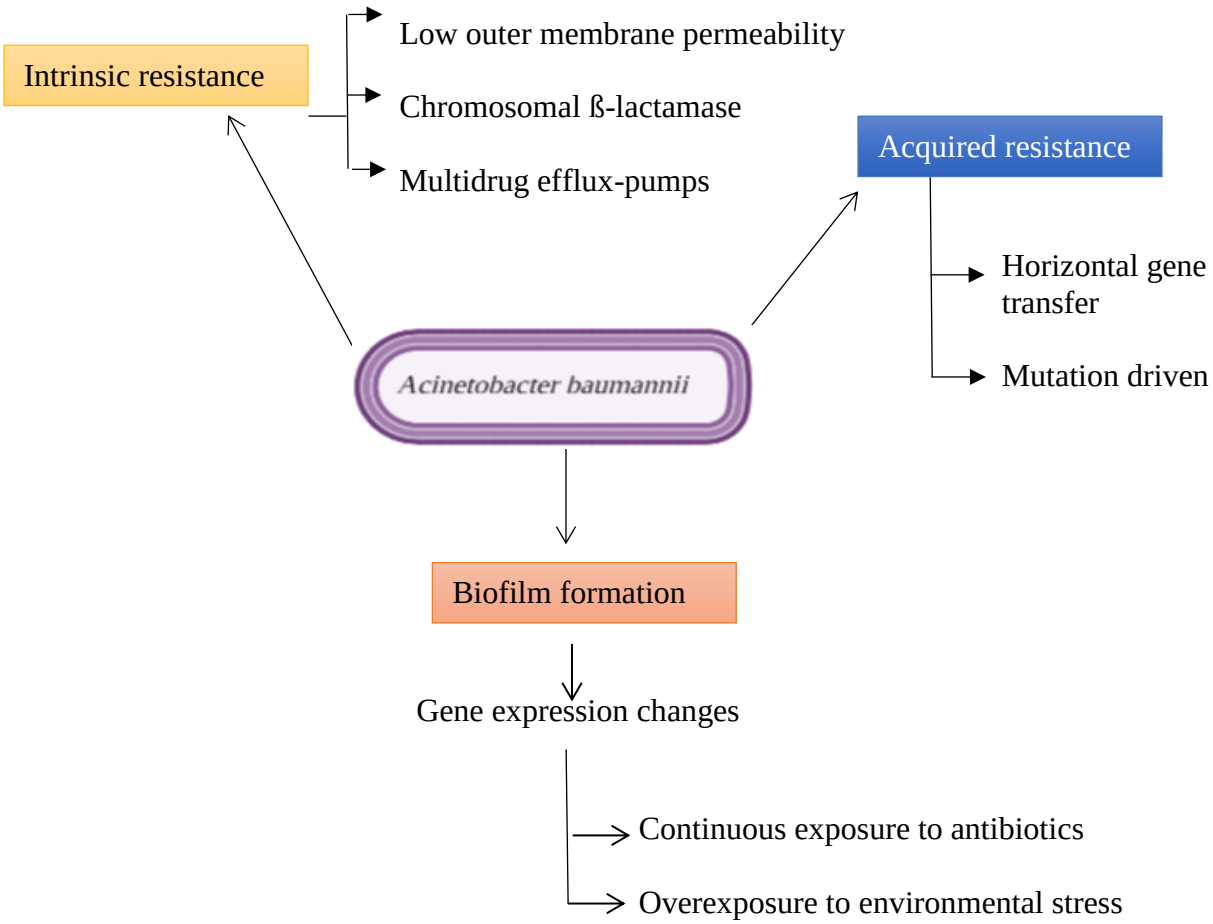


Figure 2-1. Antibiotic resistance mechanisms in *A. baumannii*.

2.6.1 Resistance to β-lactams

Several studies have explored the mechanisms of resistance to the β-lactam class of antibiotics in *A. baumannii* (Esterly et al., 2011; Evans & Amyes, 2014). Resistance to β-lactam antibiotics is mediated by both enzymatic and non-enzymatic pathways (Figure 2-2) (Asif et al., 2018; Lee et al., 2017). The enzymatic resistance mechanisms involve the production of various β-lactamases, such as extended-spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs) and oxacillinases (OXAs) (Lee et al., 2017; Noori et al., 2014). These drug-inactivating enzymes confer resistance to multiple drugs, including penicillins, synthetic cephalosporins and carbapenems.

The *bla*$_{TEM}$-type, *SHV*-type and *CTX-M*-type genes encode the production of ESBLs that mediate resistance to penicillins and third-generation cephalosporins (Fallah et al., 2014). *A. baumannii* develops resistance to most cephalosporins by producing an AmpC β-lactamase known as cephalosporinase. When an insertion sequence (ISAba1) is inserted into the AmpC gene promoter, it induces overexpression of AmpC and increased production of AmpC β-lactamase, thereby causing cephalosporin treatment failure (Bonomo & Szabo, 2006).

Carbapenem resistance is one of the most well-studied antibiotic resistance mechanisms and is primarily associated with the production of enzymes such as MBLs (Ambler class B) and OXAs (Ambler class D), which hydrolyse β-lactam ring of antibiotics (Brown et al., 1988; Thomson & Bonomo, 2005). MBLs consist of a class of potent enzymes known as carbapenemases, which mediate carbapenem resistance in *A. baumannii*. Other enzyme classes that fall under the category of MBLs are IMP-like, SIM-1- and NDM-like and VIM-like carbapenemases. MBL-encoding genes are found on integrons and can be transferred from one bacterial cell to another (Falagas & Karageorgopoulos, 2008).

The carbapenem-hydrolysing class D β-lactamase is the most prevalent carbapenemase responsible for carbapenem resistance in *Acinetobacter* species. The four subgroups of carbapenem-hydrolysing class D β-lactamase that have been reported in *A. baumannii* are *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-51}$ and *bla*$_{OXA-58}$. These predominant gene clusters encode the production of various OXAs that confer resistance to carbapenem and are either located on the chromosome or plasmids of *A. baumannii* (Azimi et al., 2015; Poirel & Nordmann, 2006).

Class D *OXA-51*-like carbapenemases are located on the chromosome and encode a carbapenem-hydrolysing class D β-lactamase that is intrinsic to *A. baumannii* (Turton et al., 2006). This weak carbapenemase imparts carbapenem resistance only if the additional promoter ISAba1 is present upstream of the structural gene. The insertion of ISAba1 upstream of bla_{OXA-51} upregulates its expression and increases the production of the encoded enzyme (Paton et al., 1993).

The product of bla_{OXA-23}, a carbapenem-hydrolysing class D β-lactamase that shares 56% homology with the product of bla_{OXA-51}, has been identified as a primary enzyme responsible for the development of imipenem resistance in *A. baumannii* (Evans & Amyes, 2014). bla_{OXA-23} is a carbapenemase-encoding gene common among carbapenem-resistant *A. baumannii* strains. The presence of this gene has been used as a marker in genotypic rapid diagnostic tests to predict carbapenem susceptibility (Lee et al., 2017; Perez-Llarena & Bou, 2009). The bla_{OXA-23}-encoded enzyme-producing *A. baumannii* strains have been isolated from different geographical regions, including Asia, America, Europe and Australia. Numerous bla_{OXA-23} variants located either on the plasmid or the chromosome have been identified and linked with transposons (Tn2008, Tn2007, & Tn2006) or insertion sequences (e.g., ISAba1) (Mugnier et al., 2010; Potron et al., 2015).

A. baumannii has also gained resistance to β-lactam via non-enzymatic mechanisms, such as the decreased permeability of membranes due to porin changes and the overexpression of efflux pumps (Lee et al., 2017). Porins are OMPs capable of forming channels for transport molecules through the lipid bilayer membrane. *A. baumannii* is unique in that it has a lower number of smaller pores, compared to the pores present in other Gram-negative bacteria. This results in the apparent impermeability of the outer

membrane (Catel-Ferreira et al., 2012; Vila et al., 2007). The reduced density of membrane porins (*CarO*, *Omp 3*3-36 and *OprD*), which occurs due to the reduced expression of porin-encoding genes, enhances carbapenem resistance. For example, disruption of the *carO* gene by inserting ISAba825 and ISAba125 leads to the loss of a 29-kDa section of the OMP and confers carbapenem resistance upon *A. baumannii* (Mussi et al., 2005).

There is a growing body of evidence on the role of efflux pump systems, which can be naturally acquired, in the development of resistance to several drugs. The *adeABC* operon belongs to the resistance–nodulation–cell division (RND) class of efflux pump systems. Overexpression of this operon confers resistance to imipenem and meropenem (Perez et al., 2007). However, other recognised efflux RND pumps, such as AdeIJK and AdeFGH, and non-RND efflux systems are not involved in carbapenem resistance (Coyne et al., 2011; Damier-Piolle et al., 2008; Rosenfeld et al., 2012).

Furthermore, the mutation of genes encoding penicillin-binding protein (PBP) 2 and PBP6b decreases the expression of these proteins and promotes carbapenem resistance. A mutation resulting from an insertion sequence element located close to ISAba125 has been shown to disrupt the PBP6b-encoding gene and enhance carbapenem resistance (Cayô et al., 2011; Perez et al., 2007).

2.6.2 Aminoglycoside resistance

Aminoglycosides such as amikacin and tobramycin remain active against various *A. baumannii* isolates (Singh et al., 2013). However, some strains have gained resistance to aminoglycosides by acquiring plasmids, transposons and integrons that carry genes encoding aminoglycoside-inactivating enzymes such as acetyltransferase, nucleotidyltransferase and phosphotransferase (Abdallah et al., 2015). In *Acinetobacter*

species, these genes are arranged as cassettes within integrons (Peleg et al., 2008). Apart from aminoglycoside-inactivating enzymes, the decreased susceptibility of *A. baumannii* to aminoglycosides is also caused by the overexpression of efflux pumps belonging to the AdeABC system (Figure 2-2) (Decré, 2012; Magnet et al., 2001).

2.6.3 Fluoroquinolone resistance

Fluoroquinolones are an alternative treatment option for *A. baumannii* infections (Chopra et al., 2010). However, the incidence of fluoroquinolone resistance has risen over time (Hujer et al., 2009). Mutations in the quinolone resistance-determining regions that encode DNA gyrase A and subunit topoisomerase IV have been documented as fluoroquinolone resistance mechanisms, as described in Figure 2-2 (Peleg et al., 2008). In addition, mutations in the regulatory systems for efflux pumps cause the overexpression of RND-type efflux mechanisms (AdeABC and AdeIJK) that prevent the accumulation of antibiotics and result in the development of quinolone resistance (Almasaudi, 2018).

2.6.4 Tetracycline resistance

Resistance to tetracycline in *Acinetobacter* strains occurs via the expression of ribosomal protection proteins and energy-dependent efflux systems (Dönhöfer et al., 2012; Ribera et al., 2003). The *tetM* gene encodes a GTPase that mimics the function of elongation factors EF-G and EF-Tu and mediates tetracycline release from bacterial ribosomes. *TetM*-encoded GTPases compete with EF-G for the same binding sites, causing tetracycline to dissociate from its ribosomal binding site (RBS) and allow translation to proceed even in the presence of tetracycline (Brown et al., 2019). Recently, an *A. baumannii* strain carrying the *tetM* gene was isolated from wastewater treatment plants and animal ponds, indicating the extensive dissemination of strains

harbouring these genes (Perez et al., 2007; Zhou et al., 2019). In *A. baumannii*, tetracycline efflux pumps fall into two categories: the energy-dependent TetA and TetB, both of which are responsible for tetracycline resistance (Guardabassi et al., 2000). TetA efflux pumps confer resistance to tetracycline but not to minocycline or doxycycline, whereas TetB confers resistance to minocycline and tetracycline. In most cases, the TetA and TetB efflux pump-encoding genes are contained in plasmids or transposons, thus facilitating gene transfer and the emergence of antibiotic-resistant strains (Roberts, 1996). Other RND efflux pumps, such as AdeABC and AdeIJK, are also involved in tetracycline and tigecycline resistance (Damier-Piolle et al., 2008; Magnet et al., 2001).

2.6.5 Colistin resistance

Colistin exerts its bactericidal effect by altering the structural components of bacteria, specifically the outer membrane. Due to the emergence of antibiotic-resistant bacteria, colistin has gained popularity as a mainstream therapeutic. However, *A. baumannii* strains resistant to colistin were reported even a decade ago (Moffatt et al., 2010). Recent work has revealed three colistin resistance mechanisms in *A. baumannii*, as shown in Figure 2-2. One mechanism involves mutations in the chromosome-encoded two-component regulatory system PmrA/PmrB in response to polymyxin exposure, which alter lipid A and confer colistin resistance (Adams et al., 2009; Beceiro et al., 2011).

Another mechanism involves the loss of LPS due to mutations in the *lpxA*, *lpxC* and *lpxD* genes, which encode enzymes that catalyse the initial stages of LPS synthesis (Adams et al., 2009; Beceiro et al., 2011; Moffatt et al., 2010). Alterations in the LPS biosynthesis pathway result in a decreased negative charge on the cytoplasmic

membrane, thus reducing the affinity of colistin for the bacterial cells (Almasaudi, 2018; Lee et al., 2007; Potron et al., 2015). Furthermore, the increased expression of *emr*-like genes (*emrAB*) that encode the EmrAB efflux pump have been shown to confer resistance to colistin in *A. baumannii* (Lin et al., 2017).

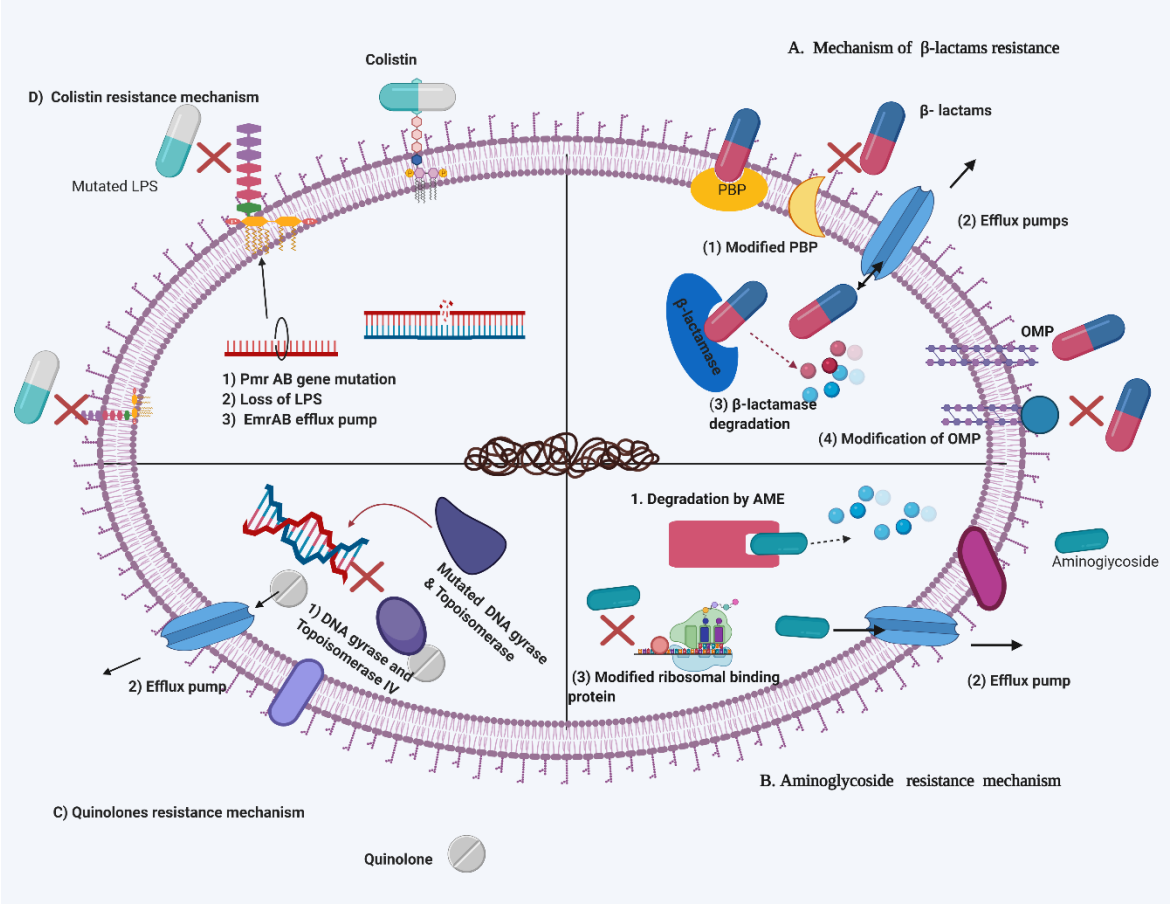


Figure 2-2. Resistance mechanisms of *A. baumannii* to different classes of antibiotics.

The figure above shows the existing mechanisms of resistance to the ß-lactam class of antibiotics (A), aminoglycosides (B), quinolones (C) and colistin (D). The figure was modified from Asif et al. (2018).

2.7 Biofilms

Biofilms are composed of a plethora of microorganisms in close contact with one another and attached to wet surfaces (Mihailescu et al., 2014; Pourhajibagher et al., 2016). The biofilm cells are embedded in a self-protective matrix composed of an extracellular polymeric substance (EPS) (Flemming & Wingender, 2010), which comprises polysaccharides, nucleic acid, lipids and proteins (Flemming & Wingender, 2010; Mihailescu et al., 2014; Pourhajibagher et al., 2016). The biofilm matrix maintains the mechanical integrity of biofilm cells, promotes surface adhesion and creates a cohesive layer that temporarily immobilises the cells (Flemming & Wingender, 2010) .

Upon maturation of the biofilm, bacterial cells switch from reversible to irreversible adhesion to the substrate. The phenotypic alterations in bacterial cells growing in a biofilm are caused by profound changes in their transcriptional profiles, which give rise to the physiological characteristics that distinguish biofilm cells from free-swimming planktonic cells (Donlan & Costerton, 2002; Stewart & Franklin, 2008). Biofilm cells are characterised by low metabolic activity and are protected by an extracellularly secreted matrix, rendering them less susceptible to antibiotics and host-intrinsic immunity (Davies, 2003). The differences between biofilm and planktonic cells are listed inTable 2-2.

Table 2-2. Comparison between biofilm and planktonic bacterial cells.

Planktonic	**Biofilm**	**References**
Single cells	Aggregated cells (multiple cells at a given interface)	(Donlan & Costerton, 2002; Mihailescu et al., 2014)
Little capsular matrix	Surrounded by extracellular polymeric substance matrix	
Physiologically homogeneous and metabolically active	Physiologically heterogeneous and contain a metabolically diverse bacterial population	(Stewart & Franklin, 2008)
Intracellular signalling is not critical for cell division	Intracellular signalling is essential for growth and higher-order architecture	
Antibiotic susceptible	Increased antibiotic resistance, up to 10–1000 times more resistant	(Mihailescu et al., 2014)
Host immune system targets cells effectively	Matrix-embedded cells are resistant to antimicrobial agents and inaccessible to host immune responses	

2.8 Biofilm formation in *A. baumannii*

The capacity of *A. baumannii* to form biofilms facilitates its long-term survival in healthcare facilities and makes it a successful hospital pathogen (Gaddy & Actis, 2009; Abdi-Ali et al., 2014). The process of biofilm formation involves a multi-step cycle of phenotypic transition from a free-swimming, planktonic cell to a sessile life-form that grows on biotic and abiotic surfaces (Figure 2-3) (Choi et al., 2009; Tomaras et al., 2003). The process of biofilm formation is affected by environmental and genetic factors and consists of four main stages, as illustrated in Figure 2-3 (López et al., 2010).

2.8.1 Initial attachment

Biofilms facilitate adhesion to both biotic and abiotic surfaces and promote the prolonged survival of *A. baumannii*. During the initial stage of biofilm development, planktonic cells approach and interact with the substrate surface (Figure 2-3). The attachment stage involves specific and non-specific adherence, depending on the surface properties of the biomaterial (Katsikogianni et al., 2004). The adherence of planktonic cells to the surface consists of reversible adhesion (phase I) and irreversible adhesion (phase II) (Hori & Matsumoto, 2010). During phase I adhesion, the cell-to-surface interaction is based on long-range physical interactions (> 50 nm). Once bacterial cells attach to the substrate surface, phase II adhesion begins, which involves short-range contacts (< 5 nm) between the bacterial cells and the surface. This close

contact is initiated via bacterial capsules, fimbriae or pili (Harris & Richards, 2006), which facilitate irreversible surface attachment.

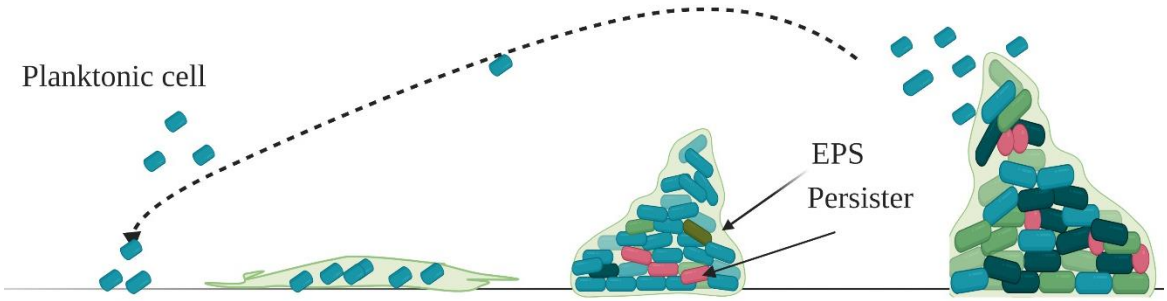
2.8.2 Accumulation and maturation

Biofilms comprise bacterial cell aggregates and microcolonies that form during the accumulation stage. The accumulation of a matrix that encloses the microbial communities occurs as the stages progress. The formation of a three-dimensional biofilm is followed by an irreversible attachment process, which indicates biofilm maturation. Cell-to-cell interactions within the biofilm may result in a heterogeneous physicochemical environment that confers unique characteristics on pathogens embedded in the biofilm (Vasudevan, 2014).

2.8.3 Biofilm dispersal

Some bacterial cells can disperse from the mature biofilm, return to the planktonic state and colonise new surfaces (Høiby et al., 2010). The dispersal stage is triggered by physical or intercellular signalling (Costerton et al., 1995). Depending on the stimulus, biofilm dispersion can occur in one of two ways (Kaplan, 2010). During active dispersion, bacteria initiate pathways by which they react to external stimuli, which are typically environmental changes that result in the release of cells into the environment (Fleming & Rumbaugh, 2017). Active dispersion stimulates phosphodiesterases (PDEs), which reduce the concentrations of cyclic-di-guanosine monophosphate (GMP) and increase the production of matrix-degrading enzymes that cause the

dispersal of biofilm cells (Petrova & Sauer, 2016). Passive biofilm dispersion or detachment occurs due to either external stimuli that prompt the release of single cells or biofilm clusters or to the enzymatic degradation of the biofilm matrix and physical triggers (Petrova & Sauer, 2016). The complex nature of biofilm formation enables the pathogen to continually contaminate medical devices and cause biofilm-specific infections (Singhai et al., 2012).



1. Attachment 2. EPS growth 3. EPS maturation 4. Dispersion

Figure 2-3. Schematic illustration of the stages of biofilm development.

Biofilm formation begins with reversible planktonic cell attachment (1), followed by monolayer formation and irreversible attachment through the formation of an extracellular matrix (2). As the biofilm matures, ‘mushroom’ structures typically composed of polysaccharides are formed (3). In the final stage, a few cells separate and disperse from the biofilm (4). EPS: extracellular polymeric substance. The figure was modified from De Oliveira et al. (2020).

2.9 Genes involved in the formation and regulation of biofilms

Previous studies have shown that various virulence factors are associated with biofilm formation in *A. baumannii* (Thummeepak et al., 2016). The genes encoding the biofilm-associated protein (BAP) (Sharon Goh et al., 2013), OMP (*OmpA*) (Gaddy et al., 2009) and the CsuA/BABCDE chaperone-usher pili assembly system (Tomaras et al., 2003) and *pgaABCD* locus (Choi et al., 2009) are involved in the processes of biofilm formation and regulation. BAP initiates the attachment of biofilm cells to specific surfaces during biofilm formation (Howard et al., 2012; McConnell et al., 2013). The *ompA* gene encodes OmpA, which binds to human epithelial cells and induces biofilm formation (Gaddy et al., 2009).

In addition, *A. baumannii* expresses the *epsA* gene, which encodes exopolysaccharide during biofilm development (Mah & O'Toole, 2001). Exopolysaccharide was shown to accumulate on the cell surface and play a significant role in shielding cells from harsh environments (Russo et al., 2010; Tayabali et al., 2012). Another study showed that exopolysaccharide plays a significant role in cell aggregation during biofilm formation in several bacterial species (Esterly et al., 2011). The expression of bla_{PER-1} increases the adherence of bacteria to the respiratory epithelium, thus initiating biofilm formation on the epithelial surface (Lee et al., 2008).

csuA/BABCDE-encoded pili are involved in the initial stages of biofilm formation by enabling the attachment of bacterial cells to abiotic surfaces and initiating the formation of microcolonies, followed by the development of fully-formed biofilm structures (Gaddy & Actis, 2009). The inhibition of *csuE*, which encodes the putative tip subunit of the pili, reduces pili production and biofilm formation (Tomaras et al., 2003).

Various environmental and genetic signals tightly regulate the formation of biofilms. The genetic regulators of biofilm formation in *A. baumannii* include the two-component regulatory system, quorum sensing (QS) systems and sRNAs. Previous studies have shown that the two-component regulatory system, which is encoded by the *BfmRS* locus and consists of a kinase sensor and a response regulator, control the expression of the *csu* operon, which is essential for biofilm formation on abiotic surfaces (Tomaras et al., 2008). The inactivation of *bfmS* was shown to reduce biofilm formation and pili production in *A. baumannii* ATCC17978 (Liou et al., 2014; Tomaras et al., 2008).

QS genes such as *abaI/abaR* are known to regulate biofilm formation in *A. baumannii*. The gene *abaI* encodes an autoinducer (AI) synthase that generates the QS molecule acyl-homoserine lactone (AHL). When the bacterial population density increases, AHL is present at high concentrations and interacts with its cognate receptor AbaR, thereby triggering downstream cellular responses (Anbazhagan et al., 2012).

In addition to the two-component and QS regulatory systems, sRNAs are involved in regulating biofilm formation and the attachment of bacteria to eukaryotic cells. A study by Alvarez et al. (2017) found that an array of sRNAs are expressed differentially in biofilm cells and planktonic cells and are involved in the regulation of biofilm formation in *A. baumannii* (Álvarez-Fraga et al., 2017).

sRNAs are non-coding bacterial RNAs (ncRNAs) that range from 40 to 500 nucleotides in length and are located between open reading frames (Ahmed et al., 2018). To survive

stressful conditions within the host or in external environments, bacteria express sRNAs that modify the expression levels of genes involved in many biological processes, such as outer membrane synthesis, QS, antibiotic resistance and biofilm formation (Guillier & Gottesman, 2006; Lenz et al., 2005; Papenfort & Vogel, 2010; Romby et al., 2006; Storz et al., 2011).

sRNAs achieve the post-transcriptional regulation of bacterial gene expression by base-pairing with target mRNAs and binding to proteins (Bronsard et al., 2017; Chambers & Sauer, 2013). Depending on the base-pairing and their positions relative to target mRNAs within the bacterial genome, sRNAs are classified into either cis or trans-acting forms. Cis-acting sRNAs are transcribed from the same genetic loci as but oriented antisense with respect to the genes they regulate (Ellis et al., 2015). They have perfect complementarity with their target mRNAs and do not require chaperones for binding.

The base-pairing of cis-acting sRNAs can either activate or inhibit mRNA degradation or translation (Ellis et al., 2015). Trans-acting sRNAs are transcribed from genetic loci that are separate from the target genes they regulate. These sRNAs share only partial complementarity with their target mRNAs and require chaperone Hfq proteins to enhance mRNA binding. Base-pairing between sRNAs and their target mRNAs can either stabilise or degrade mRNAs, thereby regulating the translation of target genes (Figure 2-4) (Aiba, 2007). Trans-acting sRNAs have multiple target mRNAs, and

therefore, even a single sRNA can modulate global physiological responses (Bejerano-Sagie & Xavier, 2007; Valentin-Hansen et al., 2007).

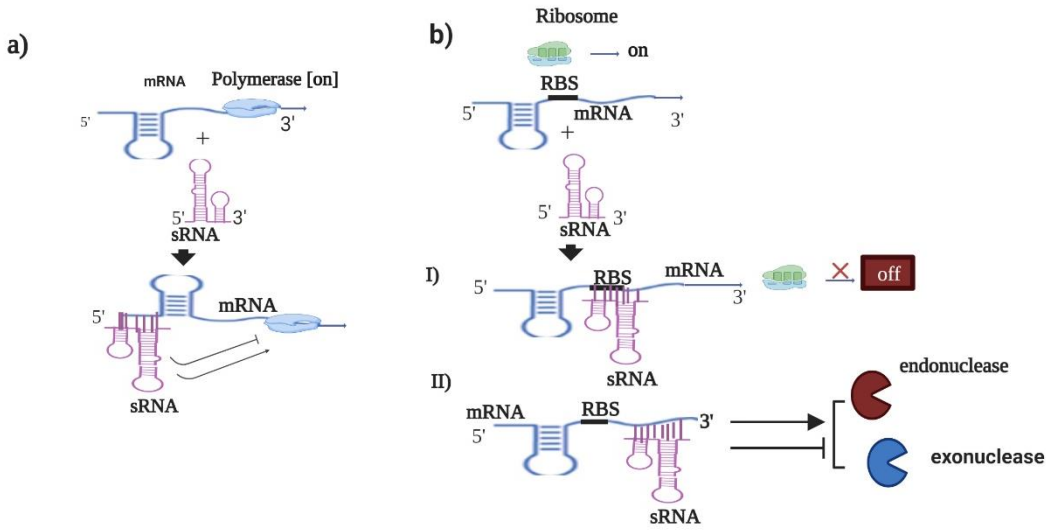


Figure 2-4. Regulatory roles of sRNA in bacteria.

The sRNA interacts with the mRNA target and, upon base-pairing, can cause structural reconfiguration that eventually promotes or suppresses the polymerase (a). Translational regulation: sRNAs regulate the translational process in several ways (b); sRNAs base-pair with target mRNAs by sequestering ribosome binding sites (RBSs) (I), which can hinder translation initiation; sRNAs interact with their mRNA targets at a distance from the RBS, causing the targeted mRNA to undergo a structural change that indirectly affects its interaction with the ribosome (II). By altering interactions with exonucleases or endonucleases, sRNAs can also facilitate or prevent the decay of mRNA. The figure was adapted from (Waters & Storz, 2009).

2.10 Biofilm-specific infections

A. baumannii biofilms play a significant role in determining host-pathogen interactions that can lead to infections (Yang et al., 2019). Biofilm-specific infections, such as ventilator-associated pneumonia and catheter-related infections, are caused by *A. baumannii* strains that are highly resistant to antibiotic treatment and render the patient at risk of reinfection (Dijkshoorn et al., 2007). In the case of pneumonia, individual *A. baumannii* cells can stick together inside the airways and coat themselves in a matrix to form biofilms that protect the bacteria against antimicrobial agents (Santos-Lopez et al., 2019). During biofilm-specific infections, planktonic bacteria from the biofilm can spread to the bloodstream or to regions around the infection site (Lewis, 2007). The combined action of antimicrobials and host immune responses can eradicate planktonic bacteria, but once a biofilm is formed, the bacteria can resist treatment and host immune reactions and thus trigger recurrent infections (Lebeaux et al., 2014).

The different types of medical devices implanted in the human body present primary colonisation sites that can serve as major sources of systemic dissemination (Lebeaux et al., 2014; Rodríguez-Baño et al., 2008). *Acinetobacter* biofilms play a vital role in infectious diseases such as cystic fibrosis, periodontitis, bloodstream infections and urinary tract infections because of their propensity to reside in medical devices (Abdi-Ali et al., 2014).

2.11 Treatment of biofilm-specific infections

The treatment for biofilm-specific infection is hampered by the increased antibiotic resistance of biofilm-forming bacterial communities (Sharma et al., 2019). Classical antibiotic chemotherapy alone cannot eradicate bacterial cells in the central regions of biofilms, suggesting that the treatment of biofilm-specific infections requires the use of new therapeutic agents (Sharma et al., 2019; Vuotto & Donelli, 2019). However, some alternative methods and novel anti-biofilm agents have been reported for treatment of biofilm infections.

Anti-biofilm treatment is based on the mechanical disruption of the biofilm using water sprays, jets and debridement of the infection site, accompanied by intensive antimicrobial treatment (Høiby et al., 2015; Percival et al., 2015). Unfortunately, irrigation-based treatment often simply fluidises and spreads the biofilm across other surfaces (Fabbri et al., 2016), which might explain the failure of pulsed lavage of infected surfaces to eradicate biofilms (Urish et al., 2014).

The use of anti-fouling or antimicrobial surfaces is another potential strategy for the prevention of biofilm formation (Francolini et al., 2014). Polymeric hydrophilic coatings, such as polyethylene glycol, are used to create anti-fouling surfaces that reduce or hinder microbial adhesion. Coatings such as silver and antioxidant nanoparticles are also used to deter biofilm formation (Antonelli et al., 2012). However, coating techniques are not sustainable, as the surface deteriorates rapidly and eventually becomes amenable to biofilm formation.

Biofilm-dissolving agents have also been used to prevent biofilm-specific infections (Donelli et al., 2007). Agents such as enzymes, peptides and antibiotic polyphenols impair biofilm formation by interfering with bacterial signalling pathways (Roy et al., 2018). Some examples of biofilm-dissolving agents are epigallocatechin gallate (EGCG) and peptide 1018. The former acts via peptidoglycan cleavage and the latter inhibits (p)ppGpp-regulated stringent responses (De la Fuente-Núñez et al., 2014; Park et al., 2011).

Despite the use of various anti-biofilm strategies, the key challenge in treating biofilm-specific infections is the persistence of biofilm cells during antibiotic exposure, which leads to the development of antibiotic resistance. The resistant bacterial cells in the biofilm are responsible for the continuous contamination of medical equipment and dissemination of bacteria in hospital environments. Furthermore, the increased resistance of biofilm cells to antibiotics renders biofilm-specific infections more persistent (Stewart & Costerton, 2001). The mechanisms by which biofilm cells display decreased susceptibility to antibiotics will be reviewed in the following section.

2.12 Mechanisms of antibiotic resistance in biofilms

Several studies have shown that the conventional antibiotic resistance mechanisms described in planktonic cells cannot fully explain biofilm-specific antibiotic resistance (Brooun et al., 2000; Walsh, 2000). The ability of a microorganism to grow in the presence of high concentrations of antimicrobial agents is known as resistance (Lewis,

2001). Planktonic cells whose minimum inhibitory concentrations (MICs) exceed the threshold level are regarded as resistant. However, no specific MIC threshold levels have been defined for bacteria in a biofilm state. Based on the description put forth by Lewis (2008), biofilms acquire antibiotic resistance when an antimicrobial agent is prevented from interacting with its intended target. In accordance with this definition, biofilm resistance mechanisms include antibiotic efflux pumps, antibiotic sequestering molecules (eDNA) and the matrix β-lactamase.

The multicellular nature of biofilms is both a significant contributor to antibiotic resistance in biofilm communities and the root of resistance mechanisms. The EPS holds bacterial cells together and facilitates the formation of the multicellular consortia that constitute the heterogeneous environments of biofilms and enable functioning as a multicellular system. During biofilm growth, an entire panel of genes and proteins is upregulated and downregulated, modulating pathway differentiation and bacterial attachment to substrate surfaces. Some of these genes have been shown to be responsible for the acquisition of biofilm-specific antibiotic resistance by altering the characteristics of bacteria in the biofilms (Hall & Mah, 2017).

2.12.1 Efflux pumps

Efflux pumps facilitate the extrusion of antimicrobial agents from their intracellular targets to the extracellular space and lead to the development of antimicrobial resistance (Poole, 2007). Efflux pumps also play a role in biofilm formation by transporting EPS,

QS and quorum quenching (QQ) molecules outside the cell and by controlling QS. The pumps also expel harmful molecules such as antibiotics and metabolic intermediates and regulate cellular aggregation by either facilitating or preventing adhesion to surfaces and other cells (Alav et al., 2018). *A. baumannii* contains RND efflux systems comprising AdeABC, AdeFGH and AdeIJK proteins that pump out antimicrobial agents from planktonic cells and facilitate the development of resistance to antibiotics (Coyne et al., 2010; Sugawara & Nikaido, 2014). RND efflux systems are also involved in biofilm formation (Wu et al., 2015). Some genes, such as *adeB* and its regulator, are involved in biofilm-specific multidrug tolerance and the development of increased antibiotic resistance (Yoon et al., 2013).

2.12.2 Extracellular DNA (eDNA)

eDNA is an essential and universal part of the biofilm matrix of most bacteria. It is derived endogenously from QS-mediated release, external membrane vesicles and biofilm cell subsets due to altruistic or fratricidal population lysis and exogenously from polymorphonuclear leukocytes at infection sites. eDNA increases biofilm resistance to some antimicrobial agents, regardless of whether the eDNA is endogenously or exogenously derived. When added to *P. aeruginosa* biofilms, eDNA from exogenous sources was shown to integrate into the matrix, resulting in three and two times greater resistance to tobramycin and gentamicin, respectively (Jakubovics et al., 2013). The alteration of the extracellular environment is another mechanism by which eDNA contributes to biofilm tolerance. As eDNA is anionic, cations (e.g., magnesium ions)

can be chelated by it; this reduces the effective Mg^{2+} concentration in the environment (Mulcahy et al., 2008).

In addition to playing a physical role in the development of antibiotic resistance, eDNA is involved in the horizontal transmission of antibiotic resistance genes between naturally competent cells within a biofilm (Bae et al., 2014). Overall, in the surface bacterial community, the sharing of antibiotic resistance genes through eDNA between certain bacterial species living in biofilms can contribute to the development of antibiotic resistance (Hall & Mah, 2017). eDNA also plays a role in biofilm matrix synthesis and acts as a scaffolding agent during biofilm aggregation or bacterial stabilisation (Sahu et al., 2012).

2.12.3 Antibiotic-modifying enzymes in the matrix

Enzymes such as β-lactamases, when present in the biofilm matrix, can degrade antibiotics and prevent them from reaching their cellular targets (Anderl et al., 2000). The β-lactamase AmpC, which is chromosomally encoded and secreted into the matrices of *P. aeruginosa* biofilms, is a clinically essential determinant of β-lactam antibiotic resistance in this pathogen (Bagge et al., 2000). The expression of AmpC in the presence of low concentrations of imipenem is limited to the biofilm periphery, even when the cells in the centre and base of the biofilm are physiologically active. However, higher concentrations of imipenem result in the complete induction of biofilm reporters, suggesting that at high doses, imipenem can overcome the

degradative potential of β-lactamases (Li & Jiang, 2009). Several studies have shown that mature *P. aeruginosa* biofilms are more resistant to ceftazidime and meropenem than younger biofilms due to increased concentrations of β-lactamase in the matrix (Bowler et al., 2012). Although the role of AmpC has been established *in P. aeruginosa*, its contribution to the development of antibiotic resistance in *A. baumannii* is not very well known.

2.12.4 Horizontal gene transfer

In addition to the acquisition of eDNA from the biofilm matrix, horizontal gene transfer by conjugation also facilitates the transfer of antibiotic resistance genes between cells in a biofilm (Ehlers & Bouwer, 1999). In fact, biofilms show higher gene transfer efficiency than their counterpart planktonic cells (Hausner & Wuertz, 1999). This higher transfer efficiency could be due to decreased shear and closer cell-to-cell contacts in biofilms (Madsen et al., 2012; Savage et al., 2013). Conjugative plasmids determine the ability of bacteria to form biofilms, implying that strains bearing these plasmids can preferentially form biofilms, thereby increasing the likelihood of biofilm-specific infections and the spread of virulence factors (Ghigo, 2001). In *Staphylococcus aureus*, for example, the conjugal transfer of a plasmid carrying the MDR gene was 10,000 times higher in biofilms than in planktonic cultures, suggesting that biofilms promote the transfer of such plasmids and result in the emergence of antibiotic resistance strains (Savage et al., 2013).

2.12.5 Mutation frequency

Classically, biofilm recalcitrance has been found to be phenotypic and has not been attributed to irreversible genetic changes. However, a recent study found that biofilm cells accumulate mutations faster than planktonic cells and that these mutations can contribute to increased antibiotic resistance (Hall & Mah, 2017). Cells grown in biofilms are intrinsically more prone to spontaneous mutations, as they experience higher levels of endogenous oxidative stress that can cause DNA damage (Boles & Singh, 2008). This can, in turn, contribute to the development of permanently hypermutable strains. In *P. aeruginosa*, the expression of ROS-detoxifying enzymes, such as superoxide dismutase and catalase, is not tightly controlled; consequently, the biofilm cell is unable to withstand oxidative stressors that affect the mutation rates (Driffield et al., 2008).

2.13 Antibiotic tolerance mechanisms in biofilms

Antimicrobial tolerance refers to the ability of a microorganism to survive in the presence of a bactericidal antimicrobial agent (Hall & Mah, 2017). These mechanisms prevent the bactericidal agent from executing its toxic downstream effects, even if the agent is bound to its target (Lewis, 2008). Following this definition, biofilm-specific antibiotic tolerance is multi-factorial and includes decreased antibiotic penetration, slow growth rate and the presence of persisters (Olsen, 2015), as shown in Figure 2-5.

Biofilm-specific antibiotic tolerance is a typical adaptive resistance mechanism (Fernández et al., 2011). Adaptive resistance is a class of non-inherited resistance mechanisms triggered in response to environmental factors or epigenetic changes that are characterised by a transient nature and analogous to mechanisms observed in persister cells. As the biofilm cells return to their planktonic states, the cells regain their original susceptibility to specific antimicrobial agents (Nickel et al., 1985). Reversible phenotypic variations in response to environmental factors imply changes in the genetic program triggered by stressful environments. These changes stimulate cell-to-surface interactions and the recruitment of molecules that facilitate biofilm formation. Such adaptive resistance strategies exhibited by biofilms eventually result in adaptive mutations or horizontal gene transfer (Cook et al., 2011; Savage et al., 2013).

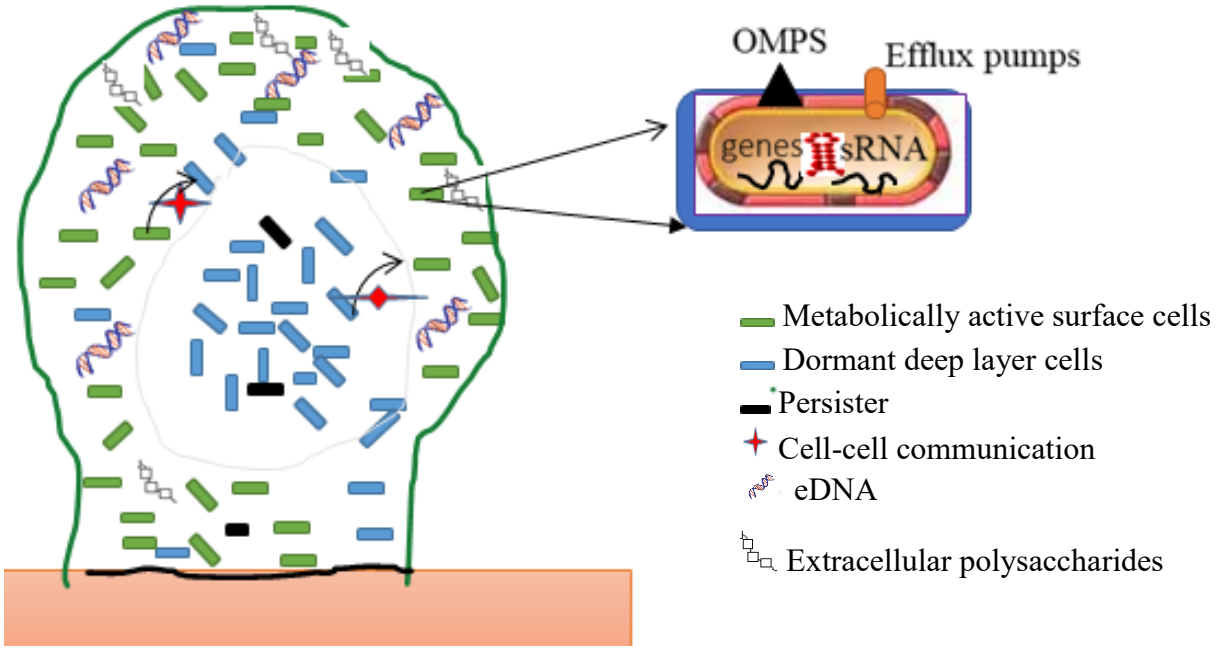


Figure 2-5. Major mechanisms of reduced susceptibility to antimicrobials in bacterial biofilms.

Biofilm cells are embedded in a matrix shaped like a mushroom. Biofilms attach to abiotic or biotic surfaces (brown rectangle). OMP: outer membrane protein; eDNA: extracellular DNA; sRNA: small RNA. The figure was adapted from Bhando et al. (2019).

2.13.1 Limited antibiotic penetration

The biofilm matrix reduces the penetration of antimicrobials or antiseptics into cells that lie deeper in the biofilm (Daddi Oubekka et al., 2012; Davies, 2003). Specifically, the EPS can act as a diffusion-limiting barrier against different antimicrobials, resulting in restricted drug access to deeper layers of the biofilm (Karygianni et al., 2014). The reduced penetration of antimicrobials due to interactions with EPS components occurs when positively charged agents bind to negatively charged polymers or when

antimicrobials are inactivated by biofilm matrix enzymes (Karygianni et al., 2014; Koo et al., 2017). Different studies have also documented how the penetration of positively charged aminoglycosides is slowed by negatively charged matrix polymers in *P. aeruginosa* biofilms (Kumon et al., 1994). *E. coli* cells that carry the biofilm-forming genetic locus *pgaABCD* exhibit enhanced tolerance to the toxic effects of antibiotics; these cells become a dominant subspecies upon antibiotic treatment and eventually evolve into resistant mutants. Gut microbiome strains that carry the *pgaABCD* genes are therefore considered to be antibiotic resistance progenitor cells (Lin et al., 2020).

Similarly, poly-(1-6)-N-acetylglucosamine (PNAG), an essential polymer required for biofilm formation, is encoded by the *pgaABCD* gene cluster. Higher expression of *pgaB* was found in a clinical strain of *A. baumannii* than in the standard strain. Furthermore, *pgaB* expression has been associated with increases in the capacity and thickness of the formed biofilms (Choi et al., 2009). The upregulation of *pgaB* supposedly results in increased extracellular matrix production, biofilm growth and antimicrobial resistance in *A. baumannii* (Choi et al., 2009). However, the precise mechanism underlying EPS-mediated tolerance of the biofilm to antibiotics has not been investigated.

Several studies have suggested that a decrease in antibiotic penetration cannot adequately justify the recalcitrance of a biofilm to antibiotics. Antibiotics such as fluoroquinolones, rifampin and ampicillin penetrate the biofilm matrix even though they do not eradicate 100% of the cells (Anderl et al., 2000; Rodríguez-Martínez et al.,

2007). Most antibiotics ultimately reach all biofilm regions, except for compounds that slowly penetrate the biofilm. Slow antibiotic penetration temporarily exposes cells in the biofilm to sub-optimal antibiotic concentrations and triggers metabolic or transcriptional alterations that change the cell physiology (Jefferson et al., 2005). The exposure of bacterial cells to sub-lethal antibiotic concentrations can thus promote the selection of antibiotic-resistant bacteria within a biofilm.

2.13.2 Slow growth rate

Bacteria in biofilms are less susceptible to antimicrobial agents due to their slower growth rates and reduced uptake of active compounds. When the effects of cetrimide on *E. coli* biofilms were studied, cells with slower growth rates were found to be more tolerant to the agent (Evans et al., 1991). Furthermore, in adverse growth environments, a subpopulation of bacterial cells in the biofilm may enter a viable but non-culturable (VBNC) state, in which the bacterial cells transiently lose culturability. Their metabolic activities decrease, and only essential activities such as respiration, rRNA biosynthesis and plasma membrane integrity are preserved (Li et al., 2014). Such physiological changes can increase the antibiotic tolerance of bacterial cells in a biofilm.

2.13.3 Persisters

Biofilms contains persister cells capable of withstanding high concentrations of antimicrobials and biocides (Figure 2-6). These cells can survive antibiotic attacks by shutting down their cellular targets and neither grow nor die in the presence of

bactericidal agents (Conlon et al., 2015). In various bacterial species, the number of persisters has been seen to increase with the bacterial culture density, reaching up to 1% in the stationary phase and higher proportions in biofilms (Keren et al., 2004) . Previous studies have also illustrated decreased antibiotic susceptibility in very thin biofilms due to the presence of these cells (Brooun et al., 2000).

Persisters are a heterogeneous population that exist in various physiological states, and thus exhibit varying levels of antibiotic tolerance (Amato & Brynildsen, 2015). Persisters can survive in antibiotic-exposed environments by invoking 'passive defence' techniques, in which the cells enter dormancy. Another technique is 'active defence', in which the cells employ efflux-mediated mechanisms to reduce intracellular antibiotic accumulation (Pu et al., 2017). High activation of the latter mechanism may incur high metabolic costs for the cell, leading to energy depletion, growth retardation and, eventually, persister formation (Orman & Brynildsen, 2015). Furthermore, a recent study revealed the presence of active efflux mechanisms in persisters that neither grew nor died, suggesting that these cells can use active defence strategies to survive in antibiotic-exposed environments (Jamal et al., 2018; Pu et al., 2017).

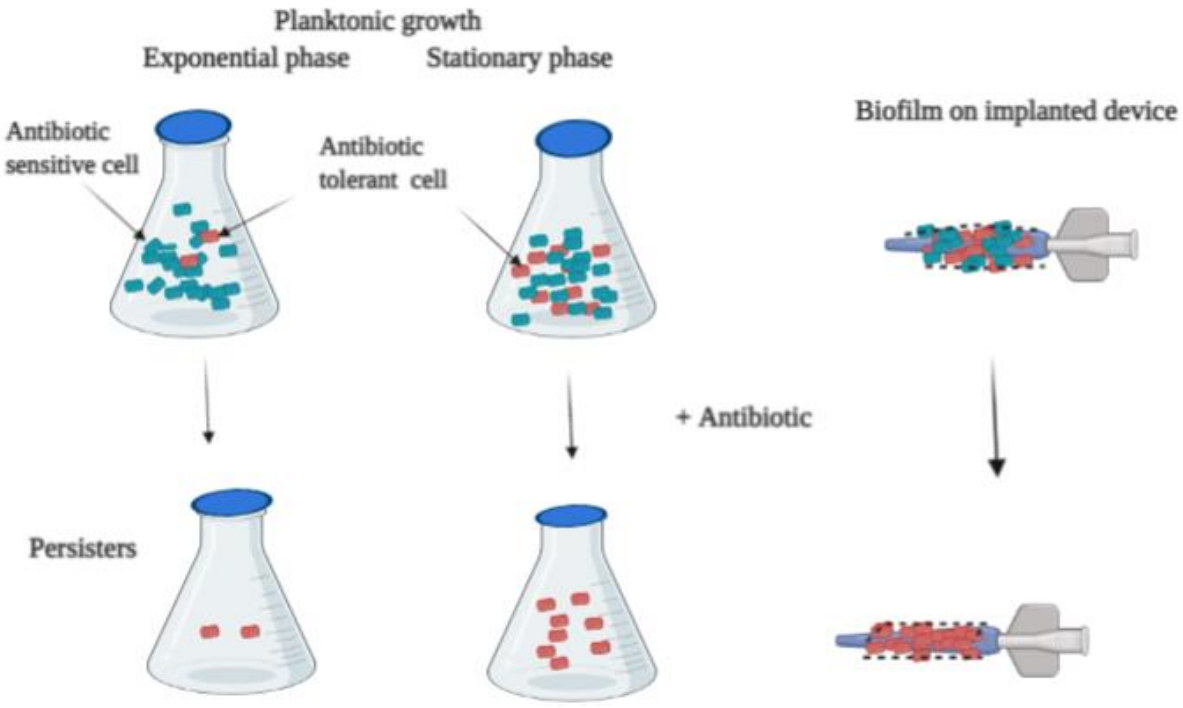


Figure 2-6. Persisters in different phases of bacterial culture.

The number of persister cells (indicated in red) is high in biofilms and in the stationary phases of planktonic cultures relative to exponential planktonic cultures. The latter are composed mostly of antibiotic-sensitive cells (shown in green).

Persisters contribute to latent clinical or chronic biofilm-specific infections (Lebeaux et al., 2014; Lewis, 2007). For example, biofilm persisters account for > 20% of catheter-related bloodstream infection relapses. These cells can withstand local antibiotic treatments at concentrations up to 1,000 times higher than the MICs of planktonic cells (Lebeaux et al., 2014). *In vivo*, antibiotic treatment can eradicate infection symptoms by destroying free-floating bacteria but cannot kill bacterial cells embedded in a biofilm. Once antimicrobial chemotherapy is completed, the biofilm can act as a reservoir for the recurrence of infection (Stewart & Costerton, 2001).

Persisters not only cause treatment failure but also lead to the development of genetic resistance (Levin & Rozen, 2006). The biofilm environment, which serves as a protective niche for *in vivo* persisters, is thought to increase mutation rates and the frequency of horizontal gene transfer, resulting in the development of genetic resistance similar to that seen in clinical strains of *A. baumannii* (Savage et al., 2013).

2.13.4 Stress responses

Biofilm cells are often under stress due to nutrient deficiencies, suboptimal pH or the accumulation of toxic substances. By altering their cellular structure and morphology through the action of stress-induced genes, biofilm cells develop survival mechanisms in response to stress by enhancing their tolerance or resistance to antimicrobial agents (De la Fuente-Núñez et al., 2013).

During biofilm formation, bacteria experience increased osmolarity and limited oxygen exposure (Sauer, 2003). Nosocomial pathogens, such as *A. baumannii*, express porins, such as OMP33 and CarO, on the outer membrane in response to highly osmolar growth environments, which indirectly lead to carbapenem resistance (Novović et al., 2018). Transcriptomic analysis of persisters has also revealed the upregulation of stress response-related genes that regulate the SOS and heat- and cold-shock responses (Keren et al., 2004). In one study, OS-deficient *E. coli* strains were unable to form persisters in the presence of certain antibiotics (Wu et al., 2015). These findings suggest that the

stress response is essential to the development of persister cells (K. Lewis, 2008; Michiels et al., 2016).

Previous studies have shown that sub-inhibitory antibiotic concentrations induce stress responses, such as the SOS response, which enhance the adaptive resistance mechanisms that decrease susceptibility to antibiotics (Fernández et al., 2011). Sub-inhibitory antibiotic concentrations also alter the regulation of gene expression and/or the expression of specific antibiotic resistance genes. For example, sub-optimal concentrations of azithromycin induce the expression of the efflux pump-encoding gene *MexCD-OprJ* in *P. aeruginosa* biofilms. However, this effect was not seen in planktonic cultures (Gillis et al., 2005).

2.14 Research gaps

A. baumannii has become a significant public health concern due to its exceptional ability to develop antibiotic resistance and form biofilms. Biofilm formation prolongs the survival of this pathogen in hospital environments and can serve as a continuous source of infections (Espinal, Martí, et al., 2012; Gaddy et al., 2009). Biofilm-specific genes, such as *csuE* and *ompA*, have been shown to regulate the expression of antibiotic resistance genes (Gaddy et al., 2009; Tomaras et al., 2003), suggesting a link between biofilm formation and antibiotic resistance.

Biofilm formation also facilitates the development of inheritable resistance through mechanisms such as adaptive mutagenesis or horizontal gene transfer (Cook et al.,

2011; Driffield et al., 2008). Bacteria grown in biofilms are 10–1,000 times more resistant to various antimicrobial agents than are bacteria grown in planktonic cultures (Davies, 2003). To understand the relationship between biofilm formation and antibiotic susceptibility, it is necessary to characterise the biofilm formation capabilities of non-MDR and MDR *A. baumannii* strains isolated from clinical samples and to investigate the influence of biofilm growth on the emergence of antibiotic resistance in these strains.

Biofilms have been recognized as a primary mode of bacterial life, as most bacteria survive as biofilm communities in diverse environments (William Costerton et al., 1995; Flemming et al., 2016). However, a large amount of information related to antibiotic resistance and virulence factors has been identified in planktonic cultures (Hua et al., 2014; Novović et al., 2018; Pi et al., 2017). Most genetic studies on *A. baumannii* biofilm cells have used the standard strains ATCC17978 and ATCC19606 (Henry et al., 2014; Rumbo-Feal et al., 2013; Trapnell et al., 2010), which were isolated 70 years ago and have been used as models for over 20 years. These standard strains might not reflect the characteristics of clinical isolates identified in the last three decades. Therefore, the transcriptional changes that occur in the biofilms of clinical strains remain primarily unexplored. In the current study, a comparative transcriptomic analysis between biofilm and planktonic cells was conducted using the hyper biofilm-producing clinical strain, *A. baumannii* ST1894. This newly identified clinical isolate is susceptible to most antibiotics in planktonic culture but has demonstrated the highest-

recorded levels of antibiotic resistance levels as a biofilm. *A. baumannii* ST1894 was therefore used to understand the transcriptional changes that occur in biofilms and in planktonic cultures.

Previous studies have shown that exposure to sub-inhibitory concentrations of colistin and carbapenem induce transcriptional changes during the planktonic growth of *A. baumannii* (Henry et al., 2014; Navidifar et al., 2019). However, these effects may not be the same as those seen in *A. baumannii* biofilms. Therefore, this study aimed to investigate the transcriptional responses of *A. baumannii* biofilms treated with sub-inhibitory concentrations of antibiotics.

Furthermore, in prokaryotes, sRNA molecules are involved in the post-transcriptional regulation of metabolic processes (Sittka et al., 2008). The interaction of sRNA with the RBS of the transcript may result in negative regulation by blocking the ribosome or in positive regulation by binding to the target mRNA and altering its secondary structure, making the RBS more accessible (Storz et al., 2011). sRNAs can also exert their effects by binding target mRNA and recruiting RNases to degrade the mRNA. sRNAs are also known to be involved in post-transcriptional regulatory networks that connect environmental signals to metabolic changes, especially during biofilm formation (Bak et al., 2015), but their biological roles in the development of biofilm-specific antibiotic resistance are not yet understood.

Based on RNA sequencing data, we found that sRNA00203 was the most significantly upregulated sRNA in *A. baumannii* ST1894 biofilms relative to planktonic cells (unpublished results). sRNA00203 also has complementary binding sites for DEGs involved in biofilm formation and antibiotic resistance. We hypothesise that deleting sRNA00203 might affect the expression of genes involved in biofilm formation and the development of biofilm-specific antibiotic resistance in *A. baumannii*. Therefore, this study aims to determine the effects of sRNA00203 on biofilm formation and the regulation of antibiotic susceptibility in *A. baumannii*. Our findings may provide insight into the mechanisms underlying reduced antibiotic susceptibility in *A. baumannii*.

2.14.1 Hypotheses

- ✓ Biofilm formation capability is associated with susceptibility to antibiotics.
- ✓ Sub-inhibitory concentrations of colistin and imipenem induce biofilm-specific antibiotic resistance genes in *A. baumannii.*
- ✓ The deletion of genes encoding sRNA00203 can affect the transcripts responsible for the formation of biofilms, antibiotic resistance and persistence of *A. baumannii*.

2.14.2 Aims

- ✓ To investigate the relationship between antibiotic susceptibility and biofilm formation capabilities in clinical *A. baumannii* strains.
- ✓ To investigate the transcriptional responses of *A. baumannii* biofilms treated with sub-inhibitory concentrations of antibiotics.
- ✓ To investigate the regulatory role of sRNA00203 in biofilm formation and the development of biofilm-specific resistance in *A. baumannii*.

3 CHAPTER THREE: BIOFILM-INDUCED ANTIBIOTIC RESISTANCE IN CLINICAL *A. BAUMANNII* ISOLATES

3.1 Abstract

To understand the role of biofilms in the development of antibiotic resistance, the biofilm formation capabilities and biofilm-specific antibiotic resistance of 104 clinical *A. baumannii* strains were investigated. The antibiotic susceptibility of selected biofilm formers was determined as they grew in the biofilm phase. The reversibility of antibiotic susceptibility in planktonic cells regrown from biofilms was also investigated. Our results showed that 59.6% of the strains were biofilm formers, of which 66.1% were non-MDR strains. The presence of the *bap*, *csuE* and *abaI* virulence genes was significantly associated with biofilm formation capabilities. The minimum biofilm eradication concentrations (MBECs) for strains grown in the biofilm state were 44, 407 and 364 times higher than the minimum bactericidal concentrations (MBCs) for colistin, ciprofloxacin and imipenem, respectively. Persisters were detected after treating the biofilms at 32–256 times the MBC of planktonic cells. The antibiotic susceptibility reversibility test revealed that biofilm development caused reversible antibiotic tolerance in non-MDR strains but a higher level of irreversible resistance in the XDR strain.

In summary, we showed that non-MDR strains were strong biofilms producers. The presence of persisters in the biofilm contributed to reduced antibiotic susceptibility. Non-MDR strains of *A. baumannii* grown as biofilms developed antibiotic tolerance, whereas XDR strains showed increased resistance when grown as biofilms. To determine the mechanisms underlying this phenomenon, analyses of the regulatory

mechanisms that determine biofilm-specific resistance and comprehensive genomic and transcriptional studies are warranted.

Reproduced in part with permission from Shenkutie, A.M.; Yao, M.Z.; Siu, G.K.-h.; Wong, B.K.C.; Leung, P.H.-m. Biofilm-induced antibiotic resistance in clinical *Acinetobacter baumannii* isolates. *Antibiotics***,** 2020, *9*, 817.

3.2 Introduction

A. baumannii is a major opportunistic pathogen responsible for a high proportion of health-related infections (Fournier & Richet, 2006). Nearly 45% of all *A. baumannii* strains isolated worldwide are MDR strains (resistant to ≥ 3 antibiotic classes). Environmental reservoirs are the primary source of MDR *A. baumannii* infections in hospital settings (Yang et al., 2019). The ability of *A. baumannii* to develop a biofilm facilitates prolonged survival and persistence in clinical environments, enabling this pathogen to spread extensively (Donlan & Costerton, 2002; Gaddy et al., 2009; Yang et al., 2019). Biofilm cells form as a result of coordinated gene expression by individual bacterial cells. The cells undergo a phenotypic switch to generate a community that can withstand adverse environmental conditions. Phenotypic switches also promote the emergence of antibiotic resistance by expressing antibiotic resistance genes or causing genetic mutations (Burmølle et al., 2006).

Virulence genes associated with biofilm development in *A. baumannii* include *bap*, the *csu* locus, *adeFGH*, *ompA* and *abaI* (Yang et al., 2019). *Bap* encodes a large bacterial surface protein composed of 8,621 amino acids (Burmølle et al., 2006). The predicted structure of BAP is similar to that of bacterial intercellular adhesins, which belong to the immunoglobulin-like fold superfamily and facilitate the development of mature biofilm structures (Loehfelm et al., 2008). The *csuE* gene is part of the *csu* operon, which encodes the chaperone-usher pili assembly system. Pili production is

required for the initial stages of biofilm formation on abiotic surfaces (Tomaras et al., 2003). The *ompA* gene encodes porin, which is involved in epithelial cell adhesion, antibiotic resistance and biofilm formation (Gaddy et al., 2009). *AdeFGH* encodes an RND antibiotic efflux system involved in the synthesis and transport of AI molecules during biofilm formation (He et al., 2015). *AbaI* encodes an AI synthase that participates in the synthesis of the QS molecule AHL. Dou et al. (2017) showed that mutating *abaI* in *A. baumannii* increases its susceptibility to meropenem and piperacillin. *AbaI* mutants treated with meropenem showed lower levels of OXA 51, *ampC*, *adeA* and *adeB* expression (Dou et al., 2017). The expression of biofilm-specific genes influences the expression of antibiotic resistance-encoding genes, suggesting a link between biofilms and antibiotic resistance formation. It has been documented that strongly biofilm-forming *A. baumannii* strains are antibiotic-resistant (Yang et al., 2019). Strong biofilm formation is beneficial to the survival and dissemination of resistant strains. However, antibiotic-sensitive strains are vulnerable to antibiotic challenges; to survive, they may form a biofilm to protect themselves from antibiotics in the surrounding environment.

To understand the relationship between biofilm growth and antibiotic susceptibility, the biofilm formation abilities of clinical *A. baumannii* strains with different antibiotic susceptibility profiles were characterised. Alterations in the susceptibility of biofilm cells to antimicrobial agents were also investigated. The results obtained from the phenotypic characterisation of biofilm production and the antibiotic susceptibility of biofilm cells will help us to understand the balance between biofilm formation and antibiotic resistance as developed by *A. baumannii* to enhance its survival. Understanding the emergence of antibiotic resistance in biofilm cells offers valuable insights into the development of preventive strategies for biofilm-specific infections.

3.3 Materials and methods

3.3.1 Clinical isolates

One hundred and four archived and non-duplicate *Acinetobacter* species isolates were obtained from various hospitals in Hong Kong. The *Acinetobacter* species were isolated from urine, blood, sputum and soft tissue samples and stored at -80 °C in Luria-Bertani broth (Oxoid Ltd., Basingstoke, UK) containing 20% glycerol. *A. baumannii* ATCC19606 and *E. coli* ATCC25922 were used as control strains in the biofilm assays and antibiotic susceptibility experiments.

3.3.2 Confirmation of bacterial identities

The identities of the *A. baumannii* isolates were confirmed using a multiplex polymerase chain reaction (PCR) assay. Three pairs of primers were used to identify strains, as illustrated in Table 3-1 (Chen et al., 2014). PCR was performed at a final reaction volume of 40 μL, which contained 5X Phusion HF buffer, 10 mM dNTP, Phusion DNA polymerase (all from New England BioLabs [NEB], Uk) and 0.5 μM of each primer. Reactions were run in a Veriti thermocycler (Applied Biosystems, Foster City, CA, USA) set to the following conditions: initial denaturation at 98 °C for 5 min and 35 denaturation cycles at 95 °C for 30 s each and annealing at 54 °C for 30 s. The PCR products were separated by 1.5% agarose gel electrophoresis containing 0.5 μg/mL RedSafe (nucleic acid staining solution) (iNtRON biotechnology, Gyeonggi, Korea). The stained gel was visualized using a Gel Doc System XR (Bio-Rad Laboratories, Richmond, CA, USA).

Table 3-1. Primers used for the identification of *A. baumannii* (Chen et al., 2014).

Gene	**Primer**	**Sequences (5´ to 3´)**	**Amplicon size (bp)**	**Species specificity**
recA	P-rA1	CCT GAA TCT GGT AAA AC	425	*Acinetobacter species*
	P-rA2	GTT TCT GGG CTG CCA AAC ATTA		
gyrB	sp4F	CAC GCC GTA AGA GTG CAT TA	294	*A. baumannii* and
	sp4R	AAC GGA GCT TGT CAG GGT TA		*A. nosocomialis*
ITS	P-Ab-ITSF	CAT TAT CAC GGT AAT TAG TG	208	*A. baumannii*
	P-Ab-ITSB	AGA GCA CTG TGC ACT TAA G		

3.3.3 Multilocus sequence typing (MLST)

The Oxford scheme of MLST targeting seven chromosomal housekeeping genes was performed in accordance with the method described in the MLST database (http://pubmlst.org/abaumannii). Genomic DNA was extracted using a Favorprep Blood Genomic DNA Extraction Mini Kit (Favorgen biotech. Corp, Taiwan). The seven housekeeping genes (*gltA*, *gyrB*, *ghdB*, *recA*, *cpn60*, *gpi* and *rpoD*) were amplified from the 104 *A. baumannii* isolates and sequenced using the primers shown in Table 3-2, following the method described by Bartual et al. (2005). The allele number of each gene was obtained by comparing its sequence with the reference sequences in the database. The sequence type of a given isolate was identified by matching the seven locus numbers obtained. If a sequence did not match any of the reference sequences in the database, it was designated as a new allele. In addition, if the seven loci did not match any existing allele combinations in the database, the isolates were regarded as new sequence types. A phylogenetic tree was constructed using MEGA X software, the neighbour-joining method and a concatenated aligned sequence of the FASTA files of the seven housekeeping genes (Kumar et al., 2018; Saitou & Nei, 1987).

Table 3-2. Primers used for the multilocus sequence typing (MLST) of *A. baumannii* under the Oxford Scheme.

Locus	Primer	Sequences	Application	Amplicon size (bp)
gltA	Citrato F1	AAT TTA CAG TGG CAC ATT AGG TCC C	PCR/sequencing	722
	Citrato R12	GCA GAG ATA CCA GCA GAG ATA CAC G		
gyrB	gyrB_F	TGA AGG CGG CTT ATC TGA GT	PCR/sequencing	594
	gyrB_R	GCT GGG TCT TTT TCC TGA CA		
gdhB	GDHB 1F	GCT ACT TTT ATG CAA CAG AGC C	PCR	774
	GDHB 775R	GTT GAG TTG GCG TAT GTT GTG C		
	GDH SEC F	ACC ACA TGC TTT GTT ATG	Sequencing	
	GDH SEC R	GTT GGC GTA TGT GC		
recA	RA1	CCT GAA TCT TCY GGT AAA AC	PCR/sequencing	425
	RA2	GTT TCT GGG CTG CCA AAC ATT AC		
cpn60	cpn60_F	GGT GCT CAA CTT GTT CGT GA	PCR/sequencing	640
	cpn60_R	CAC CGA AAC CAG GAG CTT TA		
gpi	gpi_F	GAA ATT TCC GGA GCT CAC AA	PCR/sequencing	456
	gpi_R	TCA GGA GCA ATA CCC CAC TC		
rpoD	rpoD-F	ACC CGT GAA GGT GAA ATC AG	PCR/sequencing	672
	rpoD-R	TTC AGC TGG AGC TTT AGC AAT		

Citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), glucose-6-phosphate isomerase (*gpi*), RNA polymerase sigma factor (*rpo*) (Bartual et al., 2005). PCR: polymerase chain reaction.

3.3.4 Biofilm formation

The biofilm formation capability of the isolates was tested using the Calgary Biofilm Device (CBD), with minor modifications to the procedures described by Ceri et al. (1999) and the manufacturer (Innovotech, Edmonton, AB, Canada). Briefly, 3–5 colonies were picked from an overnight Luria-Bertani (LB) agar plate and inoculated into 10 mL of maximum recovery diluent at a turbidity equivalent to 0.5 of the McFarland standard. The inoculum was mixed in a 1:1 ratio with LB broth. For each *A. baumannii* strain, 150 µL of the bacterial suspension were inoculated into a well of a 96-well microtiter plate. The plate was then covered with a plastic lid containing 96 pegs (Innovotech). The microtiter plate was incubated at 37 °C for 48 h on a platform shaker set at 110 rpm. After incubation, planktonic cells were aspirated from the wells and discarded, and the wells were washed three times with the maximum recovery diluent. The plates and lids with pegs were inverted and allowed to dry for 2 h at room temperature. The biofilm mass quantification was performed in octuplicate, and each assay was repeated on three separate days.

3.3.5 Quantification of the biofilm mass

The biofilm masses formed on the 96-well microtiter plate were quantified using the crystal violet staining method. After drying the microtiter plate and the lid with pegs, 200 µL of 0.1% aqueous crystal violet solution were added to each well of the microtiter plate and covered with the lid. The setup was incubated at room temperature for 15 min. After staining, the wells and pegs were washed three times with maximum recovery diluent to remove the excess stain and then air-dried. After drying, 200 µL of 33% (v/v) acetic acid were added to each well of the microtiter plate to extract the crystal violet bound to the biofilm. The absorbance was measured at 570 nm using a microplate

spectrophotometer (Bio-Rad). The average optical density (OD) corresponding to each *A. baumannii* isolate was calculated, and the biofilm formation capacity was interpreted based on the guidelines described by Stepanović et al. (2000)(Table 3-3). The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the uninoculated control.

Table 3-3. Categorisation of biofilm formation capacity based on the optical density (OD) value.

Category	**OD Range**
Non-biofilm producer	$OD \leq ODc$
Weak biofilm producer	$ODc < OD \leq 2 \times ODc$
Moderate biofilm producer	$2 \times ODc < OD \leq 4 \times ODc$
Strong biofilm producer	$OD > 4 \times ODc$

3.4 Detection of biofilm-specific genes

The detection of biofilm-specific genes (*bap*, *csuE*, *ompA*, *adeFGH* and *abaI)* was performed on a Veriti thermocycler (Applied Biosystems) using the sets of primers presented in Table 3-4. Each PCR was performed in a 20-µL volume containing 1 µL of extracted genomic DNA, 10 µL of Luna Universal qPCR Mastermix (NEB) and 1 µL (10 µM) of each primer. The reaction conditions were initial denaturation at 95 °C for 7 min; 35 cycles of denaturation at 95 °C for 30 s each, annealing at 60 °C for 1 min and extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. The PCR products were separated by 1.5% agarose gel electrophoresis and stained with 0.5 µg/mL RedSafe (iNtRON Biotechnology). The stained gel was visualized using a Gel Doc System XR (Bio-Rad).

Table 3-4. Primers used to detect biofilm-specific genes.

Target gene	Primers (5´- 3´)	Amplicon size (bp)	Annealing temp (°C)	References
bap	F-GGTACAAACTATGTGCCGGATT R-CTGTATTCACTCCTTGACCAGC	934	60	(Farshadzadeh et al., 2018)
csuE	F-AGACATGAGTAGCTTTACG R-CTTCCCCATCGGTCATTC	516	60	
ompA	F-CTGGTGTTGGTGCTTTCTGG R-GTGTGACCTTCGATACGTG	352	60	(Ghasemi et al., 2018)
adeFGH	F-TTCATCTAGCCAAGCAGAAG R-CCTGCTAATGGTAGGGTTAAG	201	60	(Yoon et al., 2013)
abaI	F-CCACACAACCCTATTTACTCGG R-GGCGGTTTTGAAAAATCTACGG	121	60	(Farshadzadeh et al.,2018)

3.4.1 Determination of MIC and MBC

The broth microdilution technique was used to determine antimicrobial susceptibility in accordance with the procedures described by the Clinical and Laboratory Standards Institute (Wayne, 2019). The interpretation of susceptible, intermediate and resistant to each type of antimicrobial was based on the CLSI guidelines. Thirteen types of antimicrobials representing eight categories that have been used for the treatment of *A. baumannii* infections were included to determine their MICs, as represented in Table 3-5. The strains were designated as MDR if they were resistant to at least three classes of antimicrobial agents, including penicillin and cephalosporin (including inhibitor combinations), fluoroquinolones and aminoglycosides. MDR strains resistant to carbapenem were designated as XDR (Wayne, 2019). XDR strains resistant to colistin and all other classes of antibiotics were designated as PDR. After the MIC determination, 10 μL aliquots from wells that showed no visible bacterial growth were inoculated onto LB agar plates and incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration of antibiotic that killed 99.9% of the initial bacterial population.

Table 3-5. Minimum inhibitory concentration (MIC) (μg/mL) breakpoints for the interpretation of antimicrobial susceptibility.

Antimicrobial agent	**Interpretation guide for MIC breakpoints (μg/mL)**		
	Susceptible	Intermediate	Resistant
ß-lactam/ß-lactamase inhibitor combinations			
Ampicillin-sulbactam (SAM)	≤ 4	8	≥ 16
Piperacillin-tazobactam (TZP)	≤ 16/4	32/4–64/4	≥ 128/4
Cephems (cephalosporins)			
Ceftazidime (CAZ)	≤ 8	16	≥ 32
Cefotaxime (CTX)	≤ 8	16–32	≥ 64
Carbapenems			
Doripenem (DOR)	≤ 2	4	≥ 8
Imipenem (IPM)	≤ 2	4	≥ 8
Meropenem (MEM)	≤ 2	4	≥ 8
Lipopeptide			
Colistin	≤ 2	-	≥ 4
Aminoglycosides			
Gentamicin (CN)	≤ 4	8	≥ 16
Tetracyclines			
Tetracycline (TET)	≤ 4	8	≥ 16
Fluoroquinolones			
Ciprofloxacin (CIP)	≤ 1	2	≥ 4
Levofloxacin (LVX)	≤ 2	4	≥ 8
Folate pathway antagonist			
Trimethoprim-sulfamethoxazole (SXT)	≤ 2/38	-	≥ 4/76

3.4.2 Detection of antibiotic resistance genes in *A. baumannii*

The *A. baumannii* ST195 strain was subjected to total RNA sequencing (RNA-seq), and antibiotic resistance genes were identified from the RNA-seq data. After preparing a library, RNA-seq was carried out using the Illumina HiSeq 2500 platform provided by the next-generation sequencing (NGS) service provider. After sequencing, the reads were assembled based on the reference genome of *A. baumannii* ATCC17978. The assembled contiguous sequences were annotated, and a bioinformatics analysis was conducted using the ResFinder online database (https://cge.cbs.dtu.dk/services/ResFinder/). A panel of 12 antibiotic resistance genes bla$_{TEM}$, *bla*$_{OXA-51}$, *bla*$_{OXA-111}$, *bla*$_{OXA-23}$, *bla*$_{OXA-66}$, *bla* $_{ADC-25}$, *aph(3´)-Ib*, *aph(6)-Id*, *armA*, *tet(B)*, *mph(E)* and *msr(E)*) was identified from the RNA-seq data of *A. baumannii* ST195 (ab114). This strain was used as a positive control to establish multiplex PCR and identify antibiotic resistance genes in the remaining 103 strains of *A. baumannii*, using the list of primers designed in this study and as shown in Table 3-6.

Table 3-6. Primers used to detect antibiotic resistance genes.

Target gene (Accession no.)	Sequence (5´- 3´)	Annealing temperature (°C)	Amplicon size (bp)	Mechanisms of antibiotic resistance
β-Lactam resistance				
bla$_{TEM}$ (AY560328)	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	59.9 59.3	800	Extended-spectrum β-lactamase
bla$_{ADC-25}$ (EF016355)	F: ATGTGCCAGGTATGGCTGTG R: ACGCTATATGCTGGGGCATC	60.3 60.0	346	AmpC cephalosporinase
bla$_{OXA-51}$ (KF048919)	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	56.4 56.0	353	Carbapenemase
bla$_{OXA-111}$ (EF650037)	F: CCTCAGCAAGAGGCACAGTT R: ACCCATCCAGTTAACCAGCC	60.2 59.6	182	Carbapenemase
bla$_{OXA-66}$ (AY750909)	F: TCGTGCTTCGACCGAGTATG R: GAGGCTGAACAACCCATCCA	59.9 59.9	506	β-lactam resistance
bla$_{OXA-23}$ (AY795964)	F: TCTGGTTGTACGGTTCAGCA R: AGACTGGGACTGCAGAAAGC	59.2 59.9	346	Carbapenemase
Aminoglycoside resistance				
aph(6)-Id (M28829)	F: ACTCCTGCAATCGTCAAGGG R: GGATCTATCACCAGCCAGCC	60.0 59.9	464	Aminoglycoside phosphotransferase
aph(3´´)-Ib (AF024602)	F: CCTGTCAGAGGCGGAGAATC R: CGTACTCTTGTCCTCGTCCG	59.9 59.9	414	Aminoglycoside 3´-phosphotransferase
armA (AY220558)	F: GTAGTTCGGGGGAAAAACCG R: GCTGTTTTAGCACAGGAAGCA	58.8 59.3	655	Transposase and 16S rRNA methylase
Macrolide–lincosamide–streptogramin B (MLSB) resistance				
mph(E) (DQ839391)	F: TCGTGATGGCATGAGGGAAC R: CCCAACTGAGCTTTTGCTCC	60.1 59.4	729	Macrolide phosphotransferase
msr(E) (FR751518)	F: TACCGAAAGTGCTGGACGAC R: ACTTTAGCGCCAAGCGGTAT	60.0 60.1	270	Macrolide transporter
Tetracycline resistance				
tet(B) (AP000342)	F: TTCAAGTGCGCTTTGGATGC R: TTTCGCCCCATTTAGTGGCT	60.0 59.9	554	Tetracycline efflux MFS transporter

MFS: major facilitator superfamily.

3.4.3 Determination of MBIC and MBEC

The antibiotic susceptibility test for biofilms was performed in accordance with the procedures described by Moskowitz et al. (2004). After the biofilms were formed on the CBD pegs, the pegs were rinsed three times in a 96-well microtiter plate containing 150 µL of maximum recovery diluent. The lid with the pegs was then transferred to a new standard 96-well plate, with each well containing 150 µL of Mueller-Hinton broth and serial two-fold dilutions of antibiotics (range: 2–1,024 µg/mL). The 96-well microtitre plate was incubated overnight at 37 °C. Following incubation, the turbidity in each well was examined visually. MBIC was defined as the minimum antibiotic concentration at which no bacterial growth was observed, which was also the minimum antibiotic concentration that inhibited the release of planktonic cells from the biofilm.

The MBEC was determined after performing the MBIC examination. The lid with pegs was removed and rinsed three times in a 96-well plate containing 150 µL of maximum recovery diluent to remove planktonic cells. The lid was then placed in a second 96-well microtitre plate containing 150 µL of Mueller–Hinton broth. The plate was shaken at a speed of 180 rpm for 10 min to detach biofilm cells from the pegs into the broth. The viability of each biofilm was determined by conducting a plate count after 24 h of incubation at 37 °C. MBEC was defined as the minimal antibiotic concentration required to eradicate the biofilm.

3.4.4 Confocal laser scanning microscopy (CLSM) imaging of the biofilm

CLSM was used to estimate the percentage of viable cells in the biofilm formed on each peg. After biofilm formation, the pegs were separated from the lid using sterilised

pliers and washed three times with maximum recovery diluent to remove the planktonic cells (Ceri et al., 1999). The biofilm cells on the pegs were dual-stained with a mixture of 3.35 μM SYTO-9 and 20 μM propidium iodide, as indicated by the Film Tracer Live/Dead Biofilm Viability Kit (Invitrogen, USA). CLSM images were captured using a Leica TCS SPE Confocal Microscope (Leica Microsystems, USA) with a 63× objective lens. Live and dead cells embedded in the biofilm cell subpopulations were estimated using BioFilm Analyzer tools (Bogachev et al., 2018).

3.4.5 Reversibility of antibiotic resistance of the biofilm cells

A study was conducted to evaluate the antibiotic susceptibility profile after the biofilm cells were regrown in the planktonic state. For this part of the study, two hyper biofilm-forming *A. baumannii* (ST1894 and ST373) strains and one weak biofilm-forming strain (ST195) were selected. *A. baumannii* ST1894 and ST373 are non-MDR strains, whereas ST195 is an XDR strain. The biofilm cells from these three *A. baumannii* strains were sub-cultured on LB agar. The MBICs of three antibiotics (colistin, imipenem, and ciprofloxacin) for biofilm cells and MICs for the recovered planktonic cells were determined by following the procedures mentioned above.

3.4.6 Enumeration of persister cells from planktonic and biofilm populations

Persister cells in the planktonic and biofilm communities were enumerated in accordance with the procedures described by Marques (2015), with slight modification. To isolate persister cells, the hyper biofilm-producing, non-MDR strain *A. baumannii* ST1894 was selected. The strain was streaked on LB agar plates and incubated for 24 h at 37 °C. One or two colonies were inoculated in 5 mL of LB broth and incubated at 37 °C with agitation (180 rpm) for 12 h. After incubation, the broth

culture was diluted to 1% in 20 mL of fresh LB broth and incubated at 37 °C with agitation (180 rpm) for 24 h.

To enumerate the persister cells in the planktonic population, a 10-mL sample of an overnight LB broth culture of *A. baumannii* ST1894 was collected and centrifuged at 8,000 rpm for 10 min at 4 °C, and the cells were resuspended in 10 mL of saline at 4 °C. The washing step was repeated one more time. The cell density was adjusted to an absorbance of 0.8 at 600 nm. A duplicate set of bacterial cell suspensions was prepared in the same way. Ninety-eight microliters of 2,048 µg/mL ciprofloxacin or 98 µL of saline containing 0.1% acetic acid were added to a 10-mL bacterial cell suspension incubated at 37 °C with agitation at 180 rpm for 24 h. Viable bacterial cells were enumerated using the plate count method. One hundred microliters of the experimental and control samples were inoculated onto LB agar plates containing 1% $MgCl_2.7H_2O$ to neutralize the ciprofloxacin. The plate count was performed in triplicate at 0, 1, 3, 5, 7, 9, 12, 15 and 24 h after incubation. The number of persister cells present in the planktonic population was determined using the formula shown below:

$$\text{Number of persisters/mL of planktonic cells} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume plated}}$$

To enumerate persister cells in the biofilm population, an overnight LB broth culture of *A. baumannii* ST1894 was used to grow a biofilm on a CBD, in accordance with the procedures described earlier. After incubation for 48 h, the pegs were removed from the CBD using pliers, and each peg was placed in a centrifuge tube with 1 mL of maximum recovery diluent. The biofilm cells on the pegs were detached by centrifugation at 18,000 rpm for 10 min and then resuspended in 10 mL of saline at 4

°C. The cell density was adjusted to an absorbance of 0.8 at 600 nm. Ninety-eight microliters of 2,048 µg/mL ciprofloxacin or 98 µL of saline containing 0.1% acetic acid were added to a 10-mL bacterial cell suspension incubated at 37 °C with agitation at 180 rpm for 24 h. The number of viable biofilm cells in each treated and untreated sample was counted using the steps described for planktonic cells. The number of persister cells isolated from the biofilm population was determined using the formula shown below:

$$\text{Number of persisters/mL of biofilm} = \frac{\text{Number of colonies} \times \text{dilution factor} \times \text{total sample volume}}{\text{Volume plated} \times \text{surface area of peg}}$$

3.4.7 Statistical analysis

The relationship between the biofilm formation capability and biofilm-specific genes and the association between carbapenem susceptibility and biofilm formation were evaluated using the chi-squared test. Statistical Package for Social Sciences (SPSS) v24 (IBM Corporation, USA) was used for the statistical analysis. A p value ≤ 0.05 was considered to indicate the statistical significance of the data analysed in this section of the study.

3.5 Results

3.5.1 Antibiotic susceptibility profiles of *A. baumannii* strains

The MICs of 13 antibiotics (listed in Table 3-7) from 8 major antibiotic classes were determined for the 104 *A. baumannii* isolates. The antibiotic susceptibility profiles of the isolates are summarised in Table 3-7. Our findings revealed that 79.8% (82/104) of the isolates were susceptible to colistin, but only 4.8% (5/104) were susceptible to ampicillin-sulbactam; 29.8% (31/104) to 52.9% (55/104) of the isolates were susceptible to the remaining 11 antibiotics. Of the 104 isolates, 46.2% (48/104) were non-MDR strains, 30.8% (32/104) were XDR strains and 23.1% (24/104) were PDR strains (Table 3-7 and Figure 3-1).

A. *baumannii* ST2031, ST2104, ST2028, ST2034, ST2103, ST1860, ST2032 and ST1417 were designated as PDR strains carrying 12 antibiotic resistance genes that conferred resistance to the β-lactam, aminoglycoside, macrolide and tetracycline classes of antibiotics. Aside from *A. baumannii* ST1417, the other 7 strains were new sequence types resistant to all 13 types of antibiotics tested and carrying 12 antibiotic resistance genes, as shown in Figure 3-1 and Figure 3-2.

Table 3-7. Antimicrobial susceptibility profiles of *A. baumannii* isolates (n = 104).

Antimicrobials	**Susceptible (%)**	**Intermediate (%)**	**Resistant (%)**
Colistin	79.8	-	20.2
Imipenem	48.1	-	51.9
Meropenem	48.1	-	51.9
Doripenem	39.4		60.6
Cefotaxime	29.8	13.5	56.7
Ceftazidime	39.4	-	60.6
Piperacillin-tazobactam	33.7	-	66.3
Ampicillin-sulbactam	4.8	3.8	91.3
Ciprofloxacin	48.1	-	51.9
Levofloxacin	48.1	-	51.9
Gentamycin	52.9	-	47.1
Tetracycline	48.1	-	51.9
Trimethoprim-sulfamethoxazole	50.0	-	50.0

3.5.2 Biofilm formation capabilities of *A. baumannii* isolates

Of the 104 *A. baumannii* isolates studied, 59.6% could form biofilms, of which 25% were strong biofilm producers, 14.4% were moderate biofilm producers and 20.2% were weak biofilm producers. As shown in Table 3-8, 66.1% of the biofilm-forming isolates were non-MDR strains. In addition, 83.3% of non-biofilm formers were resistant to multiple classes of antibiotics. Overall, these findings revealed that a higher number of antibiotic-sensitive *A. baumannii* isolates could form biofilms, compared with resistant isolates (p-value = 3×10^{-6}).

The biofilm formation capability of each sequence type is illustrated in Figure 3-1. Phylogenetic analysis revealed that most of the non-MDR strains that formed biofilms were genetically linked and clustered together. This was also true for the antibiotic-resistant strains that formed weak or no biofilms (Figure 3-1). We found that isolates with the same sequence type, ST2028, ST1417, ST195, ST1860, and ST2037, differed in terms of biofilm formation capabilities.

Table 3-8. Relationship between the biofilm formation capabilities and antibiotic susceptibility profiles of *A. baumannii* strains.

Biofilm formation capability	**Percentage of isolates**	**Percentage of isolates with different antibiotic susceptibility profiles**		
		Non-MDR	**XDR**	**PDR**
		46.2% (48/104)	30.8% (32/104)	23.1% (24/104)
Biofilm forming	59.6% (62/104)	66.1% (41/62)	17.7% (11/62)	16.1% (10/62)
Strong	25% (26/104)	92.3% (24/26)	7.7% (2/26)	0% (0/26)
Moderate	14.4% (15/104)	66.7% (10/15)	26.7% (4/15)	6.7% (1/15)
Weak	20.2% (21/104)	33.3% (7/21)	23.8% (5/21)	42.9% (9/21)
Non-biofilm forming	40.4% (42/104)	16.7% (7/42)	50% (21/42)	33.3% (14/42)

MDR: multidrug resistant; XDR: extensively drug resistant; PDR: pan drug resistant.

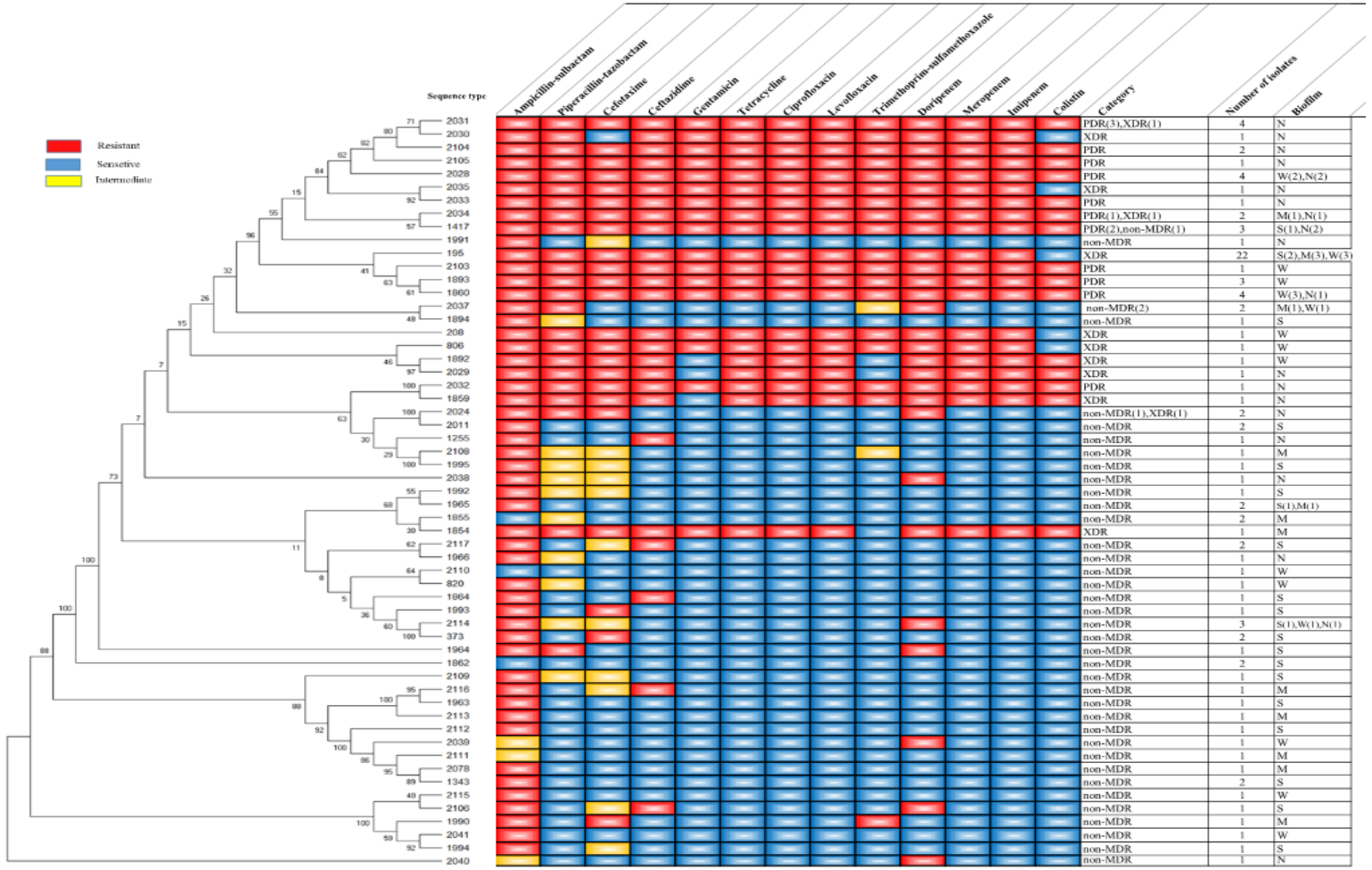


Figure 3-1. Relationship between the antibiotic resistance profiles and biofilm formation capabilities of *A. baumannii* sequence types.

An evolutionary tree was constructed using the neighbour-joining method and a concatenated FASTA aligned sequence of seven housekeeping genes. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Evolutionary distance was determined using the p-distance method and represents the number of base differences per position in the units. Evolutionary analyses were performed using MEGA X software. The antibiotic susceptibility profile of each sequence category is classified as either pan drug-resistant (PDR), extensively drug-resistant (XDR), multidrug-resistant (MDR) or non-MDR. The biofilm formation capability of each strain is classified as strong (S), moderate (M), weak (W) or non-biofilm-forming (N).

3.5.3 Distribution of biofilm-specific genes in clinical *A. baumannii* strains

The percentages of *A. baumannii* strains carrying the *bap*, *csuE*, *adeFGH*, *ompA* and *abaI* genes were 9.6%, 85.6%, 95.2%, 89.4% and 82.7%, respectively. The relationship between the presence of biofilm-specific genes and biofilm formation capabilities is presented in Table 3-9. The presence of *bap*, *csuE* and *abaI* was significantly associated with the biofilm formation capability of the *A. baumannii* strains (p = 0.005–0.033). Although 100% of the *bap*-positive isolates were biofilm formers, 55.3% of all biofilm formers were *bap*-negative. Therefore, the presence of *bap* indicates that a strain is a biofilm former, but the reverse is not true. Over 80% of the *csuE*-negative and *abaI*-negative isolates were biofilm formers, but > 50% of all biofilm formers carried both of these genes. Similar patterns were observed for the *adeFGH* and *ompA* genes, but these associations were not statistically significant (p = 0.07 and 0.193, respectively).

Table 3-9. Relationship between biofilm-specific genes and the biofilm formation capabilities of *A. baumannii* isolates.

Biofilm-specific genes	**Biofilm forming no. (%)**	**Non-biofilm forming no. (%)**	***p* Value**
bap			0.005*
Positive	10 (100)	0 (0)	
Negative	52 (55.3)	42 (44.7)	
csuE			0.02*
Positive	49 (55.1)	40 (44.9)	
Negative	13 (86.7)	2 (13.3)	
adeFGH			0.07
Positive	57 (57.6)	42 (42.4)	
Negative	5 (100.0)	0 (0)	
ompA			0.193
Positive	53 (57)	40 (43)	
Negative	9 (81.8)	2 (18.2)	
abaI			
Positive	47 (54.7)	39 (45.3)	0.033*
Negative	15 (83.3)	3 (16.7)	

* Significant *p* value.

3.5.4 Distributions of antibiotic resistance genes in biofilm- and non-biofilm-forming *A. baumannii* isolates

The distributions of antibiotic resistance genes were compared between the biofilm and non-biofilm forming isolates of *A. baumannii*, as illustrated in Table 3-10 or Figure 3-2. We found that 24% and 37.5% of the non-biofilm forming strains contained the bla_{TEM} and bla_{OXA-23} genes, respectively. The prevalence of aminoglycoside resistance-encoding genes, such as *aph (6)-Id* and *aph(3")-Ib*, was 38.5% and 37.5%, respectively, among non-biofilm forming strains of *A. baumannii*.

Furthermore, the prevalence of *tet(B*), which encodes tetracycline resistance, was 36.5% among non-biofilm forming isolates. The frequencies of the antibiotic resistance-encoding genes bla_{TEM}, bla_{OXA-23}, *aph (6)-Id*, *aph (3")-Ib* and *tet(B)* in the non-biofilm-forming strains were substantially higher than those in the biofilm-forming strains, as shown in Table 3-10. This suggests that once an isolate obtains genes that confer resistance to β-lactams, aminoglycosides and tetracyclines, it is unlikely that these strains will form biofilms.

Table 3-10. Distribution of antibiotic resistance genes in biofilm- and non-biofilm-forming *A. baumannii* isolates.

Antibiotic resistance genes	**Biofilm forming no. (%)**	**Non-biofilm forming no. (%)**	***p* value**
β-Lactam resistance			
***bla*$_{TEM}$**			0.001*
Positive	17 (16.3)	25(24.0)	
Negative	45 (43.3)	17 (16.3)	
***bla*$_{ADC-25}$**			0.397
Positive	57 (54.8)	41(39.4)	
Negative	5 (4.8)	1 (1)	
***bla*$_{OXA-51}$**			0.004*
Positive	48 (46.2)	41(39.4)	
Negative	14 (13.5)	1 (1.0)	
***bla*$_{OXA-111}$**			0.136
Positive	47 (45.2)	37 (35.6)	
Negative	15 (14.4)	5 (4.8)	
***bla*$_{OXA-66}$**			0.397
Positive	57 (54.8)	41 (39.4)	
Negative	5 (4.8)	1 (1.0)	
***bla*$_{OXA-23}$**			0.00001*
Positive	32 (30.8)	39 (37.5)	
Negative	30 (28.8)	3 (2.9)	
Aminoglycoside resistance			
aph(6)-Id			0.00004*
Positive	38 (36.5)	40 (38.5)	
Negative	24 (23.1)	2 (1.9)	
aph(3")-Ib			0.00219*
Positive	37 (35.6)	39 (37.5)	
Negative	25 (24.0)	3 (2.9)	
armA			0.000078*
Positive	45 (43.3)	9 (8.7%)	
Negative	23 (22.1)	27 (26.0)	
Macrolide–lincosamide–streptogramin B (MLSB) resistance			
***mph(E*)**			0.000001*
Positive	44 (42.3)	5 (4.8)	
Negative	24 (23.1)	31 (29.8)	
msr(E)			0.000298*
Positive	20 (19.2)	29 (27.9)	
Negative	42 (40.4)	13 (12.5)	
Tetracycline resistance			
tet(B)			0.000001*
Positive	29 (27.9)	38 (36.5)	
Negative	33 (31.7)	4 (3.9)	

* Significant *p* value.

3.5.5 Association between carbapenem resistance and biofilm formation

When we analysed the biofilm formation capabilities of the 104 *A. baumannii* strains, we found that 54.4% (37/68) of the carbapenem-resistant strains were non-biofilm formers, as shown in Table 3-11 and Figure 3-2. These were less likely to form biofilms than the carbapenem-sensitive strains ($p = 0.000054$). We also found that 86.1% (31/36) of the carbapenem sensitive strains were biofilm producers, of which 52.8% (19/31) were strong biofilm formers

Table 3-11. Association between carbapenem resistance and biofilm formation.

Biofilm formation capability	**Carbapenem-resistant no. (%)**	**Carbapenem-sensitive no. (%)**	**Total (%)**
Biofilm-forming	**31 (29.8)**	**31 (29.8)**	**62 (59.6)**
Strong	7 (6.7)	19 (18.3)	26 (25.0)
Moderate	7 (6.7)	8 (7.7)	15 (14.4)
Weak	17 (16.3)	4 (3.8)	21 (20.2)
Non-biofilm-forming	**37 (35.6)**	**21 (20.2)**	**42 (40.4)**
Total	**68 (65.4)**	**36 (34.6)**	**104 (100)**

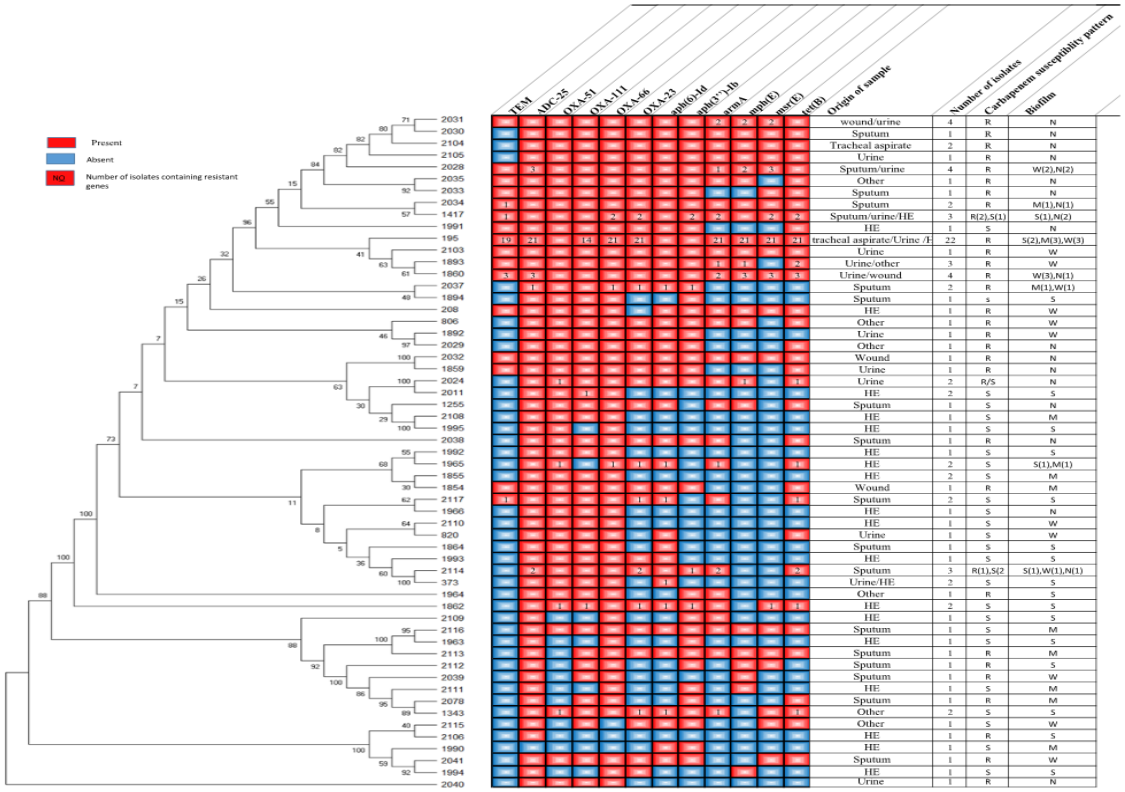


Figure 3-2. Association between antibiotic resistance genes and biofilm formation capability.

The samples were obtained from clinical specimens and hospital environments (HE); the carbapenem susceptibility pattern is shown as resistant (R) to sensitive (S); the biofilm formation capability is classified as strong (S), moderate (M), weak (W) or non-biofilm forming (N).

3.5.6 Comparison of the antibiotic resistance profiles of planktonic and biofilm cells

The MICs and MBICs of colistin, ciprofloxacin and imipenem were evaluated in nine different sequence types of *A. baumannii*. These strains were biofilm formers and susceptible to these three antibiotics in the planktonic phase. A comparison of the antibiotic resistance profiles of biofilm and planktonic cells is shown in Table 3-12. The MBICs of the biofilm cells were significantly higher than those of the planktonic cells, increasing from 2-fold to 32-fold for colistin, from 4-fold to 64-fold for ciprofloxacin and from 4-fold to 2,048-fold fold for imipenem. The MBICs were 21-fold, 31-fold and 386-fold higher than the MICs for colistin, ciprofloxacin and imipenem, respectively. The susceptibility profiles of most of the strains changed from sensitive to resistant when grown in a biofilm state. However, the biofilms formed by ST1990 and ST1417 remained sensitive to ciprofloxacin and imipenem, and the biofilm formed by ST1855 remained sensitive to colistin.

3.5.7 Determination of MBC and MBEC

Following the determination of the MICs and MBICs, we evaluated the MBCs and MBECs of the nine *A. baumannii* isolates. The MBECs of the biofilm cells increased by up to 64-fold for colistin and 1,024-fold for ciprofloxacin and imipenem in the nine *A. baumannii* isolates studied. In other words, higher concentrations of antibiotics were required to eradicate *A. baumannii* biofilm cells than to eradicate planktonic cells (Table 3-13). Although ST1990, ST1417 and ST1855 had MBICs that fell within the sensitive ranges, the MBECs were much higher than the MBICs (Table 3-12). The MBECs for colistin, ciprofloxacin and imipenem were 44-fold, 407-fold and 364-fold

higher, respectively, than the MBCs. To visualize the viability of biofilm cells after antibiotic treatment, the biofilm cells of the hyper biofilm-producing strain ST1894 were treated with antibiotics, stained using a Live/Dead BacLight Bacterial Viability Kit and examined using CLSM. Viable cells in the biofilm could be detected even after treatment with antibiotics at concentrations 32–256 times the MBC (Figure 3-3). This suggests that biofilm-producing strains of *A. baumannii* cannot be eradicated using the same concentrations of antimicrobials that are used to eradicate planktonic cells.

Table 3-12. Antibiotic susceptibility profiles of selected sequence types of *A. baumannii* planktonic and biofilm cells.

MLST	Colistin			Ciprofloxacin			Imipenem		
	MIC for planktonic cells (µg/mL)	MBIC for biofilm cells (µg/mL)	Fold change	MIC for planktonic cells (µg/mL)	MBIC for biofilm cells (µg/mL)	Fold change	MIC for planktonic cells (µg/mL)	MBIC for biofilm cells (µg/mL)	Fold change
ST1894	0.5	16	32	1	64	64	0.125	256	2048
ST1990	0.5	8	16	0.5	2	4	0.125	2	16
ST1417	0.25	8	32	0.5	2	4	0.25	4	16
ST1992	2	4	2	0.5	4	8	0.25	32	128
ST373	1	32	32	0.5	16	32	0.25	32	128
ST1862	1	4	4	1	8	8	0.015	8	533.3
ST1964	0.5	16	32	1	64	64	4	16	4
ST1855	0.5	2	4	0.5	16	32	0.015	8	533.3
ST1861*	0.5	16	32	1	64	64	0.5	32	64

* This strain is *A. baumannii* ATCC19606 and was used as the control strain in the biofilm assay. MIC: minimum inhibitory concentration; MBIC: minimum biofilm inhibitory concentration.

Table 3-13. Comparison of minimal bactericidal concentrations (MBCs) and minimal biofilm eradication concentrations (MBECs) of *A. baumannii* of different multilocus sequence types (MLSTs).

MLST	Colistin			Ciprofloxacin			Imipenem		
	MBC for Planktonic Cells (µg/mL)	**MBEC for Biofilm Cells (µg/mL)**	**Fold Change**	**MBC for Planktonic Cells (µg/mL)**	**MBEC for Biofilm Cells (µg/mL)**	**Fold Change**	**MBC for Planktonic Cells (µg/mL)**	**MBEC for Biofilm Cells (µg/mL)**	**Fold Change**
ST1894	4	256	64	8	1024	128	4	1024	256
ST1990	0.5	32	64	1	1024	1024	1	64	64
ST1417	1	32	32	2	64	32	0.5	64	128
ST1992	2	8	4	1	1024	1024	1	1024	1024
ST373	4	128	32	32	1024	32	4	32	8
ST1862	2	128	64	2	512	256	1	1024	1024
ST1964	4	256	64	2	32	16	4	512	128
ST1855	0.5	32	64	1	1024	1024	2	1024	512
ST1861 *	4	16	4	8	1024	128	1	128	128

* This strain is *A. baumannii* ATCC19606 and was used as the control strain in the biofilm assay.

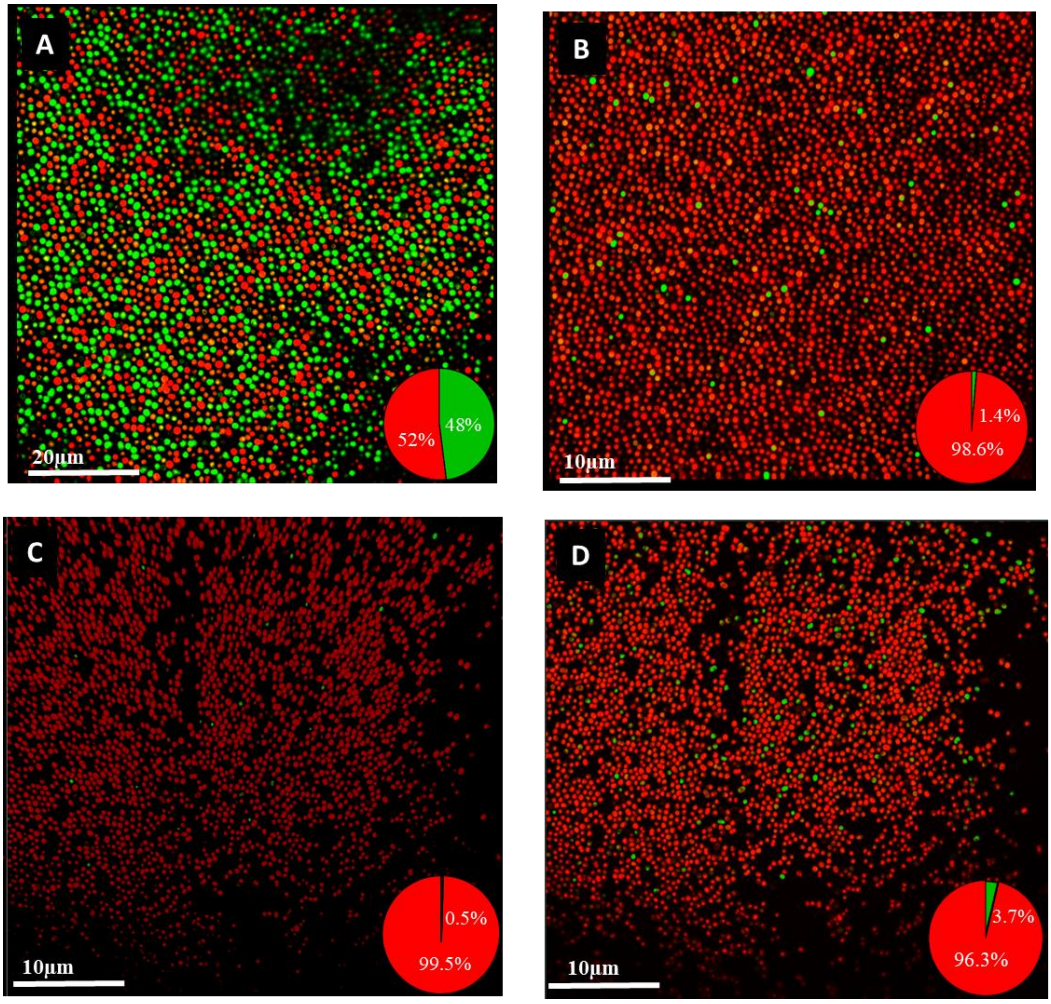


Figure 3-3. Confocal laser scanning microscopy (CLSM) images of *A. baumannii* ST1894 biofilms treated with bactericidal antibiotics.

Untreated biofilm cells (a) and biofilm cells treated with 512 µg/mL of imipenem (b), 128 µg/mL of colistin (c) or 512 µg/mL of ciprofloxacin (d). The biofilm cells were incubated with antibiotics at 37 °C for 48 h, co-stained with propidium iodide (PI) and SYTO 9 and examined under CLSM. Dead cells were stained with PI and appear red. Viable cells were stained with SYTO 9 and appear green in colour.

3.5.8 Reversibility of antibiotic susceptibility in planktonic cells regrown from biofilms

The reversibility of antibiotic resistance was analysed in two non-MDR strains (*A. baumannii* ST1894 and ST373) and one XDR strain (*A. baumannii* ST195). Biofilm cells of the three strains were regrown in the planktonic phase and then treated with colistin, imipenem and ciprofloxacin. The MICs and MBICs of the biofilm cells and MICs of the reverted planktonic cells are shown in Table 3-14. The biofilm cells of *A. baumannii* ST1894 reverted to a sensitive phenotype when the strain was regrown into planktonic cells. For *A. baumannii* ST373, reversion to the sensitive phenotype occurred with respect to colistin and imipenem but not ciprofloxacin. This suggests that the biofilm cells of *A. baumannii* ST373 might have developed a mutation associated with ciprofloxacin resistance.

Other mechanisms, such as mutations in the efflux pumps or their regulators, can also lead to ciprofloxacin resistance. *A. baumannii* ST195 was resistant to ciprofloxacin and imipenem but sensitive to colistin. The MICs of the reverted planktonic cells were the same as the MBICs for the three antibiotics, indicating that the antibiotic susceptibility of the strain did not revert to the original pattern. Additionally, the levels of resistance to ciprofloxacin and imipenem increased, and the strain did not revert to its sensitive phenotype for colistin. Together, these results imply that biofilm formation in *A. baumannii* either promotes reversible antibiotic tolerance or the emergence of irreversible antibiotic resistance.

Table 3-14. Reversibility of antibiotic resistance in biofilm cells.

MIC: minimum inhibitory concentration; MBIC: minimal biofilm inhibitory concentration; MDR: multidrug-resistant; XDR: extensively drug-resistant.

Strain	Biofilm forming	Colistin			Ciprofloxacin			Imipenem			Reason for reduced susceptibility of biofilm cells
		MIC	MBIC	MIC of reverted planktonic cells	MIC	MBIC	MIC of reverted planktonic cells	MIC	MBIC	MIC of reverted planktonic cells	
ST1894 Non-MDR	Strong	0.5	16	0.5	1	64	1	0.125	256	0.125	Tolerance
ST373 Non-MDR	Strong	1	32	1	0.5	16	16	0.25	32	0.25	Tolerance or resistant mutant for ciprofloxacin
ST195 XDR	Weak	1	16	16	4	64	64	8	32	32	Resistant mutant

3.5.9 Isolation of persister cells from among planktonic and biofilm cells

When *A. baumannii* ST1894 cells were treated with ciprofloxacin at a concentration 20 times its MIC, all of the planktonic cells were eradicated after 16 h of exposure to the antibiotic (Figure 3-4). However, 100 ± 30 CFU/peg biofilm cells survived after 24 h of exposure to ciprofloxacin, supporting our hypothesis that persister cells in the biofilm contribute to the reduced susceptibility of this strain to antibiotics. These persister cells are responsible for the regrowth of biofilm cells after antibiotic treatment.

Further examination of the biofilm using CLSM revealed persister cells amounting to 1.9% of the whole population when the biofilm was treated with ciprofloxacin at a concentration 1,024 times its MIC (Figure 3-5). Therefore, the presence of persister cells in biofilms could give rise to tolerance to different classes of antibiotics.

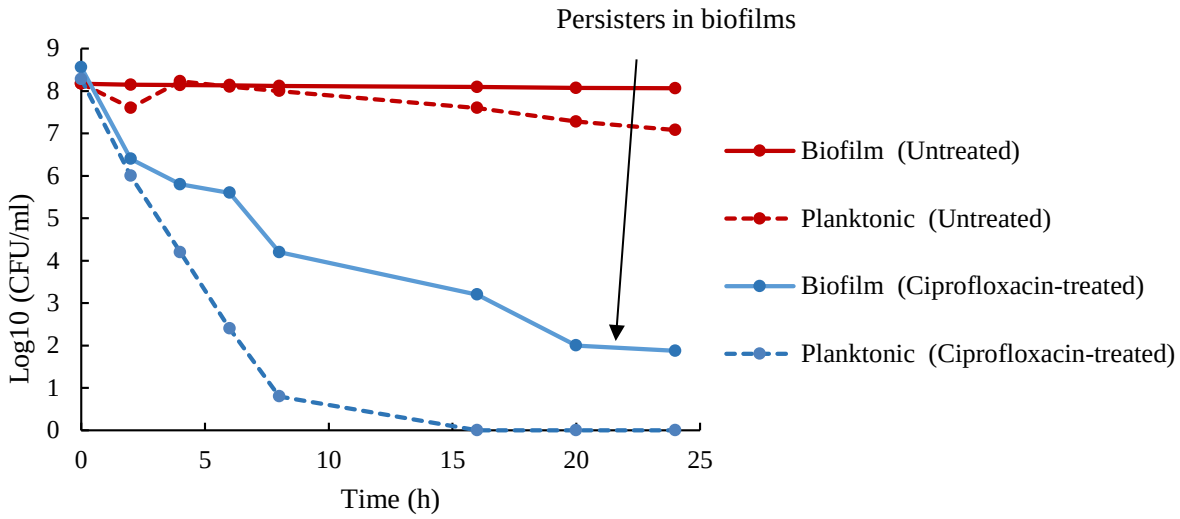


Figure 3-4. Detection of persisters from among biofilm and planktonic cells of *A. baumannii* ST1894.

The numbers of viable biofilm and planktonic cells at different time points after treatment with 2,048 μg/mL of ciprofloxacin. Red solid and dotted lines represent untreated biofilm and planktonic cells, respectively. Blue solid and dotted lines represent biofilm and planktonic cells treated with ciprofloxacin, respectively.

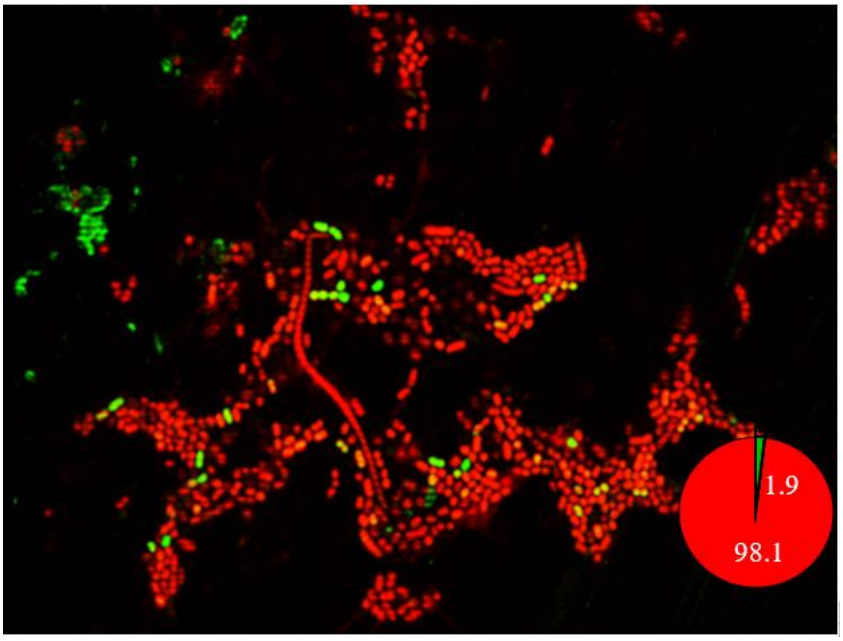


Figure 3-5. Confocal laser scanning microscopy (CLSM) image of *A. baumannii* ST1894 biofilm treated with ciprofloxacin at a concentration 1,024 times its MIC.

Viable bacterial cells in the biofilm were detected using a Live/Dead Biofilm Viability Kit. Persister cells appear green in colour, and dead cells appear red.

3.6 Discussion

The ability of *A. baumannii* to produce biofilms enhances its survival in adverse environments and increases the risk of healthcare-specific infections. The present study evaluated the biofilm formation capabilities of clinical *A. baumannii* strains and the role of biofilm production in the reduction of susceptibility to antibiotics. We found that 59.6% of the clinical *A. baumannii* isolates were able to form biofilms, similar to a report by Anghel et al. (2011) showing that approximately 63% of *A. baumannii* clinical strains were biofilm producers (Anghel et al., 2011). Another study found that more than 75% of all isolates were able to form biofilms, indicating that it is possible to control *A. baumannii* infections by developing a therapeutic agent capable of removing biofilm cells (Thummeepak et al., 2016). Therapeutic targets can thus be identified by understanding the characteristics of strains that form biofilms and the role of biofilms in establishing antibiotic resistance.

Our study showed that *A. baumannii* strains with the same sequence types, such as ST2028, ST1417, ST195, ST1860 and ST2037, can have different biofilm formation capabilities. Previous studies have documented that biofilm-forming strains are more resistant to antibiotics than non-biofilm-forming strains (Babapour et al., 2016; Yang et al., 2019). However, in this study, we found that a significantly higher proportion of biofilm-forming isolates were non-MDR strains, indicating an inverse relationship between antibiotic resistance and the biofilm formation capability of an isolate. This finding also concurs with a few other studies that demonstrated that biofilm-forming *A. baumannii* strains are more susceptible to antibiotics (Qi et al., 2016; Rodríguez-Baño et al., 2008). This might be because biofilm formation ensures the survival of susceptible strains exposed to antibiotics. However, variations in the methodologies

used to assess biofilm formation may lead to different observations. Therefore, a standardised biofilm assay is required to objectively compare different studies.

In this study, we showed that most of the carbapenem-resistant isolates were either weak biofilm or non-biofilm producers. Our results are in accordance with findings reported by other groups, which have shown that biofilm forming isolates exhibit lower carbapenem resistance rates than non-biofilm forming isolates (Rodrigues Perez, 2015; Wang et al., 2018). This is probably because the energy required to produce carbapenemase and β-lactamases might come at the expense of biofilm formation in the isolates harbouring these genes.

The antibiotic susceptibility test revealed that the *A. baumannii* strains were resistant to all 13 antibiotics tested in this study, with certain strains possessing all or most of the antibiotic resistance genes. *A. baumannii* ST2031, ST2104, ST2028, ST2034, ST2103, ST1860, ST2032 and ST1417 were identified as PDR strains bearing all 12 genes conferring resistance to β-lactams, aminoglycosides, macrolides and tetracycline. Barring ST1417, all of the other strains were new sequence types identified in this study, were resistant to all 13 antibiotics and carried all 12 antibiotic resistance genes. This indicates an incredibly worrisome level of antimicrobial resistance, implying that this pathogen is fast approaching a phase when currently used antibiotics can no longer be used to treat *A. baumannii* infections.

In addition, we observed that *A. baumannii* ST195, the predominant sequence type (22/104), was resistant to 12 of 13 antibiotics (barring colistin) and carried several antibiotic resistance genes. This finding is consistent with the observations of Lv et al. (2019), who reported the presence of multiple β-lactam-resistance genes, such as $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}66}$, $bla_{ADC\text{-}25}$ and $bla_{TEM\text{-}1D}$, in *A. baumannii* ST195 (Lv et al., 2019).

This was followed by reports of the coexistence of multiple antibiotic resistance genes against aminoglycosides, macrolides and tetracyclines in *A. baumannii* ST195 strains (Lv et al., 2019). In agreement with previous observations, we found that carbapenem-resistant *A. baumannii* strains carrying $bla_{OXA\text{-}23}$ gene also had genes that conferred resistance to aminoglycosides or tetracyclines (Qu et al., 2016).

Our findings also showed that non-biofilm forming isolates of *A. baumannii* contain a significantly higher percentage of genes associated with resistance to β-lactams (bla_{TEM}, $bla_{OXA\text{-}23}$), aminoglycosides (*aph*(3'')-Ib, *aph*(6)-Id), macrolides (*msr*(E)) and tetracyclines (*tet*(B)) than do biofilm-forming isolates. In the biofilm state, these strains are more resistant to antibiotics and promote gene transfers that contribute to the spread and emergence of more antibiotic-resistant strains. It appears that biofilms promote the transfer of antibiotic resistance genes and, once a strain acquires these genes, it may no longer need to form biofilms for additional protection against adverse environmental conditions such as exposure to antibiotics.

We also evaluated the relationship between biofilm-specific virulence genes and the biofilm formation capabilities of the *A. baumannii* strains. *Bap* was detected in a small proportion of strains tested, and all *bap*-positive strains were biofilm formers, indicating the role of *bap* in biofilm formation. BAP protein is necessary for mature biofilm formation on various abiotic surfaces, and disruption of the *bap* gene reduces the biofilm mass (Brossard & Campagnari, 2012). *csuE*, *adeFGH*, *ompA* and *abaI* were detected in 82.7–95.2% of the *A. baumannii* strains, of which almost 50% were non-biofilm formers. Therefore, it is necessary to compare the expression levels of biofilm-specific genes between biofilm producers and non-biofilm producers.

To examine the contribution of biofilms to the emergence of antibiotic resistance, the MIC, MBIC, MBC and MBEC of colistin, ciprofloxacin and imipenem were evaluated in nine selected *A. baumannii* strains that were non-MDR and strong biofilm formers. We found that biofilm cells of all nine strains developed a high level of antibiotic resistance and required antibiotics at concentrations as high as 2,048 times the MIC to inhibit the release of planktonic bacterial cells from the biofilms or at concentrations as high as 1,024 times the MBC to eradicate the biofilm cells. Of the three antibiotics tested, high average fold-increases were observed in the MBIC (386-fold) and MBEC (364-fold) of imipenem. As this antibiotic is uncharged and can penetrate the negatively charged biofilm matrix, the high level of resistance was likely not due to low penetration. Resistance might have resulted from the expression of β-lactamase, as reported by a previous study showing that the activity of the β-lactamase promoter was elevated in biofilm cells of *Pseudomonas aeruginosa* (Bagge et al., 2004). Other mechanisms for the emergence of imipenem resistance in the biofilm may include the increased expression of genes involved in drug efflux (*adeFGH*), which is responsible for pumping antibiotics out of the cells (He et al., 2015).

For ciprofloxacin, a much higher fold-increase was observed in MBEC than in MBIC (407-fold vs. 31-fold, respectively). As this antibiotic acts against metabolically active bacterial cells (Pamp et al., 2008), it would be less able to eradicate persister cells, which are metabolically dormant. The lowest average fold-increases in MBIC (21-fold) and MBEC (44-fold) were observed for colistin, which was shown to be effective against the metabolically inactive bacterial population in biofilms (Pamp et al., 2008). Furthermore, the bactericidal activity of colistin was shown to increase under anaerobic conditions, as the bactericidal action of the antibiotic was independent of hydroxyl radicals formed during aerobic respiration (Pamp et al., 2008). Overall, *A. baumannii*

was shown to develop antibiotic resistance during biofilm formation, with the level of biofilm-specific resistance varying according to the induced responses of the biofilm population and mechanisms of action of the antibiotics.

CLSM images of the non-MDR *A. baumannii* ST1984 strain showed that 0.5–3.7% of the biofilm cells were viable after treatment with colistin, ciprofloxacin and imipenem at concentrations 256–4,096 times the respective MICs. It is possible that the percentages of viable cells were higher because extracellular DNA in the biofilm might have been stained by PI, leading to an overestimation of the proportions of dead cells. The presence of persister cells is worrisome, as colistin is the last-resort treatment option for MDR *A. baumannii* infections. The emergence of biofilm-specific resistance would make infections caused by this pathogen untreatable with conventional antibiotics.

Although Qi et al. (2016) reported that antibiotic resistance is enhanced by biofilm formation, our study investigated the reversibility of antibiotic resistance developed in biofilms. We regrew biofilm cells in a planktonic state and assessed changes in the MICs of three *A. baumannii* strains. Our findings demonstrated that the reversion from resistant to susceptible forms occurred in two non-MDR strains but not in the XDR strain. The observed transient increase in antibiotic resistance in the non-MDR strains was due to the development of antibiotic tolerance by the biofilm cells. Multiple mechanisms are involved in the development of antibiotic tolerance in biofilms. The reduced metabolic activity of biofilms and induction of stress responses due to nutrient limitation in biofilm environments trigger antibiotic tolerance (Bernier et al., 2013).

The presence of persister cells in the biofilm activates toxin/antitoxin systems, which inhibit protein translation, initiate dormancy and increase tolerance to antibiotics

(Bernier et al., 2013). In this study, we demonstrated the presence of persister cells in *A. baumannii* ST1894, one of the two non-MDR strains, using CLSM imaging and persister cell isolation after treatment with a high concentration of ciprofloxacin. The presence of persister cells made the pathogen extremely multidrug tolerant and could eventually lead to the emergence of antibiotic resistance. Regarding the XDR strain (*A. baumannii* ST195), the planktonic cells regrown from biofilm cells exhibited a higher level of antibiotic resistance, possibly due to mutations of drug targets upon exposure to high antibiotic concentrations. There are two noteworthy issues with the XDR strain. First, irreversible colistin resistance developed during biofilm formation, suggesting that physicians would be left empty-handed when treating infected patients. Second, although the strain was a weak biofilm former, a high level of irreversible antibiotic resistance developed during biofilm formation. A detailed investigation at the genetic level is required to understand the regulatory mechanisms involved in the emergence of biofilm-mediated antibiotic resistance.

In this study, we showed a negative relationship between antibiotic susceptibility and biofilm formation capability. The non-MDR strains were the strong biofilm producers. We also demonstrated the presence of persisters among the biofilm cells, which accounted for the reduced antibiotic susceptibilities of the *A. baumannii* strains. We further found that biofilm growth induced reversible antibiotic tolerance in the non-MDR strains, but induced a higher level of irreversible resistance in the XDR strain, even converting colistin-sensitive cells to colistin-resistant cells. To address the regulatory mechanisms underlying biofilm-specific resistance, systematic research should be undertaken to investigate alterations of *A. baumannii* grown in the biofilm state at the genetic and transcriptional levels. A detailed understanding of these factors will facilitate the development of new therapeutics targeting biofilm-specific resistance.

4 CHAPTER FOUR: COMPARATIVE TRANSCRIPTOMIC PROFILES OF UNTREATED AND ANTIBIOTIC-TREATED BIOFILM CELLS

4.1 Abstract

A. baumannii forms biofilms that can cause both persistent and chronic infections. The hyper biofilm-producing strain *A. baumannii* ST1894 has demonstrated the highest-recorded reduction in susceptibility to antibiotics when grown in the biofilm state. The RNA sequences of untreated biofilm cells and biofilm cells treated with sub-inhibitory concentrations of antibiotics were obtained to understand the molecular mechanisms responsible for the reduced susceptibility of biofilms to antibiotics. The DEGs associated with biofilm formation and antibiotic resistance were identified and verified using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Using total RNA-seq, we observed that 51.8% (1592/3075) of the total transcribed genes were differentially expressed between mature biofilm cells and planktonic cells of *A. baumannii* ST1894. One hundred and six and 368 biofilm-specific genes were differentially expressed in biofilm cells treated with sub-inhibitory concentrations of imipenem and colistin, respectively. Seven and 11 differentially expressed novel genes were identified in biofilm cells treated with sub-inhibitory concentrations of imipenem and colistin, respectively. Similarly, 33 and 74 differentially expressed uncharacterized genes were identified in biofilm cells treated with imipenem and colistin, respectively. The DEGs induced by imipenem and colistin in biofilm cells consisted of genes encoding OMPs, RND multidrug efflux pumps, fimbrial proteins, AHL synthases and matrix synthesis proteins. The expression levels of genes encoding acinetobactin biosynthesis proteins, DNA replication and D- and L-methionine transporters were reduced when the biofilm cells were treated with imipenem and colistin. These

antibiotic-induced or repressed biofilm-specific genes and their regulatory pathways might be responsible for the decreased susceptibility of biofilms to antibiotics.

4.2 Introduction

A. baumannii is capable of forming biofilms, which play an essential role in the pathobiology of diseases (Costerton et al., 1999; Penesyan et al., 2019). A biofilm comprises a group of microorganisms embedded in an EPS matrix, which holds the biofilm cells together and binds them to substrates as they colonise surfaces (Costerton et al., 1999; Flemming et al., 2016). During the transition from planktonic cells to biofilm cells, the bacteria undergo structural and molecular changes in response to various environmental signals (Rumbo-Feal et al., 2013).

Studies have identified changes in the levels of gene expression related to various processes, such as biomolecule synthesis, metabolic activity and transcriptional regulation, during biofilm formation in *A. baumannii* (Rajamohan et al., 2009; Rumbo-Feal et al., 2013). For instance, the *A. baumannii* membrane protein OmpA has been identified as a virulence factor that promotes biofilm formation on abiotic surfaces and adherence to and invasion of epithelial cells (Gaddy et al., 2009; McConnell et al., 2013). These variations in gene expression influence the physiological features of biofilms, distinguishing biofilm cells from their planktonic counterparts (Mah, 2012). Such physiological differences in biofilm cells might play a pivotal role in the development of antibiotic resistance and environmental resilience (Anghel et al., 2011; Espinal, Martí, et al., 2012; Greene et al., 2016; Penesyan et al., 2019).

Recently, several environmental reservoirs in hospital settings were reported to be the primary sources of MDR *A. baumannii* outbreaks (Cheng et al., 2018). Biofilm

formation protects *A. baumannii* from antibacterial agents and allows the pathogen to survive on abiotic and biotic surfaces. The biofilm cells withstand the effects of antibiotics by limiting the penetration of antimicrobial agents, activating the stress response and forming slow-growing and persister cells (Høiby et al., 2010; Mah, 2012). When these biofilm cells are continuously exposed to antibiotics, antibiotic tolerance eventually evolves into antibiotic resistance (Reisman et al., 2017).

Studies have also shown that biofilm development is influenced by exposure to sub-lethal concentrations of antibiotics, which can affect various cellular processes. Sub-inhibitory concentrations of antibiotics trigger biofilm formation and the expression of biofilm-specific antibiotic resistance genes (Høiby et al., 2010; Kaplan, 2011). These genes can be either activated or repressed when low concentrations of antibiotics are used to treat cells in the biofilm state. The application of sub-inhibitory concentrations of colistin and carbapenem to biofilm cells might trigger transcriptional and post-transcriptional changes different from the changes that occur in planktonic cells. Understanding the transcriptional changes that occur upon exposure to antibiotics paves the way for the identification of genetic determinants responsible for the development of biofilm-specific antibiotic resistance in *A. baumannii*.

In our study on biofilm-induced antibiotic resistance in clinical *A. baumannii* strains, we found that strong and moderate biofilm-formers were susceptible to antibiotics in the planktonic state, but highly resistant in the biofilm state (Shenkutie et al., 2020). We also found that antibiotic concentrations up to 2,048 times those required to eradicate planktonic cells were needed to eradicate biofilm cells. This decreased antibiotic susceptibility creates a significant problem in the control of *A. baumannii* infections in clinical settings. Despite this, little attention has been paid to antibiotic

resistance mechanisms in *A. baumannii* biofilm cells compared to planktonic cells. Therefore, we aimed to understand the genetic determinants responsible for biofilm development and survival when cells are treated with sub-inhibitory concentrations of antibiotics. We conducted a comparative transcriptome study on biofilm and planktonic cells isolated from the hyper biofilm-forming, non-MDR strain *A. baumannii* ST1894. We also examined the transcriptional changes that occurred when biofilm cells were treated with sub-inhibitory concentrations of imipenem and colistin. Thus, we identified the antibiotic-induced or repressed biofilm-specific genes and their regulatory pathways to understand the development of reduced susceptibility of biofilms to antibiotics.

4.3 Materials and methods

4.3.1 Bacterial strains and growth conditions

Of the 104 A. *baumannii* clinical strains, we selected ST1894 for the comparative transcriptomic analysis of untreated and antibiotic-treated biofilms. This strain was isolated from the sputum of a patient suffering from a lower respiratory tract infection. *A. baumannii* ST1894 was susceptible to all antibiotics tested except ampicillin-sulbactam when grown in the planktonic phase. However, this strain was identified as a hyper biofilm former and displayed the highest-recorded degree of multidrug-tolerance to bactericidal antibiotics such as colistin and imipenem when grown in the biofilm phase. The isolates were grown at 37 ºC with shaking (180 rpm) and stored at -80 °C in LB broth containing 20% glycerol.

4.3.2 Sample preparation for RNA extraction

A single colony was picked from a pure culture grown on LB agar plates and then inoculated into 20 mL of LB broth to obtain a planktonic culture, as shown in Figure 4-1b. Similarly, five colonies were selected and inoculated into a larger Petri dish (150 mm diameter) containing 100 mL of LB broth, as illustrated in Figure 4-1a. The planktonic and biofilm cultures were incubated at 37 °C for 48 h. We added 62.5 mg/mL of imipenem (half of the MIC) and 250 mg/mL of colistin (half the MIC) to the biofilm cultures at 47 h and incubated them at 37 °C for another hour. After 48 h of cultivation, all of the single cells were carefully washed with maximum recovery diluent (1 g peptone and 9 g of NaCl per litre of distilled water) without disturbing the biofilm.

Subsequently, the biofilm cells attached to the Petri dish surface were removed using a cell scraper and then resuspended in maximum recovery diluent. The cells were placed on ice to prevent RNA degradation and then washed thrice with 1 mL of cold phosphate buffered saline (PBS). After washing, the biofilm and planktonic cultures were centrifuged for 15 minutes at 3,500 × *g* and 4 °C. The pellets obtained from the biofilm cells, antibiotic-treated biofilm cells and planktonic cells were stored at -80 °C until they were processed for RNA extraction.

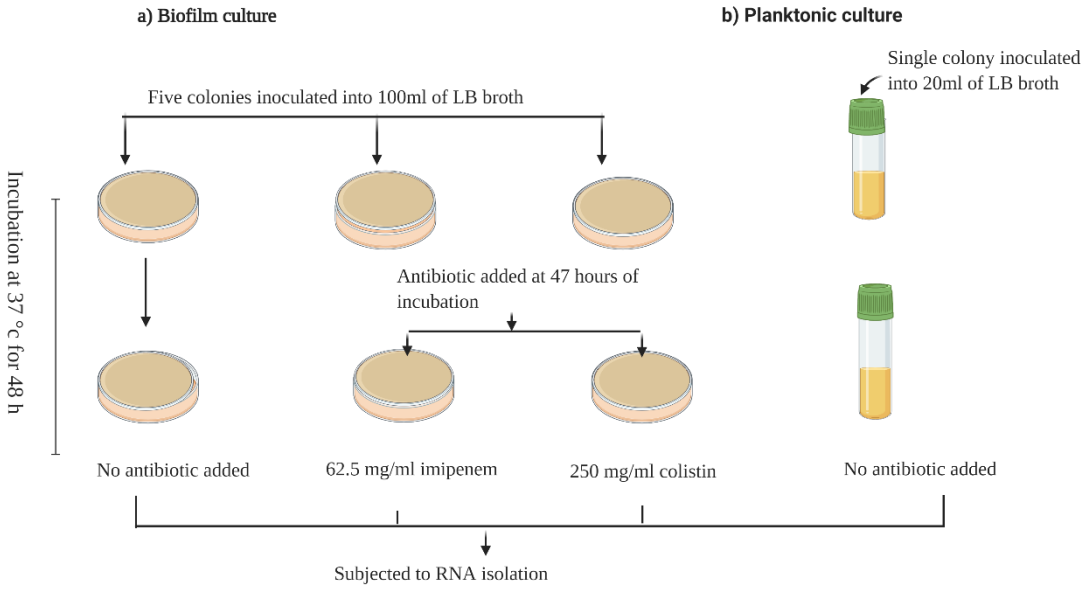


Figure 4-1. Preparation of samples for RNA extraction.

4.3.3 RNA isolation

Cell lysates obtained from the planktonic, biofilm, imipenem-treated (half of the MIC) and colistin-treated biofilm cells (half of the MIC) were processed for total RNA isolation as described in the PureLink™ RNA Mini Kit (Ambion, Life Science Technologies, Carlsbad, CA, USA). Briefly, 100 µL of lysozyme solution (10 mg/mL, Sigma Life Science, St Louis, MO, USA) were added to the prepared cell pellet and resuspended by vortexing the mixture. One microliter of 10% sodium dodecyl sulphate (SDS) was added to the solution, which was mixed and incubated at room temperature for 5 min. An aliquot of lysis buffer containing 10 µL of 2-mercaptoethanol in 1 mL of lysis buffer was prepared and mixed well.

The lysate was transferred to an RNase-free microcentrifuge tube and homogenised by passing through a 21-G needle connected to a 1-mL RNase-free syringe. The samples were centrifuged at 12,000 × g for 5 min at room temperature, and the supernatant was

transferred to a sterile RNase-free microcentrifuge tube for binding, washing and elution. We added 250 μL of absolute ethanol to each cell volume homogenate and then vortexed the mixture. After centrifugation at 12,000 × *g* for 30 s, the flow-through was discarded and the eluent was washed using wash buffers I and II. Finally, the RNA sample was eluted in 40 μL of RNase-free water. The isolated RNA was treated with DNase to eliminate contaminating genomic DNA using TURBO DNase (Life Science Technologies).

4.3.4 RNA quality assessment

The DNase-treated RNA was evaluated for concentration, purity and integrity to ensure its quality and quantity for use in transcriptomics and to achieve the specifications outlined by Wieczorek et al. (2012). The concentrations of the purified RNA samples were determined using the Qubit RNA Test Kit and the Qubit 2.0 Fluorimeter (Life Technology, CA, USA). The required concentration of each RNA sample for subsequent RNA-seq was > 50 ng/μL. The purity of the RNA samples was assessed using a NanoPhotometer spectrophotometer (IMPLEN, USA) by measuring the $OD_{260/280}$ and $OD_{260/230}$ ratios. If the OD ratios were close to 2, then the RNA samples were considered to be pure. The quality assessment results of the RNA samples used in this study are shown in Table 4-1.

RNA integrity was evaluated using an RNA 6000 Nano Assay Kit and an Agilent Bioanalyzer 2100 system (Life Technologies, CA, USA). The RNA integrity values (RINs) of the samples used are illustrated in Figure 4-2. The RINs for all RNA samples were ≥ 7 on a scale from 1 (highly degraded) to 10 (highest integrity) (Jahn et al., 2008). Finally, a 1% agarose gel was used to monitor RNA degradation and detect any contamination with genomic DNA. Once the RNA samples fulfilled all of the quality

evaluation requirements, 3 µg of RNA were prepared and submitted for total RNA-seq (Groken Bioscience, China).

Table 4-1. RNA quality assessment report for samples used in the transcriptome study.

Sample Name	**Qubit™ Fluorimeter**	**NanoDrop**		**RNA Integrity Value**
	RNA Conc. (ng/µL)	$OD_{260/280}$	$OD_{260/230}$	
a) Planktonic	712.7	2.14	1.77	9.2
b) Biofilm	388	1.87	1.32	7.9
c) Imipene-treated biofilm	627	1.92	1.78	7.3
d) Colistin-treated biofilm	106	1.71	1.77	7.10

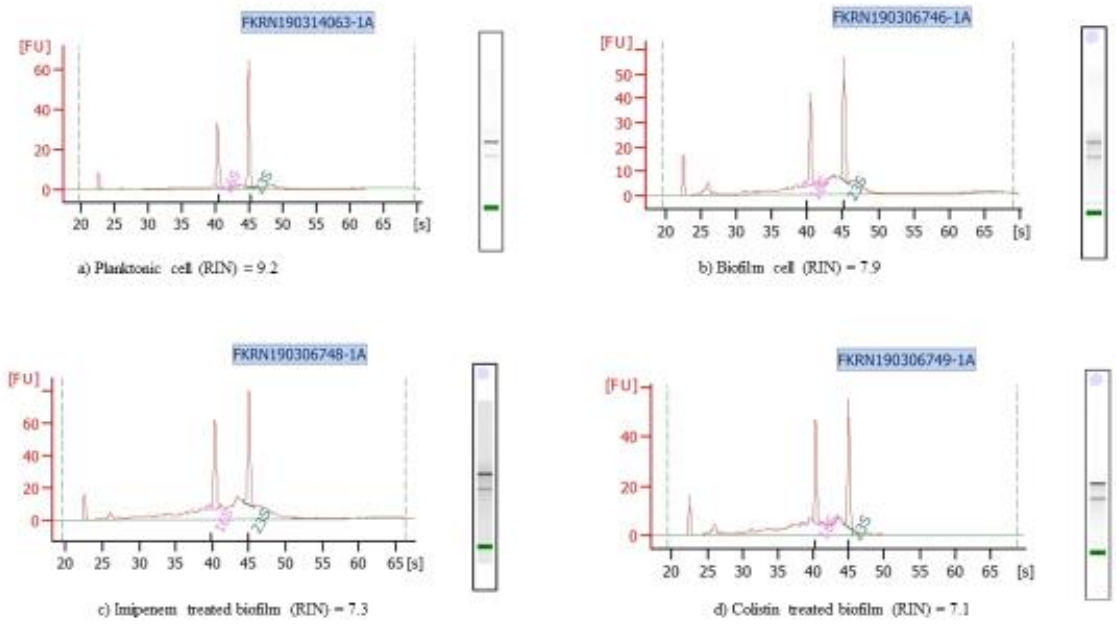


Figure 4-2. The RNA integrity values (RINs) of the samples used in the transcriptome study.

4.3.5 Library construction and sequencing

Library preparation and RNA-seq were performed by the NGS service provider Groken Bioscience Ltd. (China). The workflow is summarised in Figure 4-3; 3 μg of RNA were used as input material to prepare the sequencing libraries, in accordance with the protocol outlined by the NEBNext UltraTM Directional RNA Library Prep Kit for Illumina (NEB). 16S rRNA and 23S rRNA were eliminated from the total RNA using a Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA). In the NEBNext kit, RNA fragmentation was done by treating the samples with divalent cations at elevated temperatures. Barcoded adaptors with a 5´ phosphate and 3´, blocking group were ligated to the fragmented RNA. For first-strand cDNA synthesis, random hexamer primers were linked to the barcoded adapters and a second adapter was attached to the first-strand cDNA synthesised. M-MuLV reverse transcriptase (RNase H) was also used in this process.

Second-strand cDNA was synthesised using DNA polymerase I and RNase H in the presence of dATP, dGTP, dCTP and dTTP. The exonuclease and polymerase were used to blunt and adenylate the 3´ ends of the DNA fragments, respectively. To prepare for hybridisation, NEBNext adaptors with hairpin loop structures were ligated to the cDNA fragments, which were then purified using the AMPure XP method (Beckman Coulter, Beverly, USA) to preferentially select cDNA fragments that were 150–200 bp long.

Further, 3 μL of the USER® enzyme (Uracil-Specific Excision Reagent; NEB, UK) were added to the size-selected, adaptor-ligated cDNA fragments at 37 °C for 15 min, followed by 5 min at 95 °C before performing the PCR. The PCR was carried out using Phusion high-fidelity DNA polymerase (NEB, Uk), universal PCR primers and index (X) primers. After performing PCR, the products were purified using an Ampure XP

(Beckman Coulter) system, and the quality of the library was evaluated using an Agilent Bioanalyzer 2100 system (Life Technology, CA, USA). Clustering of the index-coded samples was conducted on the cBot Cluster Generation System using a HiSeq PE Cluster Kit cBot-HS (Illumina, San Diego, CA, USA), in accordance with the manufacturer's specifications. The prepared library was sequenced using the Illumina HiSeq2500 platform (Illumina) after cluster generation, and 150-bp paired-end reads were generated.

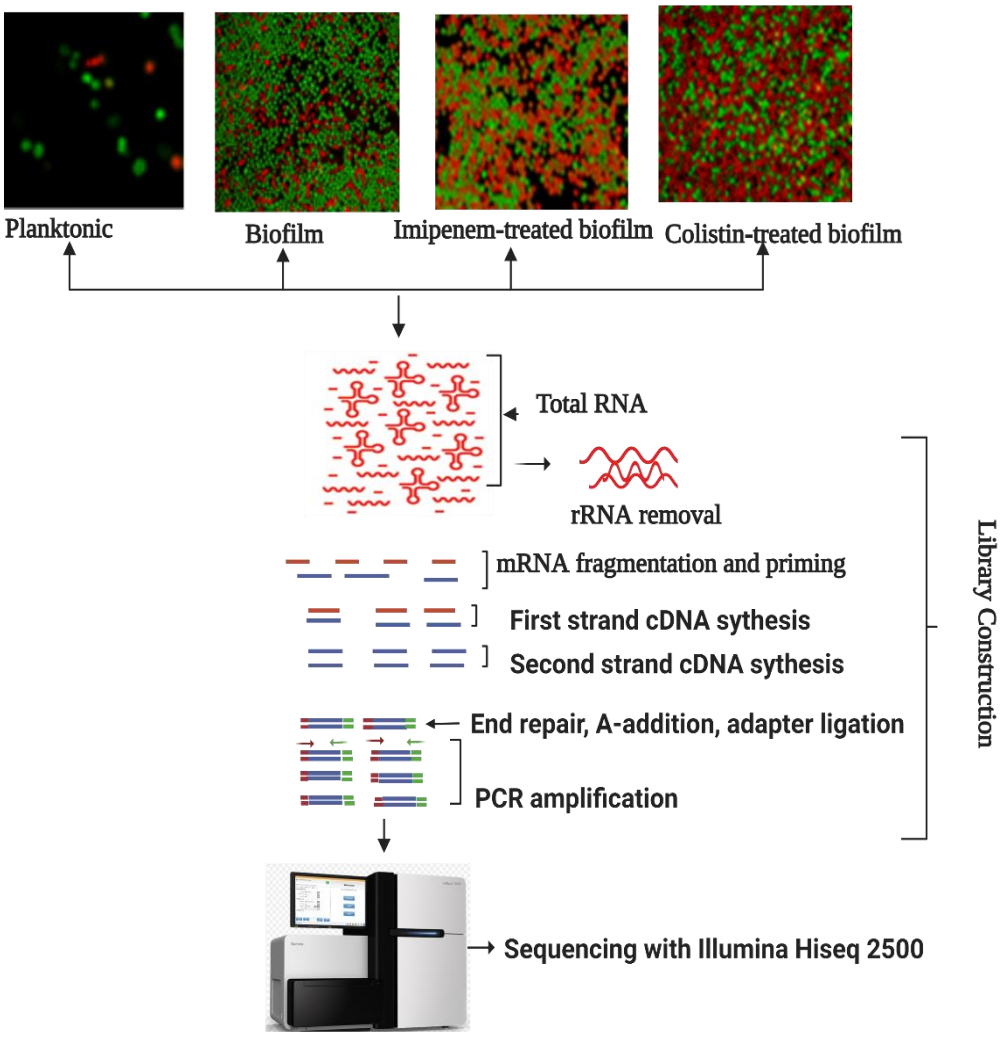


Figure 4-3. Library preparation workflow for RNA sequencing.

4.3.6 Read processing and quantification of gene expression

Sequencing data analysis was performed by following the standard pipeline used to analyse prokaryotic transcriptomes, as summarised in Figure 4-4. The raw reads in FASTQ format were first processed into perl scripts in-house. In this step, clean reads were obtained by filtering and removing reads containing the adapter (first 6 bases), discarding reads with > 10% unknown nucleotides (N > 10%) and discarding reads with > 50% low-quality nucleotides and a Qpred of 5. The low-quality sequences were trimmed using Trimmomatic v0.3032 ((Cambridge, England) with the default parameters. Simultaneously, the Q20, Q30 and GC content of the clean data were calculated. All of the downstream analyses were based on clean, high-quality sequencing data.

The reference genome of *A. baumannii* ATCC17978 with refSeq assembly accession GCF_004797155.2 (latest) was retrieved from the National Center for Biotechnology Information (NCBI) website. The reads were mapped to the reference genome using Bowtie 2 v2.2.3 with default parameters (Langmead & Salzberg, 2012). HTSeq v0.6.1(USA) was used to count the number of reads mapped to each gene, and the fragments per kilobase of transcript sequence per million base pairs (FPKM) was calculated to quantify the level of gene expression. Simultaneously, the log2 (FPKM biofilm/FPKM planktonic), which accounts for the effects of sequencing depth and gene length on the read count, was determined (Trapnell et al., 2010). The results show the number of genes with different expression levels and the expression levels of single genes. An FPKM value of 0.1 or 1 was set as the criterion for determining the expression levels of the target groups. The raw sequences were deposited in the NCBI

Sequence Read Archive (SRA) under submission ID (SUB9038432) and bio sample accession number (SAMN17808362).

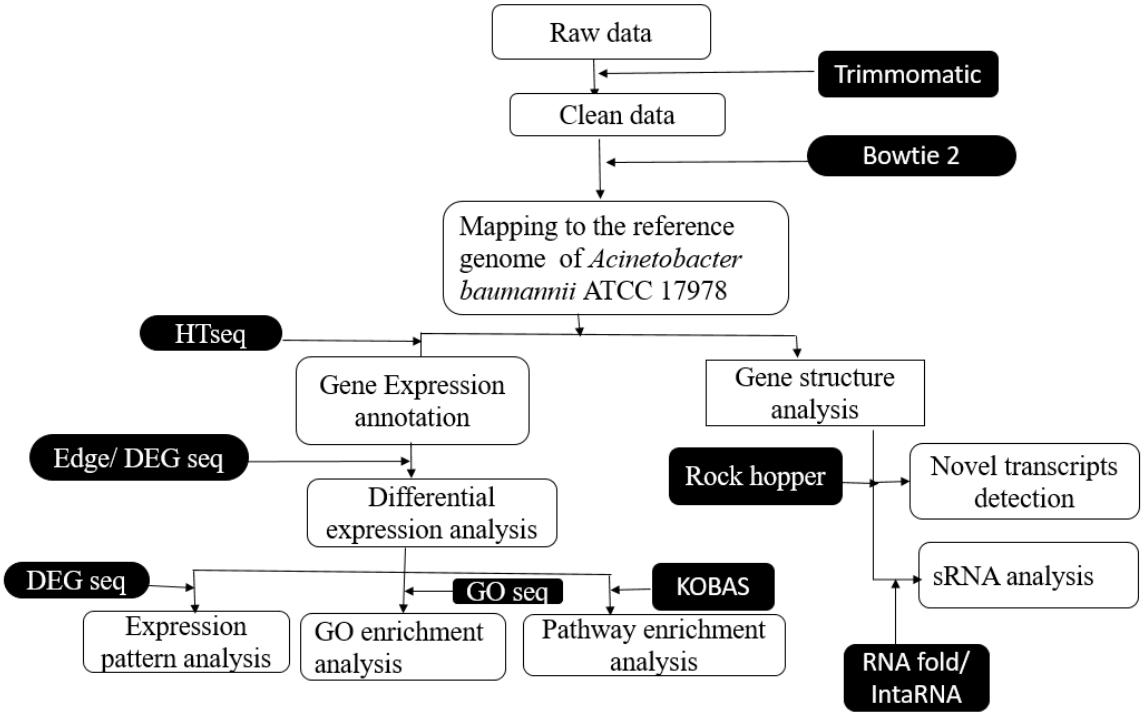


Figure 4-4. Standard sequencing data analysis workflow (Groken Bioscience, China). DEG: differentially expressed gene; sRNA: small RNA.

The sequencing reads were annotated using the R Bioconductor package (Langmead et al., 2009). Deviations in the RNA-seq analysis can arise due to variations in gene length and library sample size. Normalisation of gene expression was performed using the edge program package and a single scaling factor. The DEGseq R package (1.18.0) was used to estimate the DEGs between biofilm and planktonic cells, untreated biofilms and imipenem-treated biofilm cells, and untreated biofilms and colistin-treated biofilm cells. The fold change and level of significance, which indicate differential expression, were evaluated using a model based on the binomial distribution (which could be approximated by a Poisson distribution) (Liu et al., 2010). Genes with an expression

level of $\log_2$ fold change > 1 and a corrected p (q) ≤ 0.005 were considered to be differentially expressed. Genes were also considered to be differentially expressed if they fulfilled the following criteria: 1) the biofilm cells had a normalised gene expression value > 2-fold that of the planktonic cells; and 2) the antibiotic-treated biofilm cells had a normalised expression value > 2-fold that of untreated biofilm cells. The DEGs were then used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathway analyses.

4.3.7 Novel gene and gene structure analysis

The RNA-seq analysis helped us to identify novel transcripts and analyse transcript structures. The RNA-seq reads were mapped to the reference genome of *A. baumannii* ATCC17978 using Rockhopper, and the novel reads were compared to known gene structures (McClure et al., 2013). The novel transcripts were aligned to sequences in the NCBI non-redundant (NR) protein sequences database using BLASTx (cut-off: e value < $1e^{-5}$). Novel transcripts with NR protein sequence annotations were considered to be novel potential protein-coding transcripts. The transcription start sites (TSSs) and termination sites of operons were predicted based on the positions of the reads in the reference genome using Rockhopper. A 700-bp sequence in the upstream TSS was extracted and used to identify the promoter according to the time-delay neural network (TDNN) method. The GO and KEGG enrichment analyses of the DEGs were performed using standard software listed in Figure 4-4.

GO enrichment analysis helps us to understand the molecular functions, biological processes and cellular components of a given gene (Young et al., 2010). The GO seq R program was used to perform the GO enrichment study of the DEGs; GO terms with an adjusted p < 0.05 were considered to be enriched.

KEGG enrichment analysis, which compares DEGs with the entire genomic background, was conducted to identify significantly enriched metabolic pathways or signal transduction pathways across antibiotic-treated biofilms, untreated biofilms and planktonic cells. KOBAS (Dalian, China) was used to test the statistical enrichment of DEGs in KEGG pathways. KEGG pathways with an adjusted $p < 0.05$ were considered to be significantly enriched in DEGs.

4.3.8 Validation of DEGs

RT-qPCR was used to validate the differential expression profile of genes obtained from the RNA-seq data. Sixteen genes were selected for validation of differential expression. These genes were associated with biological functions such as matrix formation, QS, β-lactam resistance, cationic antimicrobial peptide resistance, bacterial secretion system, metabolism and the two-component system, as demonstrated in Table 4-2. The same RNA samples (technical replicates) used for transcriptomic analysis were used to synthesise cDNA and perform the RT-qPCR. In addition, new RNA samples obtained from independent experiments and isolated under the same biological conditions were used as biological replicates.

The sequences of the 16 selected genes were retrieved from the *A. baumannii* ST1894 genome and used as references for the design of primers and probes for qPCR. The primers and probes illustrated in Table 4-3 were designed using Primer3 plus (Boston, USA). A two-step protocol was used to perform RT-qPCR. We reverse transcribed 500 ng of pure RNA samples using the LunaScript RT Supermix Kit (NEB,Uk). cDNA was synthesised in a 20-µL reaction volume by mixing 4 µL of 5X LunaScript RT Supermix, 2 µL of RNA and 14 µL of nuclease-free water, followed by incubation in a thermocycler at 25 °C for 2 min, 55 °C for 10 min and 95 °C for 1 min. Control reactions

without reverse transcriptase (RT) were prepared with the same amount of cDNA synthesis reagents and subjected to the same incubation conditions; the LunaScript RT Supermix was replaced with a 5× no-RT control mix. The prepared cDNA and no-RT control reactions were diluted 1:100 in nuclease-free water to be used for RT-qPCR reactions. The SYBR Green RT-qPCR reaction was prepared as a 20-µL mixture by adding 10 µL of Luna Universal qPCR Mastermix (NEB, UK), 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 2 µL of 1:00 diluted cDNA and 7 µL of nuclease-free water. The no-RT control was prepared simultaneously. Three replicates of all of the target genes in all conditions were used for both technical and biological validation.

Table 4-2. Lists of differentially expressed genes (DEGs) and small RNA (sRNA) selected for RT-qPCR validation.

mRNA_ID	KEGG_ID	KEGG Annotation	Strand	Start	End	Length (bp)	Pathway
E5A72_RS12100	acb: A1S_0938	*PgaB*	+	2520896	2522890	1995	Biofilm matrix
E5A72_RS05130	acb: A1S_3177	Fimbrial protein	-	1078917	1079381	2538	Two-component system
E5A72_RS06870	acb: A1S_0109	Homoserine lactone synthase	+	1427610	1428176	567	Quorum sensing
E5A72_RS00640	acb: A1S_2306	RND efflux transporter	+	116590	118041	555	Multidrug efflux system
Novel00738	acb: A1S_2736	RND family drug transporter	+	617454	623989	6536	β-lactam resistance
Novel00626	acb: A1S_1965	UDP-N acetylglucosamine acyltransferase	-	3676375	3677683	1309	Cationic antimicrobial peptide resistance
Novel00171	acb: A1S_2626	DNA gyrase	-	483250	484821	1572	DNA replication
E5A72_RS17735	acb: A1S_1974	Ribosome releasing factor	-	3685962	3686516	555	Translation
Novel00584	acb: A1S_1617	30S ribosomal protein S20	-	3284582	3284881	300	

KEGG: Kyoto Encyclopedia of Genes and Genomes; RND: resistance–nodulation–cell division.

Table 4-2 continued

mRNA_ID	KEGG_ID	KEGG Annotation	Strand	Start	End	Length (bp)	Pathway
Novel00490	-	Transcription termination factor Rho OS	-	2214580	2215974	1395	Transcription
E5A72_RS14510	acb: A1S_1359	ABC-type Fe3+ transport system	+	3025424	3026461	1038	ABC transporters
E5A72_RS14240	acb: A1S_1310	K11892 type VI secretion system protein ImpK	+	2971603	2972409	807	Bacterial secretion system
E5A72_RS03500	acb: A1S_2862	Preprotein translocase subunit SecA	-	756066	758789	2724	
E5A72_RS09610	acb: A1S_0399	LysR family transcriptional regulator	+	1993774	1994670	897	Two-component system
E5A72_RS13515	acb: A1S_1182	CRP transcriptional regulator	-	2818333	2819040	708	
sRNA00203	-	--	-	1245743	1245795	53	Non-coding RNA

ABC: ATP-binding cassette: CRP: cyclic-AMP receptor protein.

The reactions were run on a ViiA 7 Real-Time PCR system (Applied Biosystems, USA) using the following parameters: 1 min at 95 °C; 40 cycles of 95 °C for 15 s and 60 °C for 30 s; and 1 min at 72 °C. A melting curve analysis was added to the thermal process to ensure the specificity of the PCR product.

To confirm the gene expression data obtained using RNA-seq and SYBR Green qPCR, TaqMan assays were performed for the novel transcripts and sRNAs using the Luna Universal Probe qPCR Mastermix (NEB). The probe-based RT-qPCR reaction was prepared in a 20-µL volume by mixing 10 µL of the Luna Universal Probe qPCR Mastermix (NEB), 0.8 µL of forward primer (10 µM), 0.8 µL of reverse primer (10 µM), 0.4 µL of probes (10 µM), 1 µL of 1:100 diluted cDNA and 8 µL of nuclease-free water.

The reaction parameters set on the ViiA 7 Real-Time PCR were: 1 min at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. For the SYBR Green and TaqMan qPCR experiments, no-RT controls were used for each target gene. We also used three biological replicates for each condition and all target genes. The expression levels of three housekeeping genes (*rpoD, gyrB* and E5A72 RS18355) were evaluated across all of the RNA-seq data. *gyrB* displayed the least variability across conditions in all RT-qPCR reactions, and therefore, the cycle threshold (CT) values of all 16 target genes were normalised using *gyrB* as an internal control.

The fold changes in the expression of all 16 target genes between biofilm and planktonic cells, untreated biofilm cells and imipenem-treated biofilms, and untreated biofilm cells and colistin-treated biofilms were determined using the Livak method, wherein the fold change of target gene expression with respect to that in the corresponding control was computed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak,

2008). First, the CT value of each target gene was normalised to that of the housekeeping gene using the formula:

$$\Delta\mathrm{Ct} = \mathrm{Ct\ target} - \mathrm{average\ Ct\ of\ the}\ gyrB\ \mathrm{gene}$$

The ΔCt of the control was determined in the same way for the target genes. The difference in the normalised CT values between the target and control was then measured using ΔΔCt. Finally, the $2^{-\Delta\Delta Ct}$ formula was used to calculate the fold change in the expression of the target gene.

Table 4-3. Primers and probes used to validate differentially expressed genes (DEGs).

Gene ID	Amplicon size (bp)	Primer /Probe	Sequence (5´ to 3´)
E5A72_RS12100 (*pgaB*)	105	E5A72_RS12100-F	CGGATGCGAATGGTTCTGC
		E5A72_RS12100-R	GCGTACGGGTTTGAATTTGC
E5A72_RS05130	217	E5A72_RS05130_4_F	CCGAAGGTACAGCTAACAGTG
		E5A72_RS05130_4_R	CCACCCACATTTGCATTTACT
E5A72_RS06870	121	E5A72_RS06870_F	GCCAGACTACTACCCACCAC
		E5A72_RS06870_R	CTACGGCTGAAAACCTTGAT
E5A72_RS00640	108	E5A72_RS00640_F	TCAGGCTTCACGTGCACTAC
		E5A72_RS00640_R	AAACCGAGTGAAGCTGGAGA
Novel00738	79	Novel00738_F	GCTGCCATTACTCGTTTACCT
		Novel00738_R	CAGGACGGCTCTCAACAAC
		Novel00738_IN	FAM-GGCAAGCTGTAGCGATGCTTGTTAAT-TAMRA
Novel00626	110	Novel00626_F	CGCATCGTTACCCATTCTT
		Novel00626_R	GAAATGCCCTTGTAGGAACTCT
		Novel00626_IN	FAM-TTGGTTGATCGTGTGACTGAAGTTACTGA-TAMRA
Novel00171	109	Novel00171_F	CATTGCCGGATGTGAGAG
		Novel00171_R	ACACGAGCAGATTTCTTGTAGG
E5A72_RS17735 (*Frr*)	98	E5A72_RS17735_F	GCGAAAGTTGCTATCCGTAA
		E5A72_RS17735_R	GCACGACGCTCATCATCT
Novel00584	114	Novel00584_F	TGCGTTCTATGGTTCGTACTT
		Novel00584_R	GCACGACGCTCATCATCT

Table 4-3 continued

Gene ID	Amplicon size (bp)	Primer /Probe	Sequence (5´ to 3´)
Novel00490	109	Novel00490_F	TTAGCCCGTGCATACAACAC
		Novel00490_R	TAGCCCGTGCATACAACAC
		Novel00490_IN	FAM-TGGTGTGGATGCACATGCTTTAGAAC-TAMRA
E5A72_RS14510	103	E5A72_RS14510_F	AGGTTTAGGCTGGGAAATGG
		E5A72_RS14510_R	ATTTGCTGCTTTGCTTACCG
E5A72_RS14240	110	E5A72_RS14240_1_F	GCACGAGTAGGCGATGAA
		E5A72_RS14240_1_R	AAAGGTAGCTCACGATGGATAA
E5A72_RS03500 (*secA*)	107	E5A72_RS03500_F	GACATTATTGCTCAGGCAGGT
		E5A72_RS03500_R	GCAAGTTTCGCTTTCCAGTT
E5A72_RS09610	86	E5A72_RS09610_3_F	AAGGTGGAACTGTGATGATGG
		E5A72_RS09610_3_R	AATTCCCAAACCTGCACAAG
E5A72_RS13515	118	E5A72_RS13515_3_F	ATCGACCTATCTTCACAACCAG
		E5A72_RS13515_3_R	ATACACGGCCAACCATTTC
sRNA00203	76	sRNA00203_4_F	GCATAAAAACCTCTTGAAACTGTTC
		sRNA00203_4_R	AGCGTTCATTTCAACCGATA
		sRNA00203_4_IN	TCAAGTTCCTTATGATCTCTTCCTTGA
gyrB	93	gyrB-F	ACGATTTACCGTGCTGGTC
		gyrB-R	GGTATTATCCGTTTCACCAATC
		gyrB_IN	FAM-TATCATCATGGTGATCCGCAATATCC-TAMRA

4.4 Results

4.4.1 RNA-seq data statistics

We obtained 8.4–12.4 million raw reads after conducting RNA-seq of *A. baumannii* ST1894 grown under 4 different conditions (Table 4-4). The percentages of clean reads obtained from the planktonic cells, biofilm cells, imipenem-treated biofilm cells and colistin-treated biofilm cells that were uniquely mapped to the reference genome of *A. baumannii* ATCC17978 were 82.0, 87.0, 84.1 and 81.6, respectively (Table 4-4). Ideally, with the right choice of reference genome and no contamination, the percentage of uniquely mapped sequences obtained from sequencing experiments should be > 70% and the number of multiple mapped reads should be < 10%. In this study, > 80% of the clean reads came from 4 different conditions that were uniquely mapped to the reference genome of *A. baumannii* ATCC17978, and < 10% of the clean reads were multiply mapped to the reference genome (Table 4-4). The RNA-seq data therefore qualified for subsequent bioinformatics analysis.

Table 4-4. Summary of the RNA-seq dataset and quality check.

Sample name	**Raw reads**	**Clean reads[a]**	**Raw reads**	**Clean bases[b]**	**Error rate (%)**	**Q20 (%)[c]**	**Q30 (%)[d]**	**Multiple mapped (%)**	**Uniquely mapped (%)**
Biofilm cells	8455924	8358208	1.3 G	1.3 G	0.03	97.44	92.80	5.77	87.08
Planktonic cells	9752102	9658282	1.5 G	1.4 G	0.03	97.34	92.43	9.47	82.02
Imipenem-treated biofilm cells	11374254	11212348	1.7 G	1.7 G	0.03	97.42	92.88	6.06	84.06
Colistin-treated biofilm cells	12466900	12330898	1.9 G	1.8 G	0.03	96.81	91.62	6.01	81.58

a: Reads from sequencing after filtering out low-quality reads.

b: Sum of nucleotides of all reads in clean data, represented as gigabytes.

c and d: Percentages of bases with Phred values of 20 and 30, respectively.

4.4.2 Differential expression of biofilm-specific genes

Following the alignment of clean reads with the reference genome *A. baumannii* ATCC17978, a list of DEGs in the biofilm cells relative to the planktonic cell controls was identified, as shown in Table 4-5 and Figure 4-5. We found that 51.8% (1592/3075) of the transcribed genes were differentially expressed in the biofilm cells compared with their planktonic counterparts; of these, 20% (614/3075) were upregulated and 31.8% (978/3075) were downregulated in biofilm cells.

In addition, 96 novel genes were differentially expressed in biofilm cells compared with planktonic cells. We classified 1,592 DEGs into different functional categories based on GO and KEGG pathways. Due to the high number of DEGs, we selected 211 that were linked to biofilms and antibiotic resistance for further analyses to identify the genes responsible for reduced antibiotic susceptibility in biofilm cells. Subsequently, we narrowed this category down to 50 DEGs that were expressed in biofilm cells and either induced or repressed upon exposure to antibiotics (Table 4-6).

Table 4-5. Summary of the numbers of differentially expressed genes (DEGs) among the three different groups.

Group comparisons	**Transcribed genes**	**Differentially expressed**			
		Total no. (%)	**Novel genes**	**Genes encoding hypothetical proteins**	**Small RNA**
Biofilm cells vs. planktonic cells	3,075	1592 (51.8)	96	431	5
Imipenem-treated biofilm cells vs. untreated biofilm cells	2,885	106 (3.7)	7	11	1
Colistin-treated biofilm cells vs. untreated biofilm cells	2,912	368 (12.6)	33	74	0

4.4.3 Gene expression profile of imipenem-treated biofilm cells

The expression patterns of genes in imipenem-treated *A. baumannii* ST1894 biofilm cells were compared with those of untreated biofilm cells of the same strain Figure 4-5b. Of the 2,885 total transcribed genes, 3.7% (106) were differentially expressed in the imipenem-treated biofilm cells (Figure 4-5e). Of these 106 DEGs, 78 were biofilm-specific, of which 48 were upregulated in treated versus untreated biofilm cells. The upregulated genes in the imipenem-treated biofilm cells included *pgaB* and genes encoding fimbrial protein, AHL synthase, the T6SS protein ImpK, preprotein translocase subunit SecA, cAMP-activated global transcriptional regulator and cyclic-AMP receptor protein (CRP), RND family drug transporter and sRNA00203 (Table 4-6). Thirty genes were downregulated in treated versus untreated biofilm cells, including genes encoding the ATP-binding cassette (ABC) transporter, DNA gyrase, ribosome release factor (RRF) and 30S ribosomal protein S20 (Table 4-6).

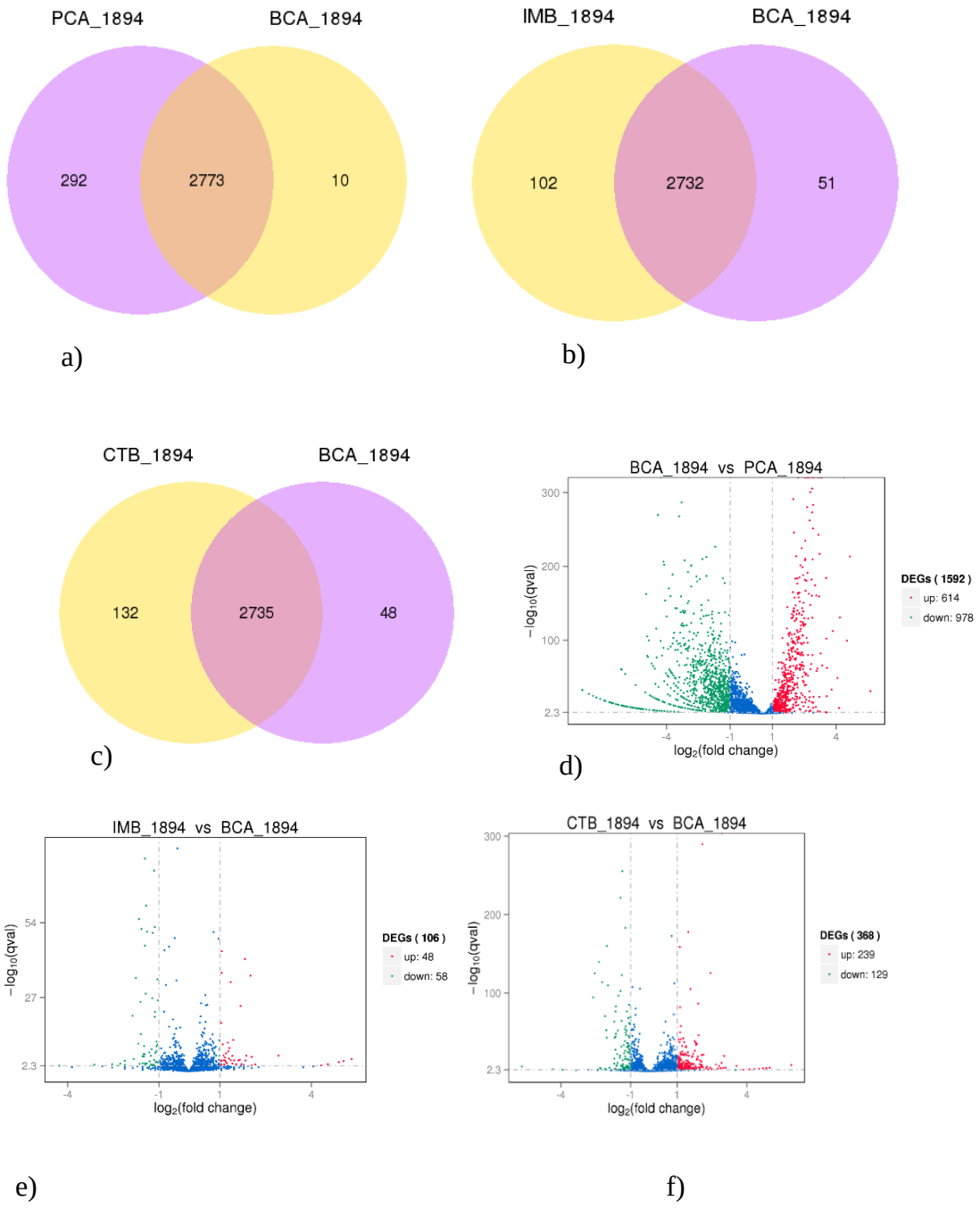


Figure 4-5. Venn diagrams and volvano plots.

Venn diagrams representing some of the genes expressed in planktonic cells(PCA), biofilm cells (BCA) ,imipenem-treated biofilm cells(IMB) and colistin-treated biofilm

cells (CTB). (a–c). Volcano plots showing the numbers of DEGs among the three growth conditions of *A. baumannii* ST1894 (d–f).

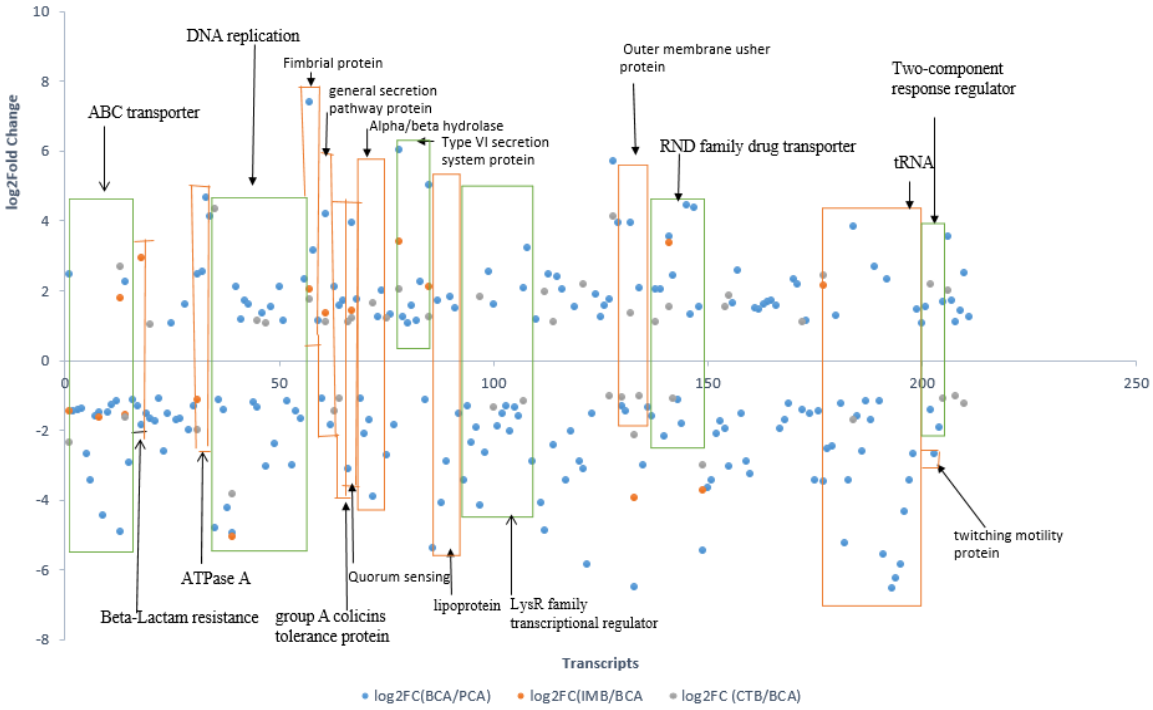


Figure 4-6. Distribution of significantly up- or downregulated genes belonging to functional categories associated with biofilms and antibiotic resistance.

The blue dots represent the DEGs between untreated biofilm and planktonic cells. The orange dots represent the DEGs between untreated and imipenem-treated biofilm cells. The grey dots represent the DEGs between untreated and colistin-treated biofilm cells.

4.4.4 Gene expression profile of colistin-treated biofilm cells

The expression patterns of genes in colistin-treated and untreated biofilm cells of *A. baumannii* ST1894 were compared Figure 4-5c. Of the 2,912 total transcribed genes, 2.6% (368) were differentially expressed in the colistin-treated biofilm cells (Figure 4-5f). Of the 368 DEGs, 285 were biofilm-specific genes, of which 197 were upregulated and 88 were downregulated compared to untreated biofilm cells. We selected 44 DEGs in the biofilm cells that were either induced or repressed when treated with colistin, as shown in Table 4-6. Of the 44 DEGs, 28 and 16 were upregulated and downregulated, respectively, when the biofilm cells were treated with sub-inhibitory concentrations of colistin (Table 4-6). The colistin-induced biofilm-specific genes included those encoding ABC-type Fe^{3+} transport proteins, fimbrial protein, lipoprotein (biofilm matrix), AHL synthase (*AbaI*), multidrug efflux pumps, OMP protein and catalase and the *csu* operon.

The biofilm-specific genes repressed by sub-optimal concentrations of colistin included genes encoding the LPS export system permease, DNA gyrase, RRF, 30S ribosomal protein S20, tRNA-i(6)A37 modification enzyme and ATPase. This indicates that a sub-inhibitory concentration of colistin can either activate or suppress biofilm-specific genes to promote the survival of biofilm cells in the presence of antibiotics.

Table 4-6. Biofilm-specific genes that were either induced or repressed by imipenem or colistin treatment.

GENE_ID	KEGG_ID	$\log_{2FC}\left(\frac{BCA}{PCA}\right)$	$\log_{2FC}\left(\frac{IMB}{BCA}\right)$	$\log_{2FC}\left(\frac{CTB}{BCA}\right)$	Function/product
Lipoprotein					
E5A72_RS12100	acb: A1S_0938	5.0363	2.09	1.23	Lipoprotein (biofilm matrix)
ABC transporter					
E5A72_RS02170	acb: A1S_2611	2.3183	2.14368	1.02	Transport protein of outer membrane lipoproteins
E5A72_RS16335	acb: A1S_1722	-1.5054	-1.639	0.095	ABC transporter ATP-binding protein
E5A72_RS14510	acb: A1S_1359	-4.9058	1.7825	2.6702	ABC-type Fe^{3+} transport system
E5A72_RS08785	acb: A1S_0229	2.0395	1.5784	-1.6349	Lipopolysaccharide export system permease protein
E5A72_RS15155	acb: A1S_1482	-3.3293	1.0455	2.0257	D-and L-methionine transport protein
β-lactam resistance					
E5A72_RS00940	acb: A1S_2367	-1.8362	2.92	1.16*	*ampC*; β-lactamase
Novel00738	acb: A1S_2736	3.5549	3.3532	-1.013	RND family drug transporter
DNA replication and repair					
Novel00171	acb: A1S_2626	-4.9582	-5.0584	-3.8428	DNA gyrase
E5A72_RS14680	acb: A1S_1389	-1.3449	-0.0409*	1.1446	DNA polymerase V component
E5A72_RS03815	acb: A1S_2918	-3.0414	-0.08*	1.0746	DNA repair protein
Fimbrial protein and *csu* operon					
E5A72_RS05130	acb: A1S_3177	7.4033	2.0455	1.7527	Fimbrial protein
E5A72_RS19200	acb: A1S_2217	5.7238	NA	2.1677	Protein CsuA
E5A72_RS19190	acb: A1S_2215	1.3111	-0.683*	1.0371	Protein CsuC
E5A72_RS19180	acb: A1S_2213	3.9517	0.2679*	4.1059	Protein CsuE
Bacterial secretion system					
E5A72_RS14240	acb: A1S_1310	6.0356	3.387	2.045	Type VI secretion system protein
E5A72_RS15605	acb: A1S_1564	4.2	1.3587	1.1006	General secretion pathway protein J

GENE_ID	KEGG_ID	$\log_{2FC}\left(\frac{BCA}{PCA}\right)$	$\log_{2FC}\left(\frac{IMB}{BCA}\right)$	$\log_{2FC}\left(\frac{CTB}{BCA}\right)$	Function/product
E5A72_RS03500	acb: A1S_2862	5.376	1.65783	2.349	Preprotein translocase subunit SecA
Two-component regulatory system					
E5A72_RS09610	acb: A1S_0399	-4.1397	-0.346*	1.8361	LysR family transcriptional regulator
E5A72_RS12380	acb: A1S_0992	1.617	-0.132*	-1.3517	
E5A72_RS13515	acb: A1S_1182	3.216	2.06	0.065*	cAMP-activated global transcriptional regulator CRP
Novel00822	acb: A1S_0685	-1.4033	1.788*	2.4156	Two-component response regulator
Quorum sensing or quenching					
E5A72_RS06870	acb: A1S_0109	3.9355	1.43253	1.21081	AHL synthase *AbaI*
E5A72_RS16835	acb: A1S_1809	-3.9015	0.267*	1.6271	Hydrolase transmembrane protein
Multidrug efflux pump					
E5A72_RS00640	acb: A1S_2306	2.0527	-0.083*	2.1244	RND efflux transporter
E5A72_RS11940	acb: A1S_0908	2.4322	-0.4375	1.0937	RND family multidrug resistance secretion protein
Outer membrane protein					
E5A72_RS11985	-	-4.8714	-0.1244*	1.9751	OprD family outer membrane porin
E5A72_RS18480	acb: A1S_2076	-2.4293	0.4473*	1.1074	Outer membrane porin receptor for Fe (III)-coprogen
E5A72_RS01795	acb: A1S_2538	-3.1257	1.4605*	2.1677	Outer membrane protein CarO precursor
Transcription and translation					
E5A72_RS17735	acb: A1S_1974	-6.5036	-3.9244	-2.1244	Ribosome releasing factor
Novel00584	acb: A1S_1617	-5.4437	-3.7365	-3.01	30S ribosomal protein S20
Novel00490	-	7.0401	1.1587	0.415	Transcription termination factor Rho OS
E5A72_RS04400	acb: A1S_3029	-3.4737	2.161	2.4156	tRNA-Arg
Peptidoglycan biosynthesis					
E5A72_RS10790	acb: A1S_1965	1.6823	-0.1303*	-1.6917	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
Novel00626	acb: A1S_1965	3.5549	NA	2.1677	UDP-N-acetylglucosamine acyltransferase

GENE_ID	KEGG_ID	$\log_{2FC}\left(\frac{BCA}{PCA}\right)$	$\log_{2FC}\left(\frac{IMB}{BCA}\right)$	$\log_{2FC}\left(\frac{CTB}{BCA}\right)$	Function/product
E5A72_RS05230	acb: A1S_3203	1.0844	-0217*	-1.032	UDP-N-acetylmuramoylalanyl-D-glutamate-2, 6-diaminopimelate ligase
E5A72_RS17730	acb: A1S_1973	2.513	-0.0783*	-1.2536	Undecaprenyl pyrophosphate synthetase
E5A72_RS07480	acb: A1S_2968	-2.7334	0.4605*	1.2183	Hypothetical protein
E5A72_RS05220	acb: A1S_3200	1.7367	0.0003*	-1.0371	Phospho-N-acetylmuramoyl-pentapeptide transferase
Transcriptional regulators and others					
E5A72_RS13590	acb: A1S_1197	-2.5865	-1.2664	0.240*	Extracellular nuclease
E5A72_RS03015	acb: A1S_2767	-1.6789	0.7044	1.0387	AraC family transcriptional regulator
E5A72_RS04330	acb: A1S_3229	3.8412	0.25977	-1.6917	tRNA-i(6)A37 modification enzyme
E5A72_RS01785	acb: A1S_2536	2.4782	-1.1416	-1.9789	ATPase
E5A72_RS14655	acb: A1S_1386	-4.8062	NA	4.3282	Catalase; K03781 catalase
E5A72_RS01830	acb: A1S_2546	-1.9701	0.9841	1.5253	Secreted trypsin-like serine protease
E5A72_RS05375	acb: A1S_3227	2.0827	-0.02825	-1.013	RNA binding protein
E5A72_RS16050	acb: A1S_1670	-3.0262	0.598*	-3.01	Secretion protein HlyD
E5A72_RS02070	acb: A1S_2592	1.5561	0.0036	-1.0789	Group A colicins tolerance protein
E5A72_RS02065	acb: A1S_2591	2.0955	-0.0461	-1.4623	Group A colicins tolerance protein
Non-coding RNA					
sRNA00203	-	5.3951	2.38935	-0.389*	

Adjusted p (pad) < 0.004; * pad > 0.005; NA: not applicable. ABC: ATP-binding cassette; RND: resistance–nodulation–cell division; AHL: acyl homoserine lactone; CRP: cyclic-AMP receptor protein.

4.4.5 RT-qPCR verification of DEGs

To verify the RNA-seq results, 16 genes were selected for qRT-PCR experiments (Figure 4-7). These DEGs were associated with adherence, biofilm matrix synthesis, QS, β-lactam resistance, multidrug efflux pumps, replication and translation, environmental information processing and ncRNAs (Table 4-6 and Figure 4-8). A correlation coefficient of 0.98 was obtained from the linear regression plotted between the RT-qPCR and RNA-seq data, suggesting a strong positive correlation (Figure 4-7). The changes in gene expression measured using RT-qPCR displayed a pattern similar to the one seen in the RNA-seq data ($p \geq 0.05$), suggesting that RNA-seq accurately detected the transcriptional changes. The small variation between the RT-qPCR expression and RNA-seq data may be due to differences in the sensitivity and specificity of the two experiments.

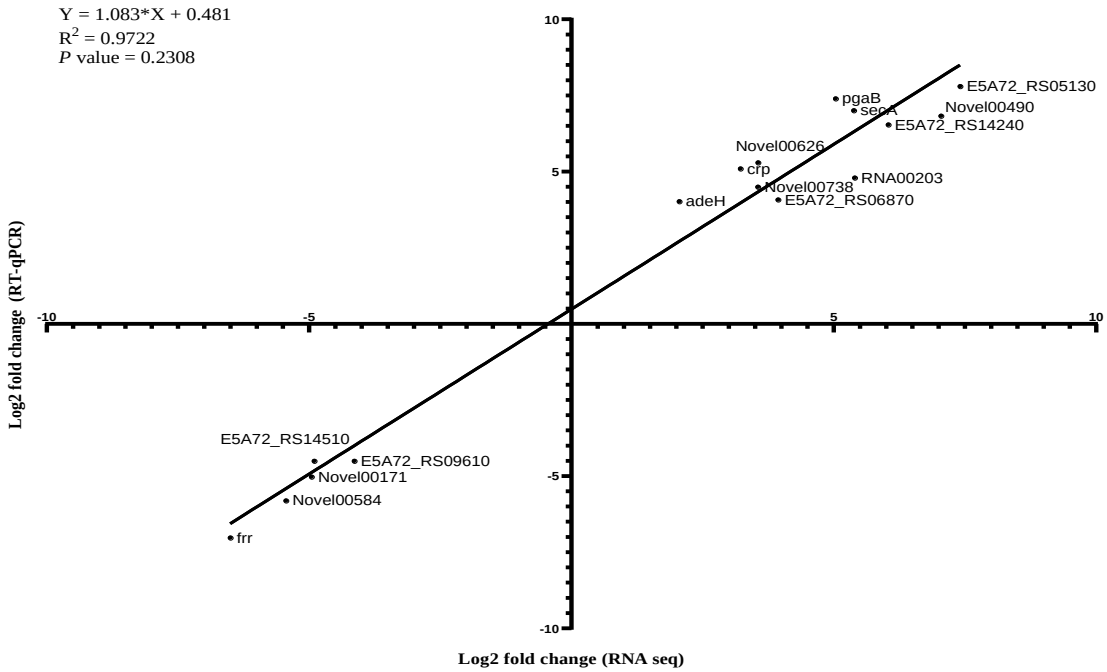


Figure 4-7. RT-qPCR assessment of the differential expression of 16 genes between biofilm and planktonic cells.

The measured $\log_2$ fold change in the expression levels of the biofilm cells relative to the planktonic cells are plotted against the RNA-seq data (statistical goodness of fit value provided).

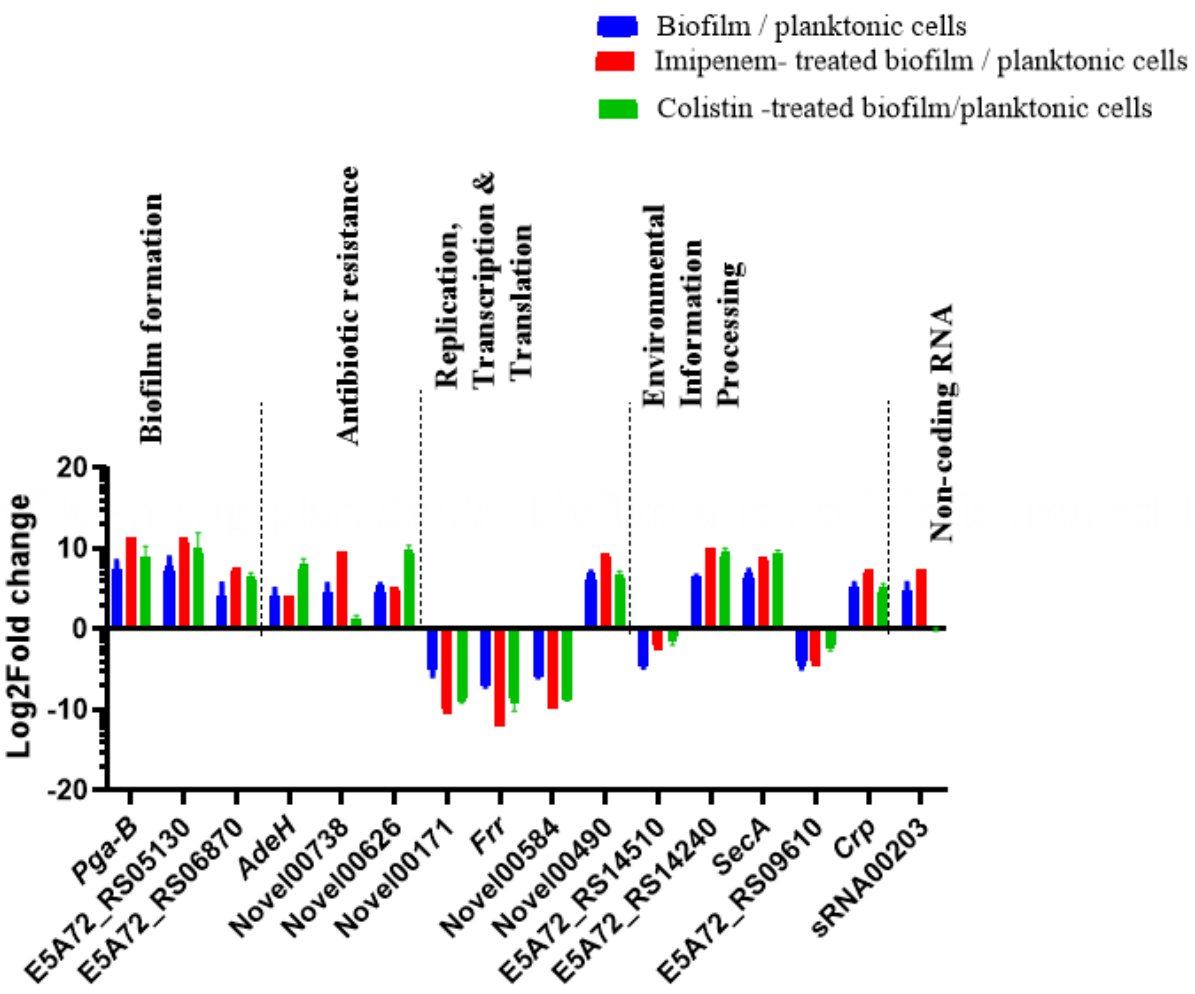


Figure 4-8. RT-qPCR results of some of the differentially expressed genes (DEGs) observed in the three pairs of samples.

pgaB: biofilm matrix synthesis; E5A72_RS05130: fimbrial protein; E5A72_RS06870: quorum sensing; *adeH*: multidrug efflux pump; Novel00738: resistance–nodulation–cell division (RND) efflux pumps; Novel00626: peptidoglycan biosynthesis; Novel00171: DNA replication; *frr*, Novel00584, Novel00490: translation; E5A72_RS14510: ATP-binding cassette (ABC) transporter; E5A72_RS14240, *secA*: bacterial secretion system; E5A72_RS09610, *crp*: two-component system,; RNA00203: non-coding RNA.

4.4.5.1 Genes involved in biofilm matrix synthesis

The expression level of *pgaB*, which encodes PNAG N-deacetylase OS, increased 169-fold when the cells were grown as biofilms versus planktonic forms (Figure 4-8). PNAG synthesis is a key component of the extracellular matrix and biofilms of several pathogenic bacteria. Genes involved in LPS and lipoprotein transport, such as A1S_1965 (*lpxA*), acb: A1S_3330 (*lpxC*), acb: A1S_1967 (*lpxD*), A1S_1557, acb: A1S_1237 and acb: A1S_1236, were also upregulated (Figure 4-9). Interestingly, all of the DEGs that play an essential role in LPS biosynthesis were upregulated, as seen in Figure 4-9. Lipoproteins and LPS are main components of the biofilm matrix, and increases in their expression levels indicate a transition from the planktonic state to the biofilm state.

In addition, *pgaB* gene expression increased 16-fold and 2.8-fold when the biofilm cells were treated with sub-inhibitory concentrations of imipenem and colistin, respectively (Figure 4-8). Upon exposure to sub-inhibitory concentrations of antibiotics, this increased expression of *pgaB* can enhance matrix synthesis and thus induce biofilm formation.

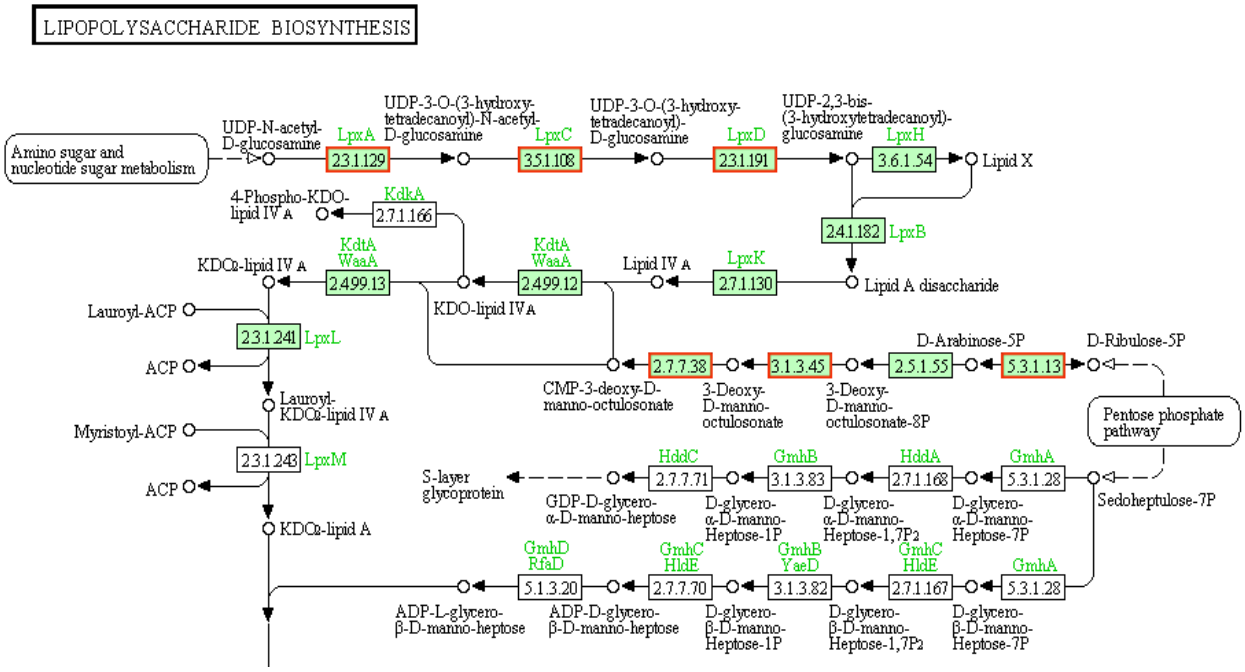


Figure 4-9. Differentially expressed genes (DEGs) involved in the lipopolysaccharide (LPS) biosynthesis pathway in biofilm cells versus planktonic cells of *A. baumannii* ST1894.

The upregulated genes are shown as green rectangles with red borders; the downregulated genes are shown as green rectangles with green borders.

4.4.5.2 Adhesion-related genes

The expression of the gene encoding fimbrial protein (E5A72 RS05130) increased 222-fold in biofilm cells compared with planktonic cells. This gene was further upregulated by 11.4-fold and 4.5-fold when the biofilm cells were exposed to sub-inhibitory concentrations of imipenem and colistin, respectively. In the biofilm state, the enhanced expression of genes encoding fimbrial proteins upon treatment with sub-inhibitory concentrations of antibiotics reflects the crucial roles of the exchange of genetic materials and of bacterial adhesion to surfaces in biofilm formation.

4.4.5.3 QS gene

The gene encoding AHL synthase (E5A72 RS06870) was upregulated 16.9-fold in biofilm cells compared with planktonic cells (Figure 4-8). The exposure of biofilm cells to sub-inhibitory concentrations of imipenem and colistin also upregulated expression of this gene by 12.2-fold and 5.8-fold, respectively.

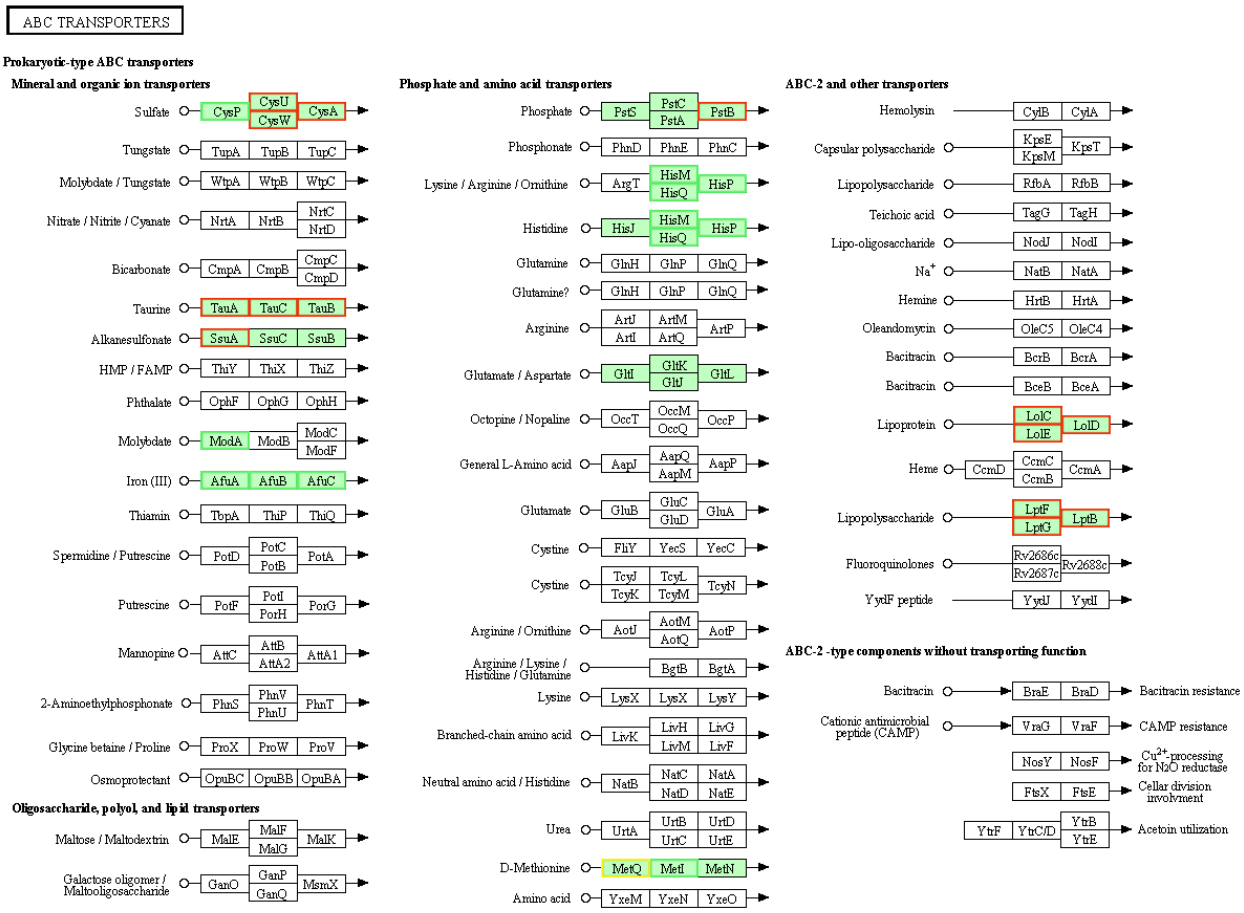


Figure 4-10. Differentially expressed genes (DEGs) involved in the ATP-binding cassette (ABC) transporter pathways that are activated during *A. baumannii* ST1894 biofilm growth.

The upregulated genes are shown as green rectangles with red borders; the downregulated genes are shown as green rectangles with green borders.

4.4.5.4 Transcriptional changes associated with efflux pumps and LPS biosynthesis

Upon profiling the transcriptome, we found that 12 genes belonging to the RND family were differentially expressed between biofilm cells and planktonic cells. Among these, only novel00738 (sequence shown in appendix 1), which putatively belongs to the RND family, was upregulated in untreated and imipenem-treated biofilm cells (Figure 4-8 and Table 4-6). Based on RT-qPCR verification, the expression of novel00738 in untreated biofilm cells was upregulated 22.6-fold relative to that in the planktonic counterparts (Figure 4-8 and Figure 4-11). This gene was further upregulated 36.7-fold when the biofilm was treated with sub-inhibitory concentrations of imipenem. However, its expression was reduced 9.2-fold when the biofilm cells were treated with sub-inhibitory concentrations of colistin.

Expression of the *adeH* gene was also upregulated 16.2-fold in biofilm cells compared with planktonic cells. Furthermore, this gene was upregulated 16.9-fold when biofilm cells were treated with sub-inhibitory concentrations of colistin, whereas no discernable changes were observed in cells treated with sub-inhibitory concentrations of imipenem (Table 4-6).

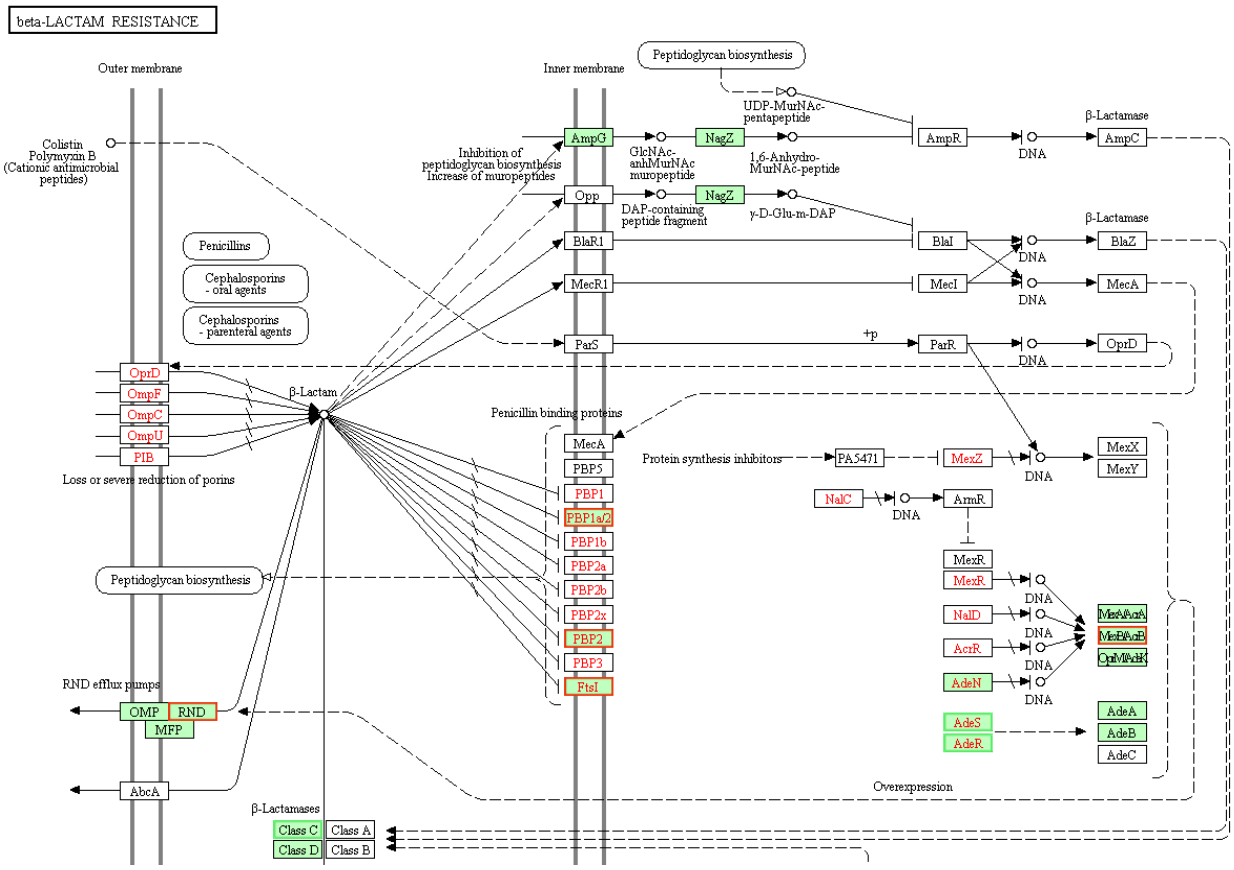


Figure 4-11. Differentially expressed genes (DEGs) involved in the β-lactam resistance pathways of *A. baumannii* ST1894 biofilm cells.

The upregulated genes are shown as green rectangles with red borders; the downregulated genes are shown as green rectangles with green borders.

We further observed that novel00626, also known as UDP-N acetylglucosamine O-acyltransferase and presumed to have a role similar to the *lpxA* gene, was highly upregulated in biofilm cells when compared with planktonic cells, as seen in Figure 4-12. Its expression level increased by 39.3-fold in biofilm cells relative to that in planktonic cells, as seen in Figure 4-8. The expression of this gene also increased by 24.2-fold when the biofilm cells were treated with sub-inhibitory concentrations of colistin. This transcriptional change could enhance LPS biosynthesis and thus increase the quantity of bacterial outer membrane and biofilm matrix production. However, novel00626 did not exhibit a significant transcriptional change when the biofilm cells were treated with sub-inhibitory concentrations of imipenem. This implies that colistin-induced changes in the expression of novel00626 may confer tolerance to cationic antimicrobial peptides as illustrated in Figure 4-12.

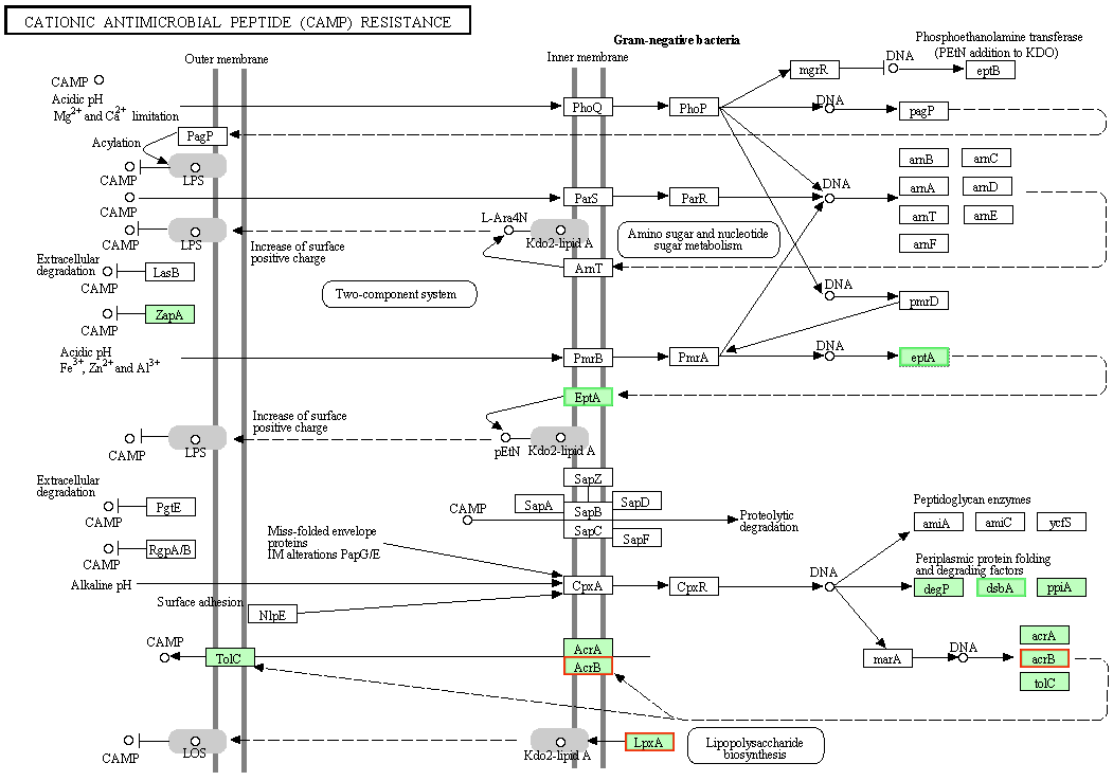


Figure 4-12. Differentially expressed genes (DEGs) involved in the cationic antimicrobial peptide resistance pathways of *A. baumannii* ST1894 biofilm cells.

The upregulated genes are shown as green rectangles with red borders; the downregulated genes are shown as green rectangles with green borders.

4.4.5.5 Transcriptional changes in genes involved in protein synthesis

Genes associated with protein synthesis are generally involved in DNA replication, transcription and translation. We observed several DEGs related to protein synthesis in biofilm cells compared with planktonic cells. Most of the genes involved in DNA replication were significantly downregulated in biofilm cells compared with planktonic cells. Among these, the expression level of novel00171, a gene that putatively encodes DNA gyrase, was reduced by 32.4-fold in biofilm cells compared with planktonic cells (Figure 4-8). When treated with sub-inhibitory concentrations of imipenem, the expression of novel00171 further decreased by 42.2-fold compared with that in untreated biofilm cells. However, there were no substantial changes in the expression of this gene when the biofilm cells were exposed to sub-inhibitory concentrations of colistin.

The expression of genes encoding the RRF (*frr*) were reduced by 129.7-fold in biofilm cells compared with planktonic cells. When biofilm cells were treated with sub-inhibitory concentrations of imipenem and colistin, the expression levels of *frr* genes further decreased by 33.3-fold and 4.5-fold, respectively (Figure 4-8). In addition, the expression of novel00584, a gene that putatively encodes the 30S ribosomal protein S20, decreased by 55.7-fold in biofilm cells compared with planktonic cells. The exposure of biofilm cells to sub-inhibitory concentrations of imipenem and colistin further reduced its expression by 16.4-fold and 7.4-fold, respectively, compared with untreated biofilm cells. Unlike the genes involved in transcription and translation, which were downregulated, the expression of novel00490, which encodes the transcription termination factor Rho OS, was upregulated by 113.7-fold in biofilm cells, and this expression increased further by 6.1-fold when the biofilm cells were treated with sub-inhibitory concentrations of imipenem.

4.4.5.6 Transcriptional changes in genes related to environmental signal processing

Genes involved in membrane transport and signal transduction are also thought to be involved in environmental signal sensing. These genes generally encode proteins associated with the ABC transport, bacterial secretion and two-component regulatory systems. As shown in Figure 4-8, the expression of genes encoding the T6SS protein ImpK and the preprotein translocase subunit SecA (E5A72 and RS14240, respectively) increased by 93-fold and 129-fold, respectively, in *A. baumannii* ST1894 biofilm cells relative to planktonic cells. Expression of these two genes was also induced when the biofilm cells were exposed to sub-inhibitory concentrations of antibiotics. We further found that genes encoding the ABC-type Fe^{3+} transport pathway (A1S 1359) were downregulated by 22.6-fold in biofilm cells (Figure 4-10).

The transcriptome study also showed that the expression of all eight DEGs encoding acinetobactin biosynthesis proteins was downregulated in biofilm cells relative to planktonic cells (Table 4-7). Genes related to iron synthesis have been shown to regulate the expression of other genes involved in the synthesis of citric acid cycle products. Indeed, as shown in Figure 4-13, all the DEGs involved in the citric acid cycle were downregulated and those involved in glycolysis were upregulated in biofilm cells relative to planktonic cells. This suggests that biofilm cells have lower metabolic rates than their planktonic counterparts.

Table 4-7. Differentially expressed genes (DEGs) involved in acinetobactin biosynthesis.

* pad > 0.005; * pad > 0.005. BCA: biofilm; PCA: planktonic cells; IMB: imipenem-treated biofilm cells; CTB: colistin-treated biofilm cells.

Gene_ID	**KEGG_ID**	**Strand**	**Start**	**End**	**Length**	**Expression Log2 Fold Change, *q* < 0.005**		
						BCA vs. PCA	IMB vs. BCA	CTB vs. BCA
E5A72_RS00970	acb: A1S_2372	-	192383	193552	1170	-2.1498	-1.5395	-0.0022*
E5A72_RS00975	acb: A1S_2373	-	193677	194432	756	-6.1366	1.460*	2.7527
E5A72_RS00980	acb: A1S_2374	-	194443	195177	735	-1.6007	-0.446*	0.167*
E5A72_RS01005	acb: A1S_2380	-	199967	200836	870	-2.2798	-0.325	-0.1542
E5A72_RS01015	acb: A1S_2383	+	202644	205586	2943	-1.486	-0.855	-0.534
E5A72_RS01050	acb: A1S_2390	+	213689	215716	2028	-1.7003	-1.4723	-0.137
E5A72_RS01055	acb: A1S_2391	-	215787	217634	1848	-3.1434	-0.681*	-0.131*
E5A72_RS01060	acb: A1S_2392	+	218027	218887	861	-1.0168	-1.1609	-0.004*
E5A72_RS07045	acb: A1S_0076	-	1465658	1468264	2607	1.6074	0.1601	-0.689*

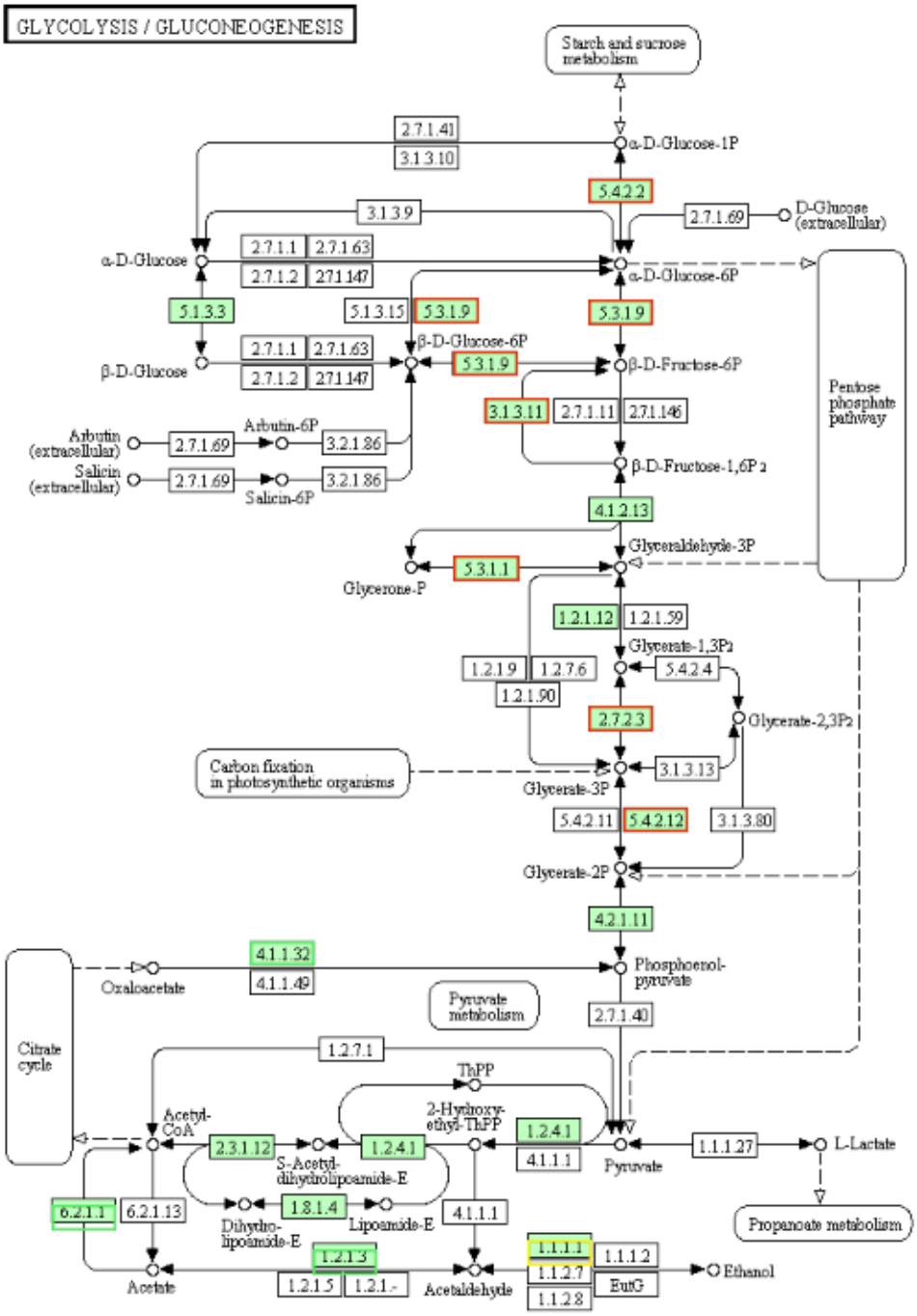


Figure 4-13. Differentially expressed genes (DEGs) involved in the glycolysis pathway of *A. baumannii* ST1894 biofilm cells.

The upregulated genes are shown as green rectangles with red borders; the downregulated genes are shown as green rectangles with green borders.

4.4.5.7 Non-coding sRNAs

Of the 138 sRNAs identified from the transcriptome, only 5 were differentially expressed in the biofilms (Table 4-8**)**. The sequences of these differentially expressed sRNAs were conserved across all sequenced strains of *A. baumannii*, as confirmed using BLASTn searches. We observed that sRNA00203 had the highest expression level in both the biofilm state and in biofilm cells treated with sub-inhibitory concentrations of imipenem (Figure 4-8). sRNA00203 has binding sites that are complementary to the antibiotic-induced biofilm-specific genes of *A. baumannii* ST1894. The sRNA00203 interacts through base-pairing with the mRNAs of the antibiotic-induced biofilm-specific genes such as *pgaB,* novel00738, novel00626, *secA,* and CRP transcriptional regulator.

Table 4-8. Differentially expressed sRNAs between biofilm and planktonic cells of *A. baumannii* ST1894.

* pad. BCA: biofilm; PCA: planktonic cells; IMB: imipenem-treated biofilm cells; CTB: colistin-treated biofilm cell; NA: not applicable.

Small RNA	**Strand**	**Start**	**End**	**Length**	**Expression Log2 Fold Change, $q < 0.005$**		
					BCA vs. PCA	**IMB vs. BCA**	**CTB vs. BCA**
sRNA00203	-	245743	1245795	53	5.40	2.39	0.241*
sRNA00207	-	1338464	1338744	281	1.30	0.411*	0.403*
sRNA00226	-	2213801	2214170	370	2.28	-0.340	0.0275
sRNA00272	+	753263	753365	103	1.82	0.653*	-1.32
sRNA00286	+	1677973	1678039	67	-3.44	NA	NA

4.5 Discussion

The capacity of *A. baumannii* to form biofilms enhances its survival in adverse environments, making it a successful nosocomial pathogen (Harding et al., 2018). In our earlier study, we identified the non-MDR and strong biofilm-forming strain *A. baumannii* ST1894, the biofilm cells of which exhibited reversible antibiotic tolerance to colistin, imipenem and ciprofloxacin. This finding indicated that biofilms play a substantial role in the survival of *A. baumannii* by modifying its responses to antibiotics. The phenotypic changes observed in this strain were a result of alterations in gene expression in the biofilm cells and not irreversible genetic mutations.

In the current study, we analysed the transcriptomic profiles between biofilm and planktonic cells, untreated biofilm cells and imipenem-treated biofilms, and untreated biofilm cells and colistin-treated biofilms of *A. baumannii* ST1894. Upon conducting RNA-seq of the biofilm and planktonic cells, we found that 51.8% of the total transcribed genes (3,075) were differentially expressed between mature biofilm and planktonic cells of *A. baumannii* ST1894; 38.6% of the DEGs were upregulated in the biofilm cells compared with the planktonic cells. Genes involved in biofilm matrix synthesis, bacterial adhesion, QS, the RND family and LPS biosynthesis were upregulated in biofilm cells. In contrast, genes involved in DNA replication, translation and acinetobactin biosynthesis were downregulated in biofilm cells compared with their planktonic counterparts. These transcriptional changes highlight the significant genetic changes that occur as bacterial cells transition from the planktonic to the biofilm state.

An increase in the expression levels of the *pgaB* gene and genes encoding LPS biosynthesis components was observed in biofilm cells relative to planktonic cells. Other research groups have reported that deletion of the *pga* locus led to the loss of the

strong biofilm formation phenotype of *A. baumannii*, demonstrating that this gene is essential for biofilm formation (Choi et al., 2009). The production of EPS indicates that biofilm cells have reached the stage of irreversible adherence to the surface. The *pgaB* gene is involved in the biosynthesis of EPS, which is a significant component of the biofilm matrix (Vu et al., 2009).

Components of the biofilm matrix can limit the penetration of antimicrobial agents, such as bleach and antibiotics, into biofilm cells by binding to or consuming the antimicrobial agent (Branda et al., 2005; Mah, 2012). However, when we studied antibiotic-treated biofilm cells using confocal imaging, we observed that most of the biofilm cells were killed by treatment with high concentrations of colistin, imipenem and ciprofloxacin. This observation suggests that the antibiotics passed through the matrix of the biofilm. Although it is possible that the biofilm matrix might have slowed the penetration of antibiotics into the biofilm cells, this possibility needs to be experimentally verified.

We also observed the increased expression of *pgaB* and the gene coding for UDP-N-acetylglucosamine acyltransferase (novel00626) in biofilm cells exposed to sub-inhibitory concentrations of imipenem and colistin. These results indicate that biofilm formation was enhanced in response to sub-inhibitory concentrations of antimicrobials. This finding is in agreement with a previous report showing that sub-inhibitory concentrations of antibiotics can induce bacterial biofilm formation *in vitro* (Kaplan, 2011). In addition, biofilm matrix formation triggers stress-induced metabolic or transcriptional changes that increase resistance in cells exposed to sub-inhibitory concentrations of antibiotics (Jefferson et al., 2005; Singh et al., 2010).

We also observed that expression of the *pilA* gene, which encodes the fimbrial protein (A1S_3177) or type IV pilus assembly protein, was upregulated 222-fold in biofilm cells compared with planktonic cells. The expression of *pilA* also increased when the biofilm cells were treated with sub-inhibitory concentrations of imipenem and colistin.

A previous study reported that this type IV pilus assembly protein, commonly found in pathogenic *A. baumannii* strains, plays an essential role in host cell adhesion, biofilm formation, microcolony formation and horizontal gene transfer (Ronish et al., 2019). In this study, the upregulated expression of type IV pili genes upon exposure of the cells to sub-inhibitory concentrations of imipenem and colistin might have activated signalling cascades associated with pathogenicity and antibiotic resistance in *A. baumannii*. When *Acinetobacter* cells form biofilms in clinical settings, overexpression of the type IV pilus assembly protein upon exposure to sub-optimal concentrations of imipenem and colistin can increase the rates of conjugative gene transfer, thereby increasing the likelihood of developing irreversible antibiotic resistance and consequent therapeutic failure. Sub-inhibitory concentrations of antibiotics also promote mutation, leading to the emergence of antibiotic resistance (Laureti et al., 2013). This sequence of events can eventually result in the evolution of sensitive strains into resistant strains.

We observed that the *csu* operon (proteins CsuA, CsuC and CsuE) was upregulated in biofilm cells. The *csu* operon encodes proteins involved in the chaperon-usher pili assembly mechanism, which is essential for the assembly of pili and the formation of biofilms (Tomaras et al., 2003). The *csu* operon has been identified in pathogenic strains of *A. baumannii*, indicating its role as a virulence factor (Peleg et al., 2012). However, we observed that exposure to sub-inhibitory concentrations of imipenem and colistin did not significantly affect the expression of the *csu* operon.

QS is a regulatory mechanism that allows bacteria to communicate cell density information through the diffusion of small molecules and adjust their gene expression profiles accordingly. All QS bacteria generate and release chemical signal molecules called AIs that increase in concentration as a function of cell density. AHLs are a class of AI signal used by Gram-negative bacteria to regulate various physiological processes such as conjugation, virulence factor production and biofilm formation (Antunes et al., 2010).

AHLs have been identified as major components of biofilm formation in *A. baumannii* cells (Brelles-Mario & Bedmar, 2001). In this study, the genes encoding AHL synthase (E5A72_RS06870) were upregulated by 16.9-fold in biofilm cells compared with planktonic cells. The exposure of biofilm cells to sub-inhibitory concentrations of imipenem and colistin also induced genes encoding AHL synthase, which were upregulated by 12.2-fold and 5.8-fold, respectively. At a particular threshold, the binding of AHL to receptors within the cell promotes a signal transduction cascade that eventually changes the expression levels of specific genes involved in virulence and antibiotic resistance (Rao et al., 2008). These changes in gene expression enable this pathogen to survive in many adverse environmental conditions by promoting biofilm formation. When treated with sub-inhibitory concentrations of imipenem and colistin, the increased expression of genes encoding AHL synthase in biofilm cells suggests that a higher concentration of AIs is required to counteract adverse conditions encountered in the biofilm state.

Efflux pumps actively eliminate antimicrobial agents from intracellular targets, facilitating the reduced antibiotic susceptibility of biofilm and planktonic cells (Poole, 2007). In the current study, we observed that 12 RND family MDR genes were differentially expressed in biofilm cells. Among these genes, novel00738 was

upregulated in untreated biofilms and in biofilm cells exposed to sub-inhibitory concentrations of imipenem. The product of this gene is functionally similar to the MexB-AcrB efflux pump, which confers resistance to β-lactams and cationic antimicrobial peptides. Poole et al. (1993) previously reported that deleting the genes encoding the MexAB-OprM efflux pathway in *P. aeruginosa* resulted in hypersensitivity to many antimicrobial compounds (Poole et al., 1993).

The gene novel00738 encodes as a multidrug efflux pump that is active in β-lactam resistance pathways and is overexpressed when treated with sub-inhibitory concentrations of imipenem. The overexpression of novel00738 can result in tolerance to imipenem when biofilm cells are treated with high concentrations of β-lactam antibiotics. This finding is similar to one reported by He et al. (2015), who demonstrated the role of the AdeFGH efflux pump in biofilm formation in response to low-concentration antimicrobial therapy (He et al., 2015). The increased expression of genes encoding efflux systems could reduce the cytoplasmic concentrations of bactericidal antibiotics to below the threshold required for antibacterial activity.

One of the main mechanisms underlying the development of antibiotic resistance is the pumping of antibiotics out of cells by efflux systems. Efflux pumps not only can provide resistance to antibiotics used in clinical therapy but also can drive bacterial pathogenicity and persistence (Abel Zur Wiesch et al., 2015; Pu et al., 2017). The increased expression of novel00738 in biofilm cells might be responsible for antibiotic tolerance and not resistance, as this strain was seen to revert to the susceptible form after treatment with a high concentration of antibiotics.

Analysis of the cationic antimicrobial resistance peptide path also revealed that novel00738 is functionally similar to *acrB.* The expression of a*crB* gene affects the

expression of a group of efflux pump genes such as *tolC*, which is believed to confer tolerance to cationic peptides. The *tolC* gene has been shown to be expressed at significantly higher levels in persister cells than in normal viable cells (Pu et al., 2017).

We also found that the *adeH* (acb: A1S 2306) gene was upregulated in biofilm cells and was further induced by treatment with sub-inhibitory concentrations of colistin. AdeH can pump colistin out of the cell and thereby confer tolerance to this antibiotic. Overexpression of AdeFGH has also been reported to facilitate the synthesis and transport of AHLs in *A. baumannii* during biofilm production (He et al., 2015). We further observed a positive correlation between the expression of AIs and the upregulation of *adeH*, indicating that this gene might be involved in the transport of QS molecules, in addition to the expulsion of antibiotics.

We also observed that novel00626, which encodes UDP-N acetylglucosamine O-acyltransferase, appears to play a role similar to that of *lpxA*. This novel gene was highly upregulated in biofilm cells compared with their planktonic counterparts, and its expression level also significantly increased when biofilm cells were treated with sub-inhibitory concentrations of colistin. Previous studies have demonstrated that strains mutant for LOS production cannot survive desiccation, implying that the development of desiccation resistance is dependent on the composition of the outer membrane (Boll et al., 2015). However, the processes that mediate desiccation resistance have not been studied and are currently being characterised. The upregulation of genes encoding UDP-N acetyl glucosamine O-acyltransferase during biofilm formation and in cells treated with sub-inhibitory concentrations of colistin are attributable to the role of LPS in the synthesis of the biofilm matrix.

The expression of the extended-spectrum β-lactamase-encoding gene *ADC-26* was reduced in biofilm cells compared with planktonic cells. However, gene expression was induced when the biofilm cells were treated with sub-inhibitory concentrations of imipenem. These expression patterns indicate that the gene is likely responsible for conferring resistance to β-lactam antibiotics in planktonic cells (Bhattacharya et al., 2014). Our result contradicts previous research by Bagge et al. (2004) and Giwercman et al. (1991), who found that chromosomally encoded AmpC β-lactamases are overexpressed and secreted into the matrix, thereby conferring resistance to β-lactam antibiotics in *P. aeruginosa* biofilms (Bagge et al., 2004; Giwercman et al., 1991). Possibly, the energy required to express β-lactamases may reduce biofilm formation in isolates carrying these genes, whereas *A. baumannii* isolates can use this energy to express genes essential for biofilm formation and biofilm specific antibiotic resistance (Rodrigues Perez, 2015; Wang et al., 2018).

Previous studies have shown that when *ampC* genes were expressed, imipenem was present in the biofilm periphery at low concentrations but the biofilm centre and base were unaffected (Bagge et al., 2004). However, our confocal imaging results showed that most of the biofilm cells were dead after treatment with high concentrations of imipenem, barring a few antibiotic-tolerant cells. This indicates that high concentrations of imipenem can bypass the biofilm matrix and the β-lactamases contained therein, resulting in the death of most of the biofilm cells. This finding is consistent with a report by Bagge et al. (2004), who observed that high concentrations of imipenem could withstand the degrading effect of β-lactamases, allowing the antibiotic to penetrate deeper layers of the biofilms (Bagge et al., 2004).

We found that genes involved in DNA replication, transcription and translation were differentially expressed in biofilm cells compared with planktonic cells. The genes

involved in DNA replication were significantly downregulated in biofilm cells. The novel00171 gene, thought to encode DNA gyrase, was downregulated 32.4-fold in biofilm cells compared with planktonic cells. When treated with sub-inhibitory concentrations of imipenem, the expression of novel00171 decreased by 42.2-fold relative to untreated biofilm cells. Such downregulation of DNA gyrase genes during biofilm formation has never been reported previously.

DNA gyrase is necessary for the replication and transcription of DNA. Reductions in intracellular gyrase proteins by > 50% have been shown to affect cell growth (Guha et al., 2018). The altered supercoiling of DNA due to gyrase depletion causes subsequent changes in the density of RNA polymerase in the transcription units, thereby altering transcription. The consequently reduced transcriptional activity generates a high number of slow-growing biofilm cells, which can also occur due to the limited availability of nutrients. The presence of slow-growing cells in biofilms may lead to the development of decreased susceptibility to several antibiotics. We also observed no substantial changes in the expression level of the novel00171 gene when the biofilm cells were exposed to sub-inhibitory concentrations of colistin.

The expression level of the gene encoding the RRF (*frr*) was reduced by 129.7-fold in biofilm cells. When the biofilm cells were treated with sub-inhibitory concentrations of imipenem and colistin, the expression level of *frr* decreased by 33.3-fold and 4.5-fold, respectively. The RRF is sometimes referred to as ‘ribosome recycling factor’, as its primary purpose is to recycle ribosomes for subsequent rounds of protein synthesis; it is thus essential for bacterial growth (Guha et al., 2018). The downregulation of genes encoding RRFs in untreated biofilms and in antibiotic-treated biofilms might be due to the presence of slow-growing cells, which can confer antibiotic tolerance.

In addition, the expression level of novel00584, which is thought to encode the 30S ribosomal protein S20, decreased significantly by 55.7-fold in the biofilm cells compared with the planktonic cells. The exposure of biofilm cells to sub-inhibitory concentrations of imipenem and colistin reduced the expression of novel00584 by 16.4-fold and 7.4-fold, respectively, compared with untreated biofilm cells. The small subunit ribosomal protein S20 is thought to mediate translation; therefore, its downregulation could directly impact protein synthesis and slow down metabolism. This could, in turn, inactivate antibiotic targets, implying that antibiotics would no longer be effective in clearing these cells.

The expression of novel00490, which encodes the transcription termination factor Rho OS, was 113.7-fold higher in biofilm cells compared with planktonic cells. Its expression increased significantly by 6.1-fold when the biofilm cells were treated with sub-inhibitory concentrations of imipenem. The transcriptional changes observed in both untreated biofilm cells and those exposed to sub-inhibitory concentrations of imipenem and colistin may shut down metabolic activity. Such a shutdown could directly inhibit other vital cellular activities and inactivate antibiotic targets.

We further observed that all of the DEGs involved in the citric acid cycle were downregulated, while all of those involved in glycolysis were upregulated. The downregulation of the citric acid cycle suggests that biofilm cells have lower metabolic rates than planktonic cells. This metabolic quiescence might contribute to the reduced susceptibility to antibiotics observed in hyper biofilm-forming strains such as *A. baumannii* ST1894. It also implies that the persisters observed in the biofilms of *A. baumannii* ST1894 after treatment with high concentrations of bactericidal antibiotics might have emerged a result of reduced metabolic activity.

The transcriptomic analysis revealed that genes encoding acinetobactin biosynthesis proteins were downregulated in biofilm cells relative to their planktonic counterparts. The identified genes encode iron-induced proteins, such as the iron storage protein Bfr, metabolic proteins, such as AcnA, AcnB, GlyA, SdhA and SodB, and lipid biosynthesis proteins (Nwugo et al., 2011). The reduced expression of these genes can further downregulate the expression of genes involved in aerobic respiration. Similar patterns were observed in our current study: the downregulation of genes involved in the citric acid cycle suggests slower metabolic rates in biofilm cells. This reduced metabolic activity could further increase the antibiotic tolerance of biofilm cells compared with planktonic cells.

Overall, we observed that the hyper biofilm-producing strain *A. baumannii* ST1894 demonstrated a high degree of reduced susceptibility to antibiotics. The capacity of *A. baumannii* ST1894 to survive the effects of bactericidal antibiotic exposure during biofilm formation might emanate from genetic changes that arise due to this exposure in the biofilm state.

Several novel findings of this study are reported here. It is the first study to characterise transcriptional changes in biofilm cells in response to treatment with imipenem and colistin at sub-inhibitory concentrations. The consistent upregulation of genes involved in biofilm matrix synthesis (*pgaB*), multidrug efflux pump (novel00738) and LPS synthesis (novel00626) in *A. baumannii* in response to treatment with sub-inhibitory concentrations (half of the MIC) of imipenem and colistin may lead to increased biofilm production. This finding illustrates the possible relationship between low-dose antimicrobial therapy and enhanced biofilm production, which can occur during *Acinetobacter* infection. This study also showed the reduced expression of genes linked to acinetobactin biosynthesis and protein synthesis (novel00171, RRF, novel00584)

during biofilm formation, leading to slowed metabolism in these cells. Transcriptional analysis of both untreated and antibiotic-treated biofilm cells revealed novel genes that belong to the RND multidrug efflux pump family. In addition, we found that protein synthesis is essential for the survival of biofilm cells exposed to antibiotics. These changes at the genetic level can enhance biofilm-specific antibiotic resistance and environmental resilience when the cells are exposed to antibiotics. Based on our observation, it appears that several genetic factors account for the decreased susceptibility of biofilm cells to antibiotics.

In addition to protein-coding transcripts, total RNA-seq data obtained in this study showed that 5 of 138 sRNAs were differentially expressed in biofilm cells relative to planktonic cells. Of these sRNAs, sRNA00203 showed the highest expression levels in biofilm cells that were both untreated and treated with sub-inhibitory concentrations of imipenem. sRNAs regulate protein expression by complementing target mRNAs and interacting with mRNA transcripts at or near the RBS (Mehta et al., 2008; Richards & Vanderpool, 2011).

The association of sRNAs with the RBS can have either a negative regulatory effect by blocking the ribosome or a positive regulatory effect by binding to the target mRNA and altering its secondary structure, thereby making the RBS more accessible (Storz et al., 2011). sRNA00203, an ncRNA, is thought to play roles in various cellular processes, including the regulation of gene expression. sRNAs are genetic regulators that enable biofilm cells to recognise environmental signals and transfer information in the form of metabolic changes with significant physiological effects during biofilm formation (Bak et al., 2015). Given that these sRNAs can potentially regulate gene expression associated with biofilm formation and biofilm specific antibiotic resistance, further research is needed to validate their biological functions in *A. baumannii*.

5 CHAPTER FIVE: IMPACT OF SMALL NON-CODING RNA00203 ON BIOFILM-SPECIFIC ANTIBIOTIC RESISTANCE IN A. BAUMANNII

5.1 Abstract

sRNAs are known to regulate gene expression during biofilm formation. We found that sRNA00203 was the most significantly upregulated sRNA in biofilm cells relative to planktonic cells. Therefore, we aimed to evaluate the underlying mechanisms by which sRNA00203 regulates the expression of genes involved in the development of biofilm-specific antibiotic resistance in *A. baumannii*.

sRNA prediction and differential expression analyses were performed using total RNA-seq data obtained from biofilm and planktonic cells. Expression of the gene encoding sRNA00203 was verified using SYBR Green and TaqMan assays. The sRNA00203-mutant strain was constructed using double allelic exchange facilitated by suicidal vectors (pMo130-kanR). The mutant strain was also complemented with a gene encoding sRNA00203 using the pWH1266-telR system. The antibiotic susceptibility profiles of biofilm and planktonic cells of the mutant, wild-type and sRNA00203-complemented strains were obtained. The regulatory effects of sRNA00203 on the expression of CRP transcriptional regulator, PNAG N-deacetylase, RND family drug transporter, UDP-N-acetylglucosamine acyltransferase and preprotein translocase subunit SecA were compared across the sRNA00203-mutant, wild-type and sRNA00203-complemented *A. baumannii* ST1894 strains.

In this study, 2.6% (138/5249 genes) of the total genes analysed in *A. baumannii* ST1894 were annotated as sRNAs. Among these, five sRNAs were differentially expressed in biofilm cells compared with planktonic cells. The expression of sRNA00203 was 45 times higher in biofilm cells than in planktonic cells. Deletion of

the gene encoding sRNA00203 substantially decreased the biomass of the biofilm by 85%. The MBICs for imipenem and ciprofloxacin were 1,024-fold and 128-fold lower, respectively, in the sRNA00203-mutant strain than in the wild-type strain. Inactivation of the gene encoding sRNA00203 sensitised the biofilm cells to imipenem and ciprofloxacin, but not to colistin. The MBEC of imipenem was 128-fold lower in the sRNA00203-mutant than in the wild-type strain. Deletion of the gene encoding sRNA00203 substantially decreased the expression of genes involved in matrix synthesis (*pgaB*) and encoding efflux pumps (novel00738), preprotein translocase subunit SecA and the CRP transcriptional regulator, indicating that the suppression of sRNA00203 in *A. baumannii* ST1894 impairs biofilm formation and sensitises biofilm cells to imipenem and ciprofloxacin. The sequence-specific inhibition of sRNA00203 presents a new strategy for treating biofilm-specific infections or for resensitising biofilms to imipenem and ciprofloxacin. This is the first study to show the effects of sRNA00203 on the emergence of biofilm-specific antibiotic resistance, which could be used as novel drug targets for treating *Acinetobacter* biofilm-specific infections.

5.2 Introduction

Biofilm formation is known to alter gene expression and result in the development of adaptive resistance to antibiotics (De la Fuente-Núñez et al., 2013). The developmental process of biofilm formation requires the concerted expression of multiple genes that are controlled by complex regulatory systems (Gaddy & Actis, 2009; Tomaras et al., 2003). In *A. baumannii*, these regulatory pathways consist of two-component (*bfmR*) and QS systems (*abaI*) (Kröger et al., 2017). The *bfmRS* system regulates the expression of the *csuA/BABCDE* usher-chaperone assembly system, which is required for the attachment of cells to surfaces and thereby facilitates biofilm formation on abiotic surfaces (Tomaras et al., 2008). Similarly, the *abaI* and *abaR* genes regulate QS and biofilm formation (Niu et al., 2008). The *abaI* gene produces AHLs, which are diffusible molecules that act as AIs when they bind to the AbaR transcription factor (Anbazhagan et al., 2012; Niu et al., 2008).

NcRNAs, such as sRNAs, are a class of gene regulators that influence various aspects of bacterial physiology and virulence (Bobrovskyy et al., 2015). sRNA regulators have been classified as negative (Viegas et al., 2011) or positive (Soper et al., 2010) based on their type, binding pattern and the extent to which they influence post-transcriptional processes. During negative regulation, sRNAs base-pair at or near the RBSs of mRNA targets (Bobrovskyy et al., 2015). This sRNA-mRNA interaction inhibits translational initiation and, in most cases, destabilises the mRNA target. Even binding of the sRNA to the first few mRNA codons can inhibit translation (Bouvier et al., 2008). Negative regulation can also occur when RNases are used to degrade the mRNA targets, reinforcing irreversible translational repression. For several sRNAs and their target mRNAs, translational inhibition without mRNA degradation is adequate to achieve negative regulation of gene expression, while RNase E cleavage occurs in a later step

(Maki et al., 2008). Positive regulation occurs when sRNAs bind to target mRNAs and modify their secondary structures, making the RBSs more available (Storz et al., 2011). The base-pairing of sRNAs with their targets can either stabilise target mRNAs or activate translation. Translational activation typically occurs via base-pairing within the 5′-untranslated region (UTR), which changes the folding of the 5′-UTR to allow ribosomal entry and translation.

sRNA analysis of the RNA-seq data of the *A. baumannii* ATCC17978 and *A. baumannii* AB5075 strains revealed 31 and 78 putative sRNA candidates, respectively (Sharma et al., 2014; Weiss et al., 2016). Nevertheless, the physiological functions of these candidate sRNAs have not been reported. A study conducted by Álvarez-Fraga et al. (2017) identified sRNA13573 as being involved in biofilm formation and in the attachment of bacteria to A549 alveolar epithelial cells (Álvarez-Fraga et al., 2017). However, the biological roles of sRNAs in the development of biofilm-specific antibiotic resistance in clinical *A. baumannii* strains have not yet been documented. The comparative transcriptome study described in the previous chapter revealed sRNA00203 to be the most significantly upregulated of the five differentially expressed sRNAs identified in biofilm cells compared with planktonic cells. sRNA00203 also contains complementary binding sites for genes involved in biofilm formation and antibiotic resistance in *A. baumannii* ST1894. Therefore, we hypothesised that the deletion of sRNA00203 in *A. baumannii* ST1894 might affect the formation of biofilms and the development of antibiotic resistance. This study aimed to investigate the impact of sRNA00203 on the expression levels of genes involved in these processes.

5.3 Materials and Methods

5.3.1 Bacterial strains and culture conditions

A. baumannii ST1894 was cultured on LB agar at 37 °C and stored at -80 °C in LB broth containing 20% glycerol. LB agar with kanamycin (50 µg/mL) and tetracycline (25 µg/mL) was used to select transformants.

5.3.2 Prediction of sRNA

Total RNA was isolated from untreated planktonic cells, untreated biofilm cells, imipenem-treated biofilm cells (half of the MIC), and colistin-treated biofilm cells (half of the MIC), as described in Section 4.3.3 and Figure 5-1. RNA-seq data were obtained from the transcriptome profiling study described in Chapter 4. Computational analysis of the sRNAs was performed in accordance with the standard pipeline used to analyse prokaryotic sRNAs from transcriptomic data, as presented in Figure 5-2. Accordingly, ncRNAs and unannotated RNAs with lengths of 50–500 nucleotides were identified using the software shown in Figure 5-2 (McClure et al., 2013). The identified sequences were searched against the NCBI NR database using BLASTx. Novel transcripts without annotation (NR) were regarded as sRNA candidates. BLASTn searches against the NCBI databases were used to evaluate whether the sRNA00203 sequences were conserved across the *Acinetobacter* genus.

The IntaRNA algorithm was used to determine sRNA target genes (Busch et al., 2008). The algorithm provides accurate and rapid predictions of RNA-RNA interactions, even delineating the accessibility of target sites and the presence of user-defined seeds. IntaRNA predicts sRNA-RNA interactions based on the minimisation of the extended hybridisation energy used to base-pair the sRNA to a target mRNA (Tjaden et al., 2006; Vogel & Wagner, 2007). IntaRNA has been shown to be more accurate than other

existing computing programs in terms of predicting bacterial sRNA targets and determining the exact locations of the interactions (Busch et al., 2008). RNAfold was used to predict and display the secondary structures associated with the minimum free energy for the RNA sequences (Hofacker & Stadler, 2006). In addition, quantitative estimates of sRNA expression were determined from the total RNA-seq data of *A. baumannii* ST1894 cells grown in the biofilm state and of biofilm cells subjected to sub-inhibitory concentrations of antibiotics, following the standard sRNA prediction pipelines used for prokaryotic sRNA analyses as illustrated in Figure 5-2.

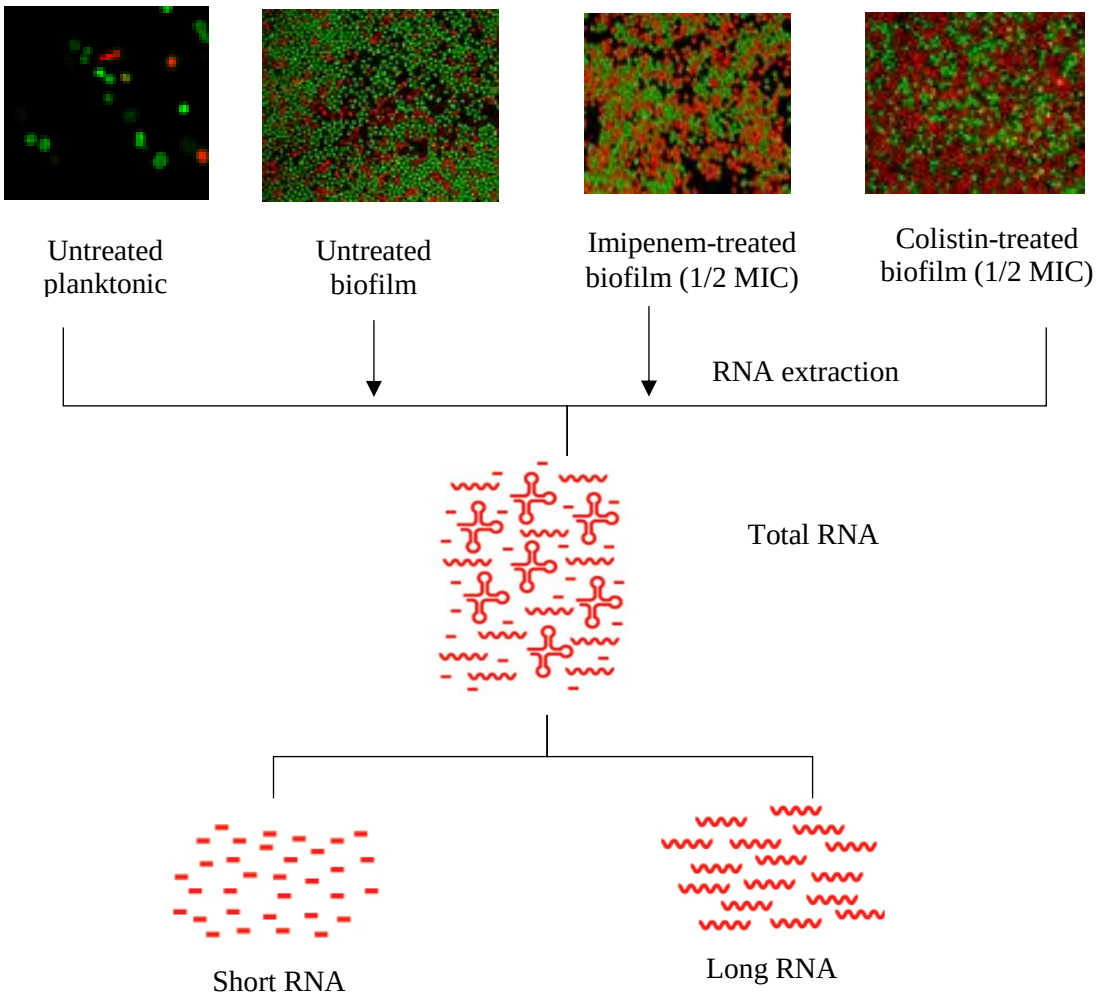


Figure 5-1. Conceptual framework of total RNA isolation from target samples.

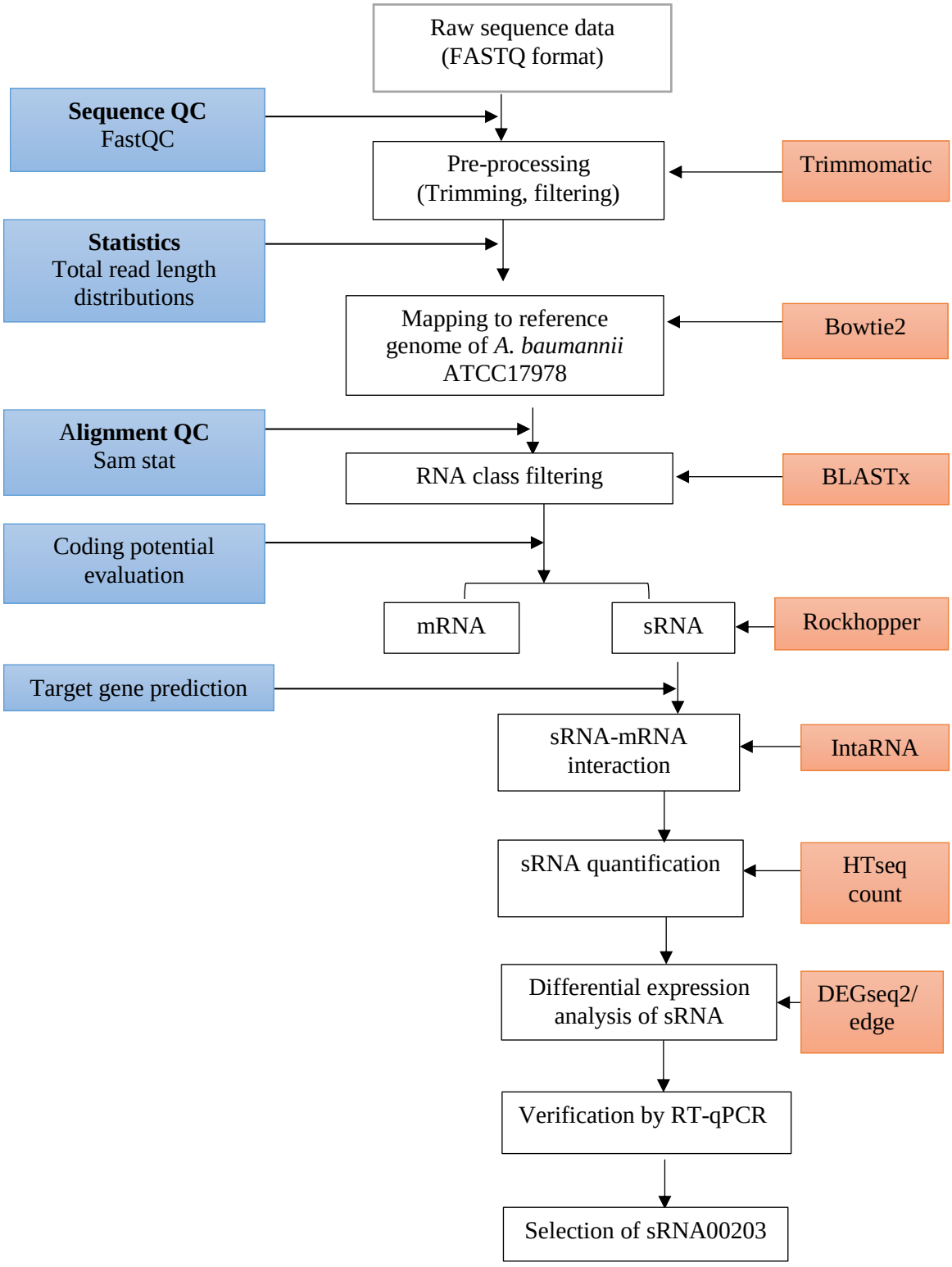


Figure 5-2. Conceptual framework for the prediction of sRNA. QC: quality control.

5.3.3 Quantification of the expression of sRNA00203

Rockhopper software was used to predict ncRNAs from the total RNA-seq data, as illustrated in Figure 5-2. One hundred thirty-eight sRNA sequences were identified from the total RNA-seq data of untreated biofilm cells, imipenem-treated biofilm cells, colistin-treated biofilm cells and planktonic cells. The number of read counts mapped to the intergenic regions in the genome was estimated using HTSeq V0.6.1 software (Trapnell et al., 2010). The FPKM was calculated to quantify the sRNA expression levels. Simultaneously, the log2 (FPKM biofilm/FPKM planktonic) change, which accounts for the effects of sequencing depth and gene length on the read count, was determined for each identified sRNA (Trapnell et al., 2010).

An sRNA was considered to be differentially expressed if the fold change was > 2 and a significant difference was observed in the number of sequencing read counts in biofilm cells relative to planktonic cells (corrected $p < 0.005$). Based on these criteria, only five sRNAs were found to be differentially expressed between biofilm cells and planktonic cells of *A. baumannii* ST1894. Of these, sRNA00203 was found to be most highly upregulated in biofilm cells compared to planktonic cells, as shown in Table 5-1.

Table 5-1. Differentially expressed sRNAs between biofilm cells and planktonic cells of *A. baumannii* ST1894 (RNA-sequencing data).

sRNA ID	**Start**	**End**	**Strand**	**Length (bp)**	**No. of target mRNA**	**Fold change**	***p* value**	***q* value**
sRNA00207	1338744	1338464	-	281	3,763	2.465705	0.001151	0.000106
sRNA00272	753263	753365	+	103	3,762	3.52861	0.004289	0.000379
sRNA00286	1677973	1678039	+	67	3, 762	-10.8807	0.010378	0.000886
sRNA00203	1245795	1245743	-	53	3, 749	42.08	5.09E-16	7.68E-17
sRNA00226	2214170	2213801	-	370	3,763	19.44328	4.81E-07	5.22E-08

5.3.4 Validation of sRNA00203 expression

The computational prediction pipeline revealed sRNA00203 to be the most significantly upregulated of the five differentially expressed sRNAs. sRNA00203 is also co-expressed with a target gene associated with biofilm formation and antibiotic resistance, the expression of which was verified using SYBR Green and TaqMan RT-qPCR assays. The gene sequence encoding sRNA00203 was retrieved from the total RNA-seq data of *A. baumannii* ST1894 and used as a reference for designing primers and probes for the RT-qPCR assay. cDNA was synthesised from the same RNA samples used for transcriptomic analysis to validate the differential expression profile of sRNA00203. RT was performed using a LunaScript RT Supermix Kit (NEB,Uk). The SYBR Green RT-qPCR reaction was performed using the Luna Universal qPCR Master Mix (NEB,Uk) in accordance with the manufacturer's protocol. SYBR Green RT-qPCR was used to quantify the copy number of cDNA synthesised from the total RNA samples of biofilm and planktonic samples and thus measure relative gene expression.

The sRNA00203 expression data obtained using the RNA-seq and SYBR Green qPCR techniques were verified using the TaqMan assay using the Luna Universal Probe qPCR Master mix (NEB,Uk). The *gyrB* gene was used as an internal control to normalise the expression levels of sRNA00203 across different samples. The relative expression of sRNA00203 in untreated or antibiotic-treated biofilms and planktonic cell controls was determined using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008).

5.3.5 Construction of sRNA00203 knockout strain

The gene encoding sRNA00203 was targeted for deletion using site-directed mutagenesis, specifically by the double allelic exchange method. A plasmid (pMo130)

was used to create chromosomal deletions of the genes encoding sRNA00203 by slightly modifying the protocol described in Amin et al. (2013). The plasmid pMo130-TelR is a suicide vector containing kanamycin and tellurite markers that can be used to delete genes in MDR *A. baumannii* strains (Amin et al., 2013).

Three DNA fragments harbouring the gene cassette encoding kanamycin resistance (*aph(3´)-I*, 795bp), the left flanking region (1000 bp upstream of sRNA00203) and the right flanking region (1001 bp downstream of sRNA00203) were amplified by PCR, as illustrated in Figure 5-3. The primers used to amplify these three fragments were designed using Vazyme software (CE Design; http://www.vazyme.com); the primer sequences are shown in Table 5-2. The restriction sites for the enzymes BamHI-HF and NdeI were incorporated at the 5´ termini of the amplified fragments on the left and right flanking regions. The same restriction enzymes were used to digest and linearise the plasmid vector (pMo130-TelR) (Figure 5-3). All three purified PCR products and linearised vectors were mixed in appropriate ratios to construct a plasmid using the ClonExpress Ultra-One Step Cloning Kit (Vazyme Biotech Co. Ltd, China), as shown in Table 5-3 and Figure 5-3. The optimal volumes of the inserts and linearised vectors were determined using the formula given in the ClonExpress Ultra One Step Cloning Kit manual (http://www.vazyme.com). The volumes and concentrations of the fragments used for the reactions are summarised in Table 5-3.

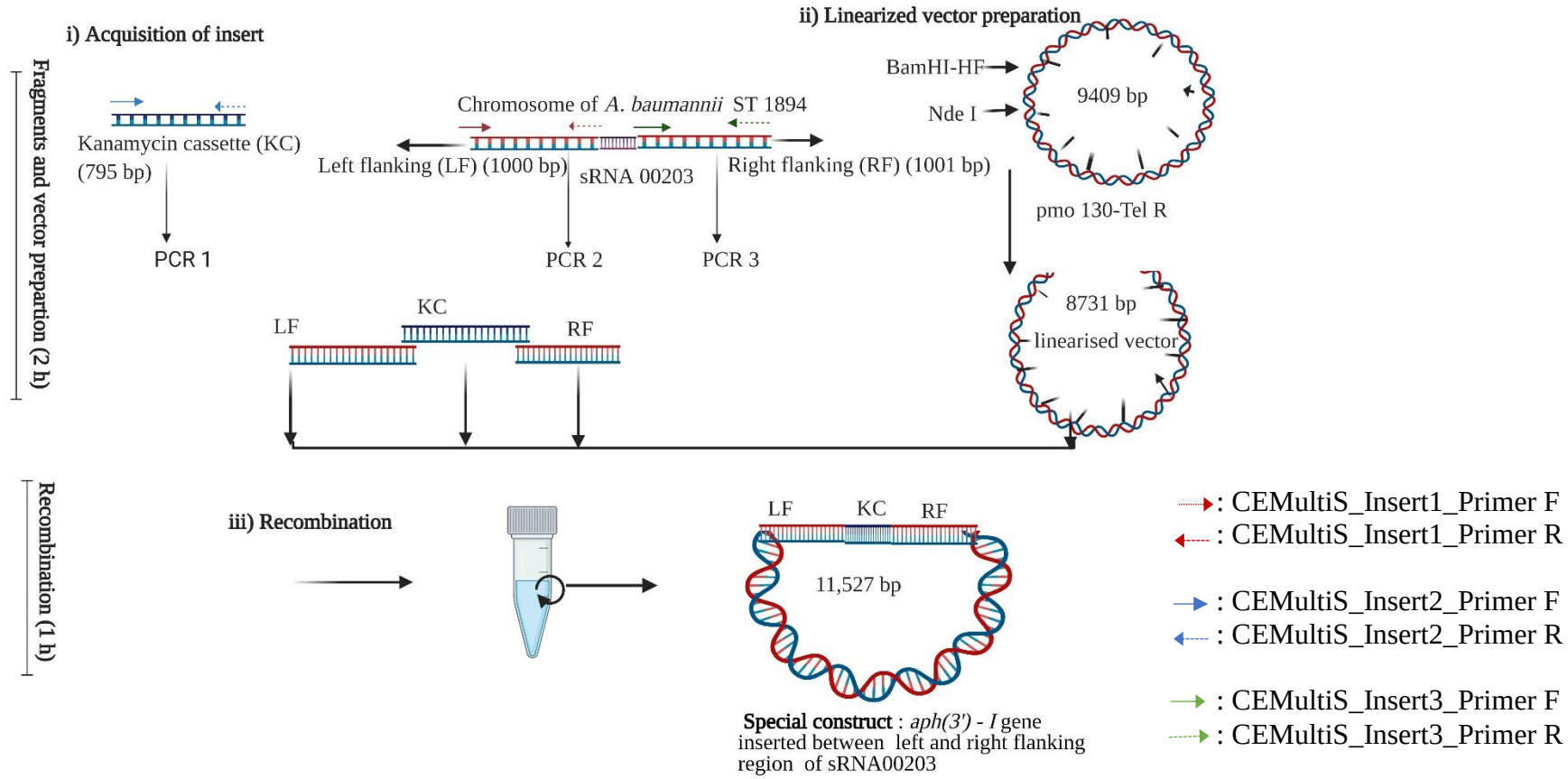


Figure 5-3. Construction of plasmid pMo130-TelR using the ClonExpress Ultra-One Step Cloning Kit.

Left flanking region (LF) (1000 bp upstream of sRNA00203), kanamycin resistance (KC) (*aph(3´)-I*, 795 bp), right flanking region (RF) (1001 bp downstream of sRNA00203).

Table 5-2. Primers used in the present study. Underlined sequences show restriction enzyme recognition sites.

Primer name	**Sequence (5´- 3´)**	**Use in the present study**
CEMultiS_Insert1_Primer F (NdeI)	agcgggatctgatggcatatgCATTTGCCAAAGAGTATCAGTCTGC	1 kb UP fragment: Construction of ΔsRNA00203 strain
CEMultiS_Insert1_Primer R	cttgacgagttcttctgTTGAAATGAACGCTTAAGATTAGGA	
CEMultiS_Insert2_Primer F	caaCAGAAGAACTCGTCAAGAAGGCG	795 bp *aph(3´)-I* gene:Construction of ΔsRNA00203 strain
CEMultiS_Insert2_Primer R	cgacgcgtgtgcataaaaaATGATTGAACAAGATGGATTGCAC	
CEMultiS_Insert3_Primer F	tTTTTTATGCACACGCGTCGT	1001 bp DOWN fragment: Construction of ΔsRNA00203 strain
CEMultiS_Insert3_Primer R (*Bam*HI-HF)	cgcatgcatctagagggatccGCATCTTGCCATTCTTGGGTT	
KMRF203_F	gctgcctcgtcttggagt	Confirmation of deletion in the ΔsRNA00203 strain
KMRF203_R	TAGCTGTCGTTGTGCCGTA	
LFKM203-F	CACACGACTTGCCATTGTTC	
LFKM -203R	CGGACCGCTATCAGGACATA	
sRNA 203 Fw-ndeI	CATATGCCGATATCAAGGAAGAGATCATAAGG	Cloning the sRNA00203-encoding gene into the pWH1266 plasmid to complement the ΔsRNA00203 strain
sRNA 203 Rv-ndeI	CATATGCCTCTTGAAACTGTTCTTACTCAAGTTC	

Table 5-3. Volumes of linearsed vectors and inserts used to construct plasmids.

Components	**Recombination**	**Negative control 1**	**Negative control 2**
Linearised vector PMO 130	6 μL	6 μL	0
Insert (n = 3), 1 μL each	3 μL	0 μL	3 μL
2× ClonExpress Mix	10 μL	10 μL	10 μL
ddH_2O	1 μL	4 μL	7 μL

The reactions comprising the linear vector pMo130-TelR and all inserts were gently mixed and incubated at 50 °C for 50 min. Electrocompetent *A. baumannii* ST1894 cells were prepared using the protocol described by Richard Lab in OpenWetWare (https://openwetware.org/wiki/Richard_Lab:Preparing_electrocompetent_cells) and transformed with the recombinant plasmid as shown in Figure 5-4. Subsequently, 100 μL of the inoculum were transferred to 900 μL of LB broth and incubated at 37 °C with agitation at 200 rpm for 1 h, after which 1 mL of the inoculum was centrifuged at 5,000 rpm for 5 min. We discarded 900 μL of the supernatant and inoculated the remaining 100 μL containing sediment onto LB agar containing kanamycin (50 μg/mL), which was incubated at 37 °C with agitation at 200 rpm for 72 h.

Potential transformant colonies were grown on LB agar plates containing kanamycin and used for DNA extraction. To confirm the complete deletion of sRNA00203, PCR and Sanger sequencing were performed to detect the overlap region of the kanamycin resistance-encoding genes and the two flanking regions of sRNA00203 (Figure 5-5)

.

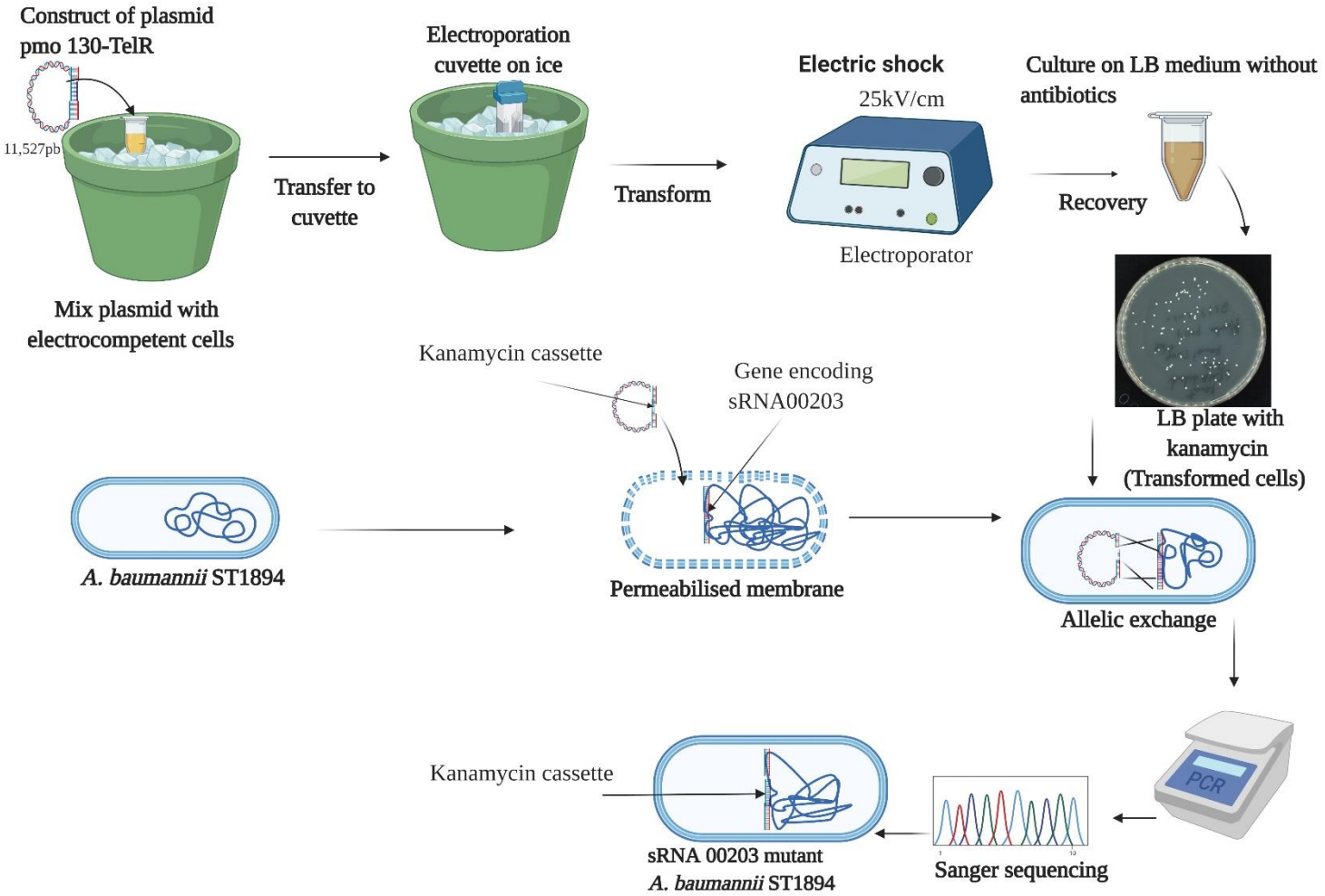


Figure 5-4. Workflow for the deletion of sRNA00203 gene using allelic exchange.

5.3.6 Confirmation of sRNA00203 knockout strain

The plasmid constructed from pMo130-TelR facilitated allelic exchange in the *A. baumannii* ST1894 genome and resulted in the insertion of the *aph(3´)-I* gene and deletion of the gene encoding sRNA00203. LB agar containing kanamycin (50 µg/mL) was used to select the sRNA00203 knockout strain. Single colonies grown on selective media were picked and sub-cultured on LB broth for 24 h and 10 passages to eliminate the plasmid construct (pMo130TelR). A broth culture of the tenth sub-culture was used to prepare cell pellets from which DNA was extracted using a Favorprep Blood Genomic DNA Extraction Mini Kit (Favorgen biotech. Corp, Taiwan). The extracted genomic DNA was used to validate the deletion of sRNA00203 using PCR and Sanger sequencing.

To confirm the insertion of the *aph(3´)-I* gene into the genome of A. *baumannii* ST1894, the junctions between the flanking regions and the *aph(3´)-I* gene were amplified and sequenced (Figure 5-5). The primers LFKM203-F and LFKM -203R were used to amplify the sequence containing the junction of the left flanking region of the gene encoding sRNA00203 and *aph(3´)*-I. Similarly, the primers KMRF203_2_F and KMRF203_2_R were used to amplify the junction of the right flanking region of the gene encoding sRNA00203 and *aph(3´)-I* (Table 5-2); the expected PCR product was 461 bp long. For PCR verification, a 50-µL reaction containing 25 µL of 2× Phanta Max buffer *(*Nanjing, China), 1 µL of dNTP mix (10 mM each), 2 µL of forward primer (10 µM), 2 µL of reverse primer (10 µM), 1 µL of Phanta Max Super-Fidelity DNA Polymerase (1 U/µL), 2 µL of DNA and 17 µL of nuclease-free water was used.

The PCR reaction conditions were pre-denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, and extension at

72 °C for 30 s and a final extension of 72 °C for 5 min. The PCR products were separated using 1.5% agarose gel electrophoresis and stained with 0.5 μg/mL of RedSafe nucleic acid staining solution (iNtRON Biotechnology, Korea). The gel was visualised using a Gel Doc Method XR (Bio-Rad Laboratories).

The specific PCR product generated using the primers KMRF203_2_F and KMRF203_2_R was purified using the Monarch® PCR and DNA Cleanup Kit (NEB). The purified product was subjected to Sanger sequencing to confirm the insertion of *aph(3´)-I* between the left and right flanking regions of sRNA00203 (sequencing service provided by Tech Dragon, Hong Kong). *A. baumannii* ST1894 with the *aph(3´)-I* gene inserted between the left and right flanking regions of sRNA00203 was confirmed to be the knockout strain and was stored in 20% glycerol at 80 °C until further use.

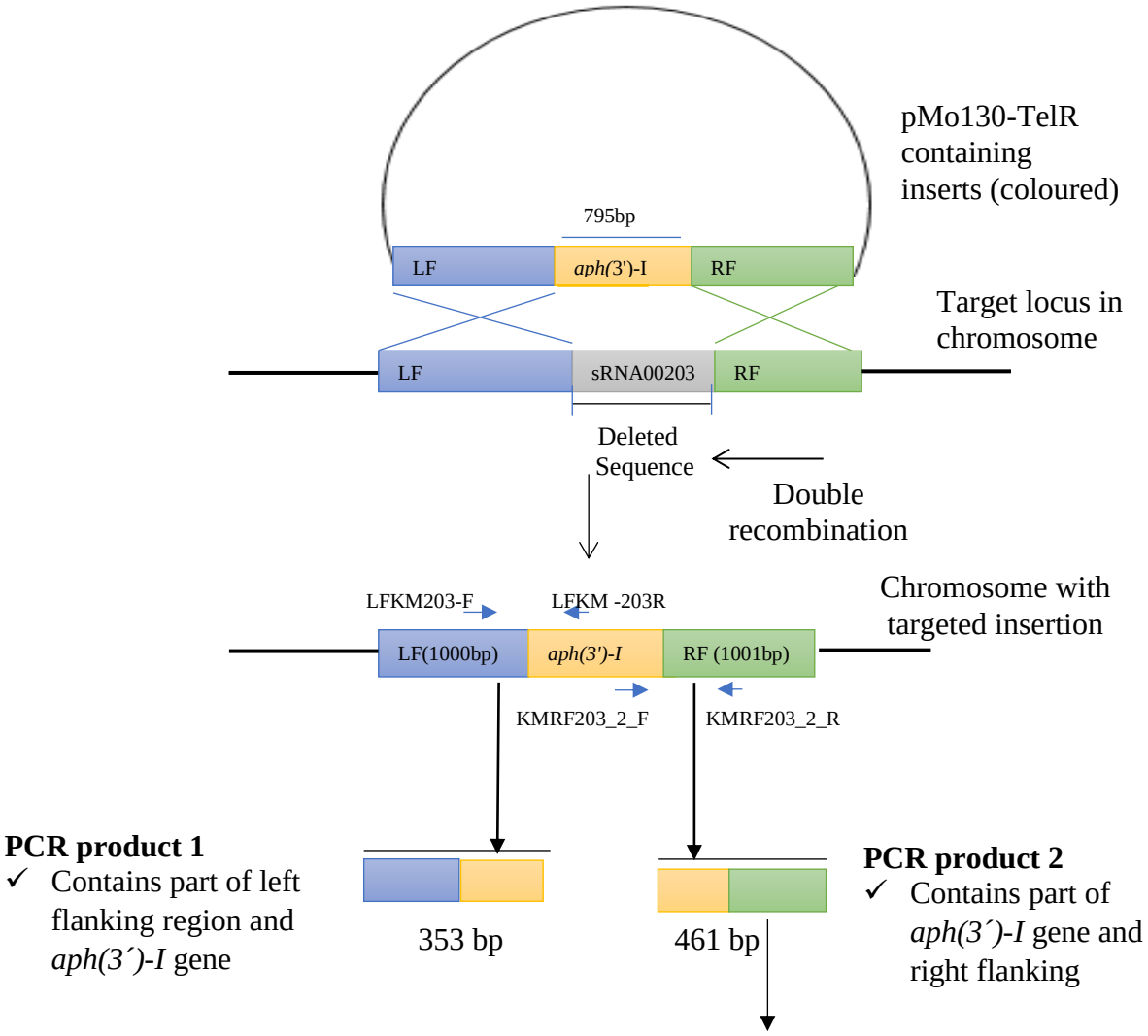


Figure 5-5. Workflow for the confirmation of sRNA00203 knockout in *A. baumannii* ST1894.

The inserted sequence contains the kanamycin encoding gene (*aph(3´)-I*) (795 bp) used to replace the gene encoding sRNA00203 (53 bp). LF: Left flanking region; RF: right flanking region.

5.3.7 Complementation of stable mutant with sRNA00203

The gene encoding sRNA00203 was complemented into the sRNA0023 mutant strain of *A. baumannii* ST1894. This process required the preparation of inserts and linearised vectors, ligation and transformation into competent *A. baumannii* ST1894 cells mutant for sRNA00203. The gene encoding sRNA00203 was amplified from the *A. baumannii* ST1894 genomic DNA. The primer used to amplify this insert incorporated the NdeI restriction site at its 5´ end, as shown in Table 5-2. The PCR product was purified using a Monarch® PCR and DNA Cleanup Kit (NEB). A 2-µg aliquot of purified PCR product was digested with NdeI in accordance with the manufacturer's protocol (NEB,Uk). The NdeI-digested PCR products were then purified using the Monarch® PCR and DNA Cleanup Kit (NEB,Uk).

The pWH1266-TelR plasmid (a generous gift from Professor Margarita Poza Dominguez, Complexo Hospitalario Universitario, Spain), which contains tellurite and tetracycline markers, was used as a shuttle vector. The plasmid was imported from Spain in *E. coli*; immediately after delivery, the bacteria were grown in LB media containing potassium tellurite (50 µg/mL). A single *E. coli* colony was selected and sub-cultured in LB broth containing potassium tellurite for 24 h. The plasmid was then extracted from the broth culture using a QIAfilter Plasmid Midi Kit (Qiagen, Center Mainz, German), purified and linearised by digestion with NdeI, in accordance with the manufacturer's protocol (NEB,Uk). After digestion, 3 µg of the linearised plasmid (pWH1266-TelR) was treated with quick calf-intestinal alkaline phosphatase (CIP) (NEB,Uk) to prevent plasmid self-ligation and enable the successful ligation of the target fragment with the sRNA00203-encoding gene. CIP-treated and linearised pWH1266-TelR was purified using a Monarch® PCR and DNA Cleanup Kit (NEB).

The NdeI-digested PCR products of the sRNA00203-encoding gene were cloned into a vector (pWH1266-TelR) using a T4 DNA ligase-dependent ligation protocol (NEB, UK), as outlined by the manufacturers (M02020). Ten microliters of the ligation reaction were mixed with 100 µL of competent cells prepared from sRNA00203-mutant strains of *A. baumannii* ST1894, using the protocol described by Richard Lab in open Wetwarehttps://openwetware.org/wiki/Richard_Lab:Preparing_electrocompetent_cells. Transformants were selected by growing cells on LB agar plates with tetracycline (25 µg/mL) and kanamycin (50 µg/mL) for 48 h at 37 °C. PCR was performed to confirm the success of sRNA00203 complementation by using a pair of primers (illustrated in Table 5-2) to detect the gene encoding sRNA00203 (sRNA203 Fw-ndeI and sRNA203RV-ndeI). The expression of sRNA00203 was also evaluated to validate the sRNA00203-complemented strain of *A. baumannii* ST1894.

5.3.8 Biofilm formation assay

Qualitative biofilm formation by the wild-type, sRNA00203-mutant and sRNA00203-complemented strains of *A. baumannii* ST1894 was evaluated using the CBD in accordance with procedures described in Section 3.3.4 of this thesis.

5.3.9 Quantification of the biofilm mass

Biofilm masses formed on a 96-well microliter plate were quantified using the crystal violet staining method as described by Stepanović et al. (2000) and in Section 3.3.5. The mean ODs of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were determined, and biofilm formation capability was defined in accordance with the guidelines mentioned in Stepanović et al. (2000).

5.3.10 Antibiotic susceptibility test for biofilms

Biofilms were subjected to antibiotic susceptibility tests in accordance with procedures described in Moskowitz et al. (2004). The MBICs for imipenem, colistin and ciprofloxacin in the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were determined using broth microdilution techniques, as described in Section 3.4.3 of this thesis. In addition, the MBECs for imipenem, colistin and ciprofloxacin in the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were measured using the CBD, as described in Section 3.4.3 of this thesis.

5.3.11 Impact of sRNA00203 deletion on DEGs

Samples of planktonic and biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were prepared and processed for total RNA isolation in accordance with the protocol described in the PureLink^{TM} RNA Mini Kit (Ambion, USA). Once the isolated RNA was found to fulfil all of the quality assessment criteria described in Section 4.3.4, 500 ng of each RNA sample were processed for cDNA synthesis, which was conducted using a LunaScript RT SuperMix Kit (NEB) as described in Section 4.3.8. To determine the effects of sRNA00203 deletion on DEGs, RT-qPCR was conducted to evaluate the expression levels of the antibiotic-induced biofilm-specific genes listed in Table 5-4. The effect of sRNA00203 on the expression of DEGs involved in matrix synthesis, antibiotic resistance, bacterial secretion and regulatory pathways was investigated before and after deleting the sRNA00203-encoding gene. The expression levels of these genes were also assessed after complementation with sRNA00203. The primers and probes used to validate the expression of these genes are listed in Table 4-3.

SYBR Green and TaqMan assays were conducted using a Luna Universal qPCR Mastermix (NEB,Uk) and Luna Universal Probe qPCR Mastermix (NEB,Uk), respectively. The expression levels in the biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were compared with those in the corresponding planktonic cells. The *gyrB* gene was used as an internal control to normalise the expression of target genes between planktonic and biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains. The expression levels of the antibiotic-induced biofilm-specific genes listed in Table 5-4 were calculated for the wild-type, sRNA00203-mutant and sRNA00203-complemented strains relative to the wild-type strain planktonic cells using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008).

5.3.12 Statistical analysis

RT-qPCR gene expression data were analysed using GraphPad Prism v7.0 (GraphPad Software Inc, California, USA) and are presented as means ± standard deviations (SD). The Shapiro-Wilk test was used to test the normality of data distribution. The mean OD at 570 nm, MBIC/MIC, MBEC/MBC and fold changes in the expression levels of the target genes were compared between the wild-type, sRNA00203-mutant and sRNA00203-complemented strains using one-way analysis of variance (ANOVA). If the difference was significant ($p < 0.05$), a *post-hoc* test was conducted between datasets to identify pairs with significant differences. Tukey's *post-hoc* test was used to analyse the data and compare differences between each paired category. Differences were regarded as statistically significant at a p value < 0.05.

Table 5-4. Antibiotic-induced biofilm-specific genes targeted to study the regulatory effects of sRNA00203.

mRNA_ID	KEGG_ID	KEGG Annotation	Strand	Start	End	Length (bp)	sRNA00203 position	mRNA position	Pathway
E5A72_RS12100 (*pgaB*)	acb: A1S_0938	Poly-beta-1,6-N-acetyl-D glucosamine N-deacetylase	+	2520896	2522890	1,995	31–41	1584–1594	✓ Biofilm matrix
Novel00738	acb: A1S_2736	RND family drug transporter	+	617454	623989	6,536	6–27	461–492	✓ β-lactam resistance ✓ Cationic antimicrobial peptide resistance
Novel00626 (*lpxA*)	acb: A1S_1965	UDP-N-acetylglucosamine acyltransferase	-	3676375	3677683	1,309	25–34	265–274	✓ Cationic antimicrobial peptide resistance ✓ Lipopolysaccharide biosynthesis
E5A72_RS03500	acb: A1S_2862	Preprotein translocase subunit SecA	-	756066	758789	2,724	7–50	2653–2695	✓ Quorum sensing ✓ Protein export ✓ Bacterial secretion system
E5A72_RS13515	acb: A1S_1182	CRP transcriptional regulator	-	2818333	2819040	708	8–48	204–244	✓ Two-component system ✓ Quorum sensing

RND: resistance–nodulation–cell division; CRP: cyclic-AMP receptor protein.

5.4 Results

5.4.1 Differentially expressed sRNAs

sRNA-encoding genes accounted for 2.6% (138) of the 5,249 genes identified from the transcriptomic data of *A. baumannii* ST1894. The identified sRNAs were 50–420 bp long, as illustrated in Figure 5-6. Among these sRNA-encoding genes, 3.6% (5/138) were differentially expressed in biofilms relative to planktonic cultures (Table 5-1). The sequences of these five differentially expressed sRNAs (shown in Appendix 2) were conserved across the 465 *Acinetobacter* genera deposited in the NCBI database. sRNA00203 was the most highly upregulated of the five differentially expressed sRNA. Therefore, the effects of sRNA00203 on the expression of antibiotic-induced biofilm-specific genes were studied.

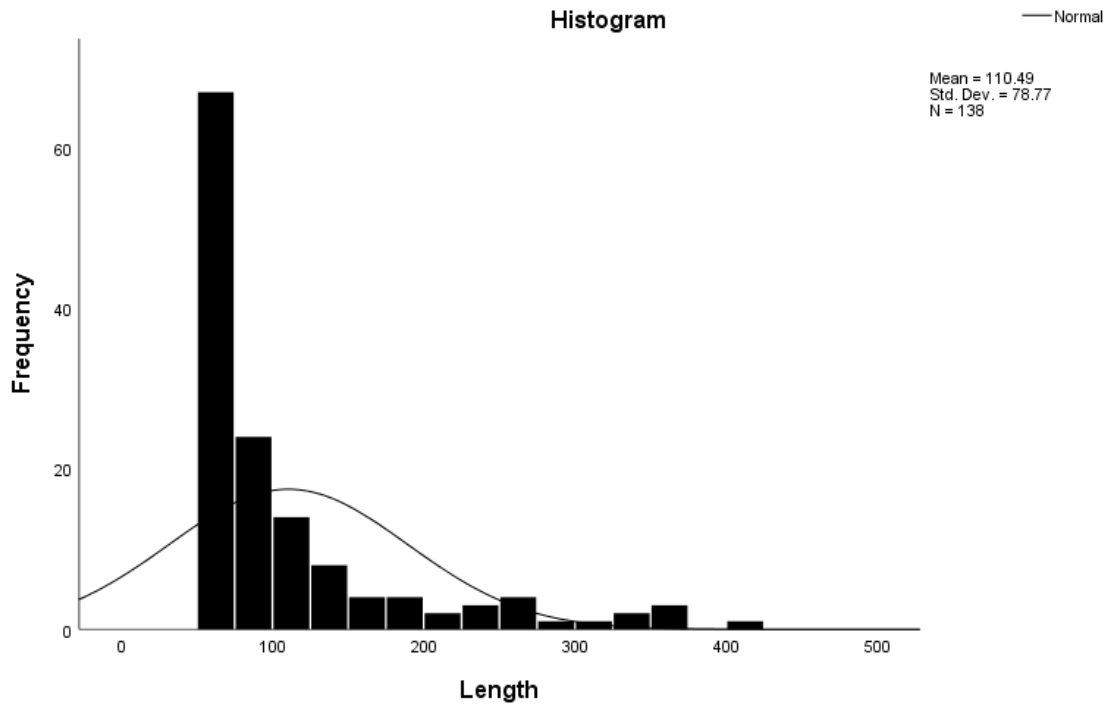


Figure 5-6. Distribution of sRNA sequence lengths identified in *A. baumannii* ST1894.

5.4.2 Confirmation of sRNA00203 expression

The expression level of sRNA00203 in biofilms relative to planktonic cultures was assessed using RT-qPCR. The expression of sRNA00203 was upregulated 45-fold in biofilm cells compared to planktonic cells (Figure 5-9), which is similar to the RNA-seq results, as shown in Section 4.4.5 of this thesis. The BLASTp search revealed that the sRNA00203 sequence lacks any recognisable peptide-coding sequence, implying that it does not express any active biological peptides.

5.4.3 Construction of the ΔsRNA00203 strain

The gene encoding sRNA00203 was replaced by the *aph(3´)*-I gene through allelic exchange using the pMo130 TelR plasmid. The success of the knockout was confirmed by amplifying and sequencing the junction of the *aph(3´)*-I gene and the right flanking region of sRNA00203 (Figure 5-7). The insertion of *aph(3´)-I* was verified by Sanger sequencing to identify the sequence of the junctions containing part of the *aph(3')-I* gene and the right flanking region of sRNA00203, as indicated in Figure 5-8. Furthermore, the expression level of sRNA00203 in the knockout strains was close to zero, as confirmed by RT-qPCR (Figure 5-9). Based on these findings, it was concluded that the gene encoding sRNA00203 was deleted from *A. baumannii* ST894.

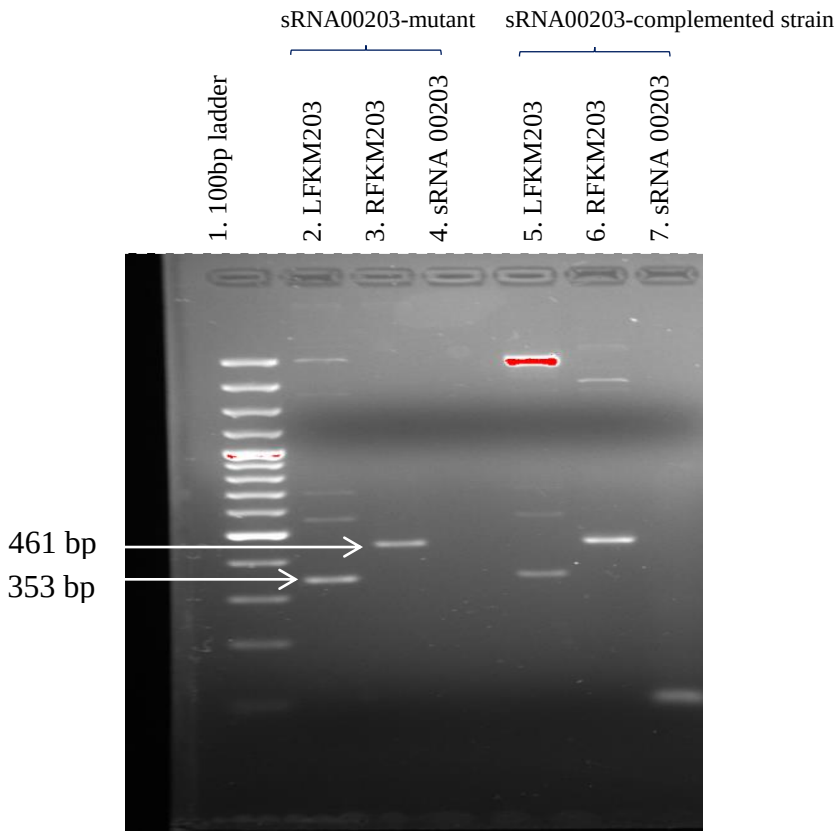


Figure 5-7. PCR products of the overlapping regions of the sRNA00203-mutant and sRNA00203-complemented strains of *A. baumannii* ST1894.

Lane 1: 100bp+ DNA ladder. Lanes 2–4: samples obtained from the genomic DNA of the sRNA00203-mutant strains of *A. baumannii* ST1894; Lane 2: left flanking region and kanamycin cassette (353 bp); Lane 3: right flanking region and kanamycin cassette (461 bp); Lane 4: sRNA00203. Lanes 5–7: samples obtained from the genomic DNA of sRNA00203-complemented strains of *A. baumannii* ST1894; Lane 5: left flanking region and kanamycin cassette (353 bp); Lane 6: right flanking region and kanamycin cassette (461 bp); lane 7: sRNA00203.

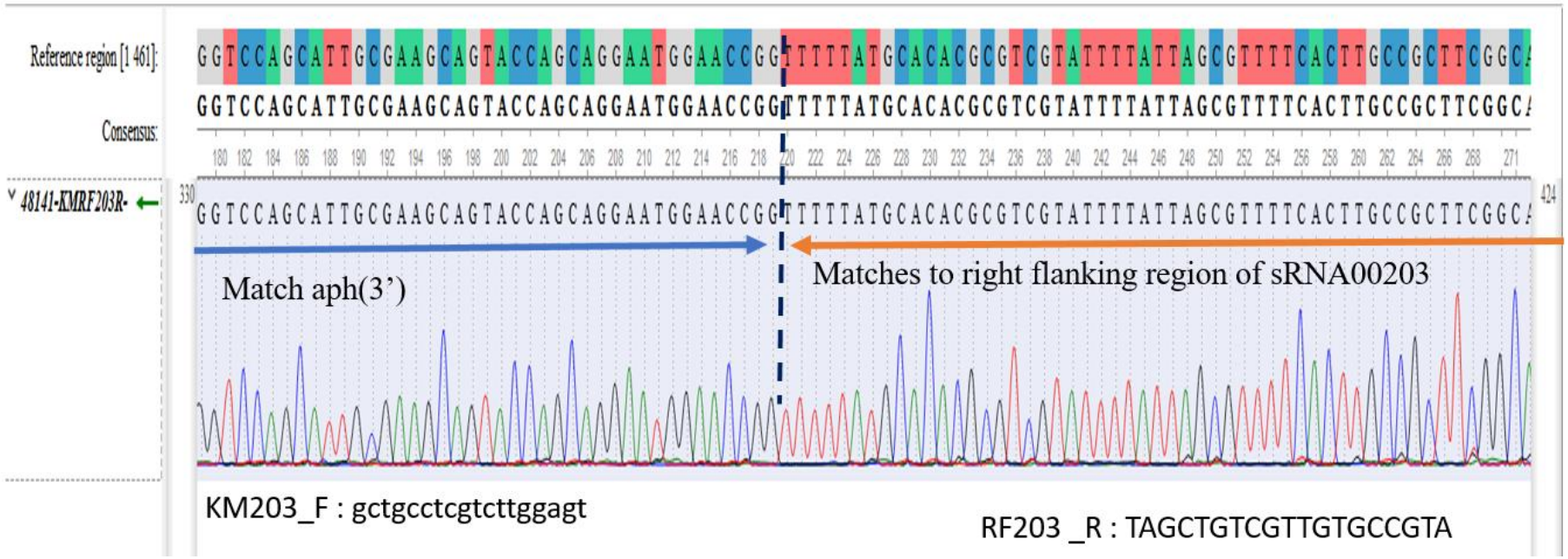


Figure 5-8. Verification of sRNA00203 knockout in *A. baumannii* ST1894.

The success of the transformation and deletion of sRNA00203 by Sanger sequencing was determined using the KMRF203 F and RF203 R primer pairs.

5.4.4 Construction of sRNA00203-complemented strain

The sRNA00203-mutant strain was complemented with the gene encoding sRNA00203 to assess whether the function of this sRNA could be restored. The success of complementation was ensured by growing transformants on LB agar plates containing tetracycline (25 μg/m:) and kanamycin (50 μg/mL) incubated at 37 °C for 48 h. The plasmid DNA was extracted, and PCR was performed to confirm the success of complementation by detecting the sRNA00203-encoding gene (Figure 5-7). In addition, the expression level of sRNA00203 in the biofilm of the sRNA00203-complemented strain was found to be 28.4 times higher than that in the planktonic cells of wild-type strains, as shown in Figure 5-9.

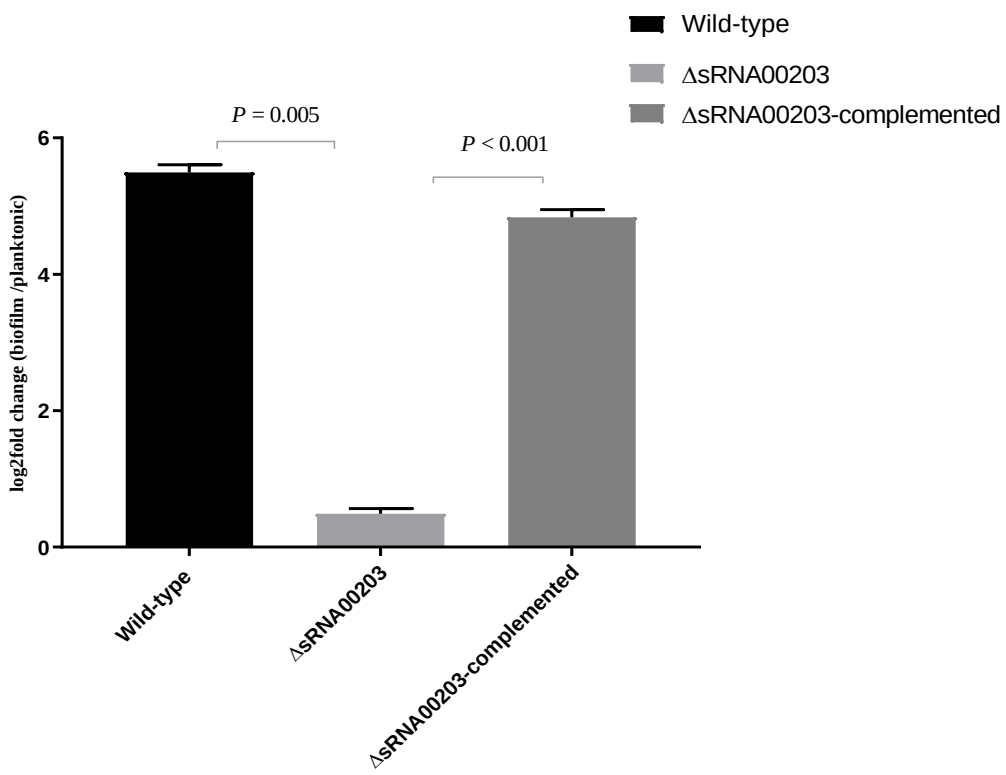


Figure 5-9. Expression levels of sRNA00203 in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented *A. baumannii* ST1894 strains.

The mean $\log_2$ fold changes in the three biofilm groups relative to planktonic cells of the wild-type strain are shown. Three independent biological replicates were evaluated for each group. The error bars represent standard deviations between different replicates. Analysis of variance (ANOVA) and a *post-hoc* test were performed to evaluate the statistical differences between the three groups. A p value < 0.05 was regarded as a significant difference.

5.4.5 Effect of sRNA00203 inactivation on biofilm formation

The sRNA00203-mutant strain was created to understand the impact of this sRNA on the expression of genes associated with biofilm formation and antibiotic resistance. The biofilm formation capabilities of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were evaluated quantitatively using the crystal violet stain elution method. The quantitative biofilm assay confirmed the presence of a significant difference in the biofilms formed by the wild-type and sRNA00203-mutant strains (Figure 5-10). Deletion of the sRNA00203-encoding gene substantially decreased the biomass of the biofilm cells by 85% compared with that of wild-type strains ($p < 0.0001$). The deletion also converted the hyper biofilm-producing strain *A. baumannii* ST1894 into a weak biofilm producer. The biofilm formation capability of the sRNA00203-complemented strain was restored and comparable to that of the wild-type strain (Figure 5-10). These findings imply that sRNA00203 is involved in the regulation of genes responsible for biofilm formation in *Acinetobacter* strains.

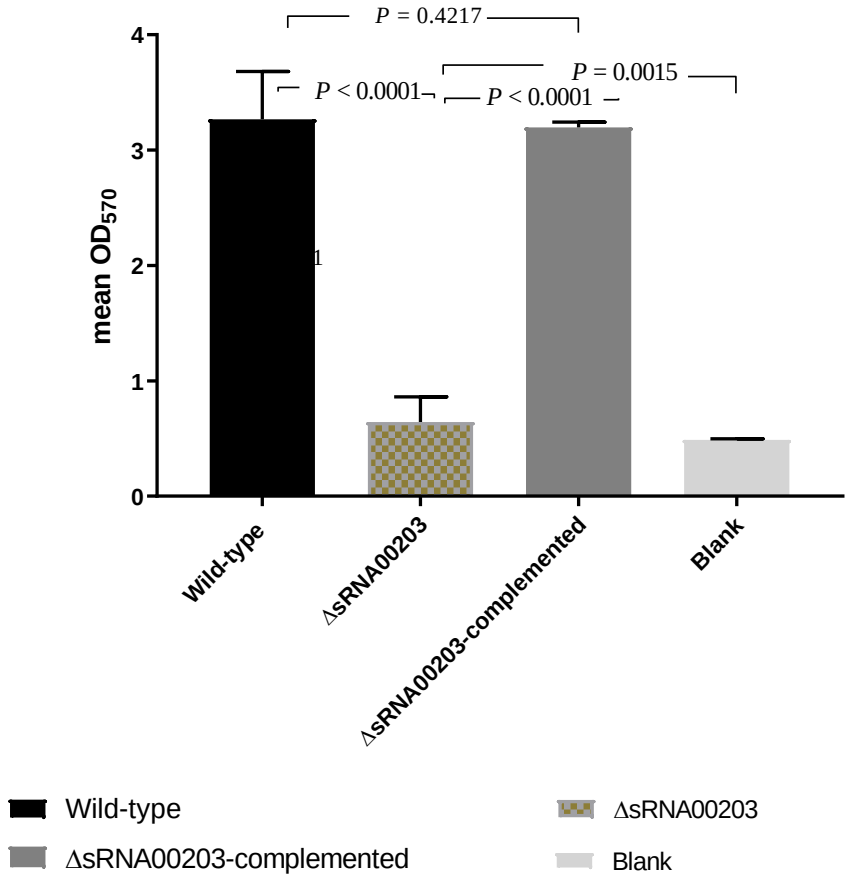


Figure 5-10. Effect of sRNA00203 inactivation on the biofilm formation capabilities of the wild-type, sRNA00203-mutant and sRNA00203-complemented *A. baumannii* ST1894 strains.

Biofilm formation was quantitatively assessed using the crystal violet stain elution method. Twenty-four independent biological replicates were performed for each group of strains and blanks. The error bars represent standard deviations between different replicates. Analysis of variance (ANOVA) and *post-hoc* tests were performed to analyse the statistical differences between the three groups. A p value < 0.05 was regarded as statistically significant.

5.4.6 Impact of sRNA00203 on the antibiotic susceptibility of biofilms

The MICs and MBICs for colistin, ciprofloxacin and imipenem were assessed in the wild-type, sRNA00203-mutant and sRNA00203-complemented strains. All three strains were susceptible to all three antibiotics when tested in the planktonic phase. The antibiotic susceptibility profiles of the biofilm and planktonic cells of three strains for colistin, ciprofloxacin and imipenem are summarised in Table 5-5. The MBIC of ciprofloxacin for the sRNA00203-mutant strain decreased substantially by 128-fold and 64-fold compared with the wild-type and sRNA00203-complemented strains, respectively.

Similarly, the MBIC of the sRNA00203-mutant strain for imipenem decreased by 1,024-fold and 512-fold compared with the wild-type and sRNA00203-complemented strain, respectively. When the gene encoding sRNA00203 was deleted, the *A. baumannii* ST1894 biofilm became susceptible to ciprofloxacin and imipenem. Deleting the gene also reduced the MBIC of colistin by half compared with the wild-type and sRNA00203-complemented strains, as summarised in Table 5-5. However, the sRNA00203-mutant strain remained resistant to colistin in the biofilm state, even though its susceptibility was comparable to the MIC of planktonic cells.

Table 5-5. Impact of sRNA00203 deletion on the antibiotic susceptibility of *A. baumannii* ST1894 biofilms.

	Colistin			**Ciprofloxacin**			**Imipenem**		
***A. baumannii* ST1894 strain**	**MIC for planktonic cells (µg/mL)**	**MBIC for biofilm cells (µg/mL)**	**Fold change**	**MIC for planktonic cells (µg/mL)**	**MBIC for biofilm cells (µg/mL)**	**Fold change**	**MIC for planktonic cells (µg/mL)**	**MBIC for biofilm cells (µg/mL)**	**Fold change**
Wild-type	0.5	32	64	1	64	64	0.125	256	2048
Mutant	0.5	16	32	1	0.5	0.5	0.125	0.25	2
sRNA00203-complemented	0.5	32	64	1	32	32	0.125	128	1,024

MIC: minimum inhibitory concentration; MBIC: minimum biofilm inhibitory concentration.

5.4.7 Effect of sRNA00203 deletion on the antibiotic tolerance of biofilms

The MBEC assay was performed to evaluate the antibiotic concentrations required to eradicate biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented *A. baumannii* ST1894 strains (Table 5-6). For the sRNA00203-mutant strain, the MBECs for colistin and ciprofloxacin were 256 μg/mL and 1024 μg/mL, respectively (Table 5-6); this value for colistin was the same as that in the wild-type strain and higher than that in the sRNA00203-complemented strain. The MBEC for imipenem in the sRNA00203-mutant was 8 μg/mL, which corresponded to an approximately 128-fold reduction compared with the wild-type and sRNA00203-complemented strains

Table 5-6. Impact of inactivation of sRNA00203 on biofilm eradication by antibiotics

	Colistin			Ciprofloxacin			Imipenem		
A. baumannii ST 1894 strains	**MBC (µg/mL)**	**MBEC (µg/mL)**	**Fold Change**	**MBC (µg/mL)**	**MBEC (µg/mL)**	**Fold Change**	**MBC (µg/mL)**	**MBEC (µg/mL)**	**Fold Change**
Wild-type	4	256	64	8	>1,024	>128	4	>1,024	>128
sRNA00203-mutant	4	256	64	8	1,024	128	4	8	2
sRNA00203-complemented	4	128	32	8	512	128	4	1,024	128

MBC: minimum bactericidal concentration (performed in planktonic cells); MBEC: minimum biofilm eradication concentration.

5.4.8 Involvement of sRNA00203 in the regulation of antibiotic-induced biofilm-specific genes

Analysis of the RNA-seq data obtained from untreated biofilm cells and antibiotic-treated biofilm cells of *A. baumannii* ST1894 revealed several antibiotic-induced biofilm-specific genes. sRNA00203 interacts through base-pairing with the mRNAs of these antibiotic-induced biofilm-specific genes, as shown by the positions of the sRNA-mRNA interactions listed in Table 5-4. Therefore, we assumed that sRNA00203 could regulate the expression of genes involved in biofilm formation or the development of antibiotic resistance.

5.4.8.1 Impact of sRNA00203 on the expression of *pga*B

The expression levels of the *pgaB* gene were compared across the biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented *A. baumannii* ST1894 strains. The level of *pgaB* expression was 12.6 times higher in biofilm cells than in planktonic cells of the wild-type strain. When sRNA00203 expression was suppressed, the expression of *pgaB* in biofilm cells increased only by 1.42-fold compared with that in planktonic cells (Figure 5-11). In the sRNA00203-complemented biofilms, the *pgaB* expression was 9.4 times higher than in the planktonic cells of the wild-type strain. These results indicate that the deletion of sRNA00203 significantly reduces the expression of *pgaB* , which might reduce synthesis of the biofilm matrix ($p = 0.007$).

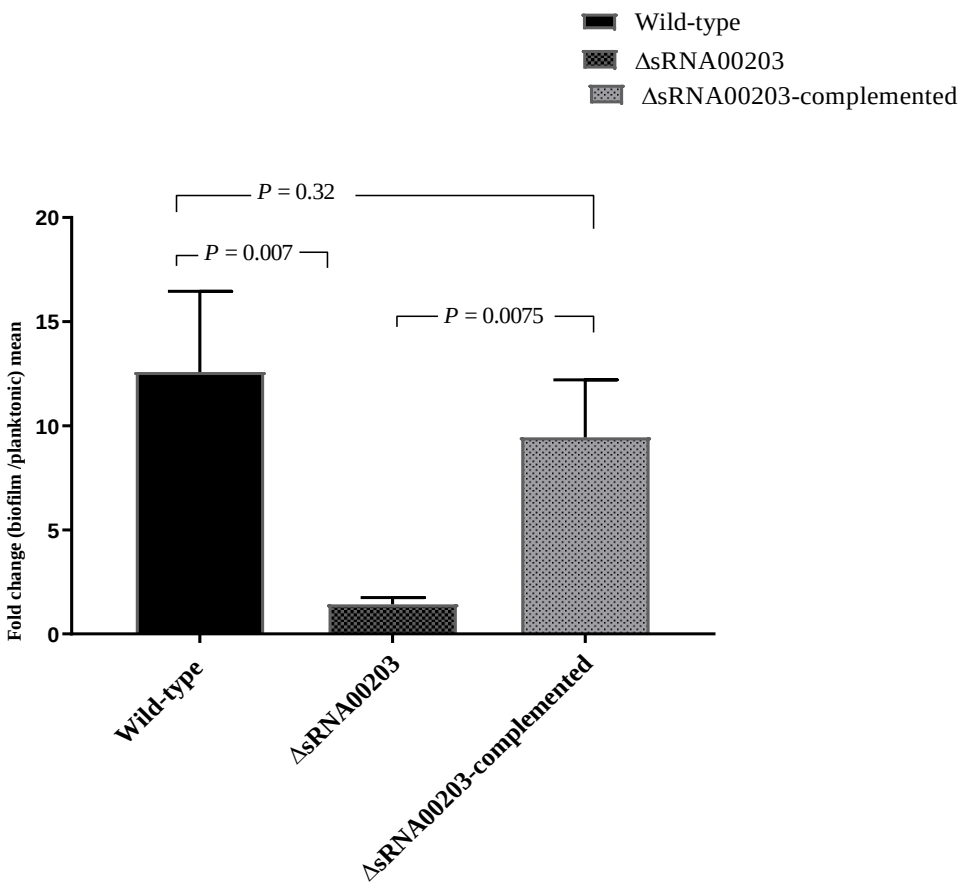


Figure 5-11. Expression levels of *pgaB* in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented *A. baumannii* ST1894 strains.

The mean fold changes in the three biofilm groups relative to planktonic cells of the wild-type strain are shown. Three independent biological replicates were analysed for each group. The error bars represent standard deviations between different replicates. Analysis of variance (ANOVA) and *post-hoc* tests were performed to assess the statistical differences between the three groups. A p value < 0.05 was regarded as being statistically significant.

5.4.8.2 Impact of sRNA00203 on the expression of efflux pump-encoding genes

The novel00738 gene, which is believed to associate with RND family drug transporters, was differentially expressed in *A. baumannii* ST1894 biofilm cells compared with planktonic cells. Transcriptomic data analysis revealed that the novel00738 gene was uniquely expressed in biofilms, indicating its involvement in the efflux of antibiotics during biofilm growth. sRNA00203 can control the expression of the novel00738 gene by binding to the corresponding mRNA. The expression of novel00738 in biofilms was 24.9-fold, 3.83-fold and 30.4-fold higher than in planktonic cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains, respectively. This finding indicates that deletion of the sRNA00203 gene significantly reduces the expression of novel00738 in the mutant strain compared with the wild-type ($p = 0.003$) and sRNA00203-complemented strains ($p = 0.0025$), as shown Figure 5-12.

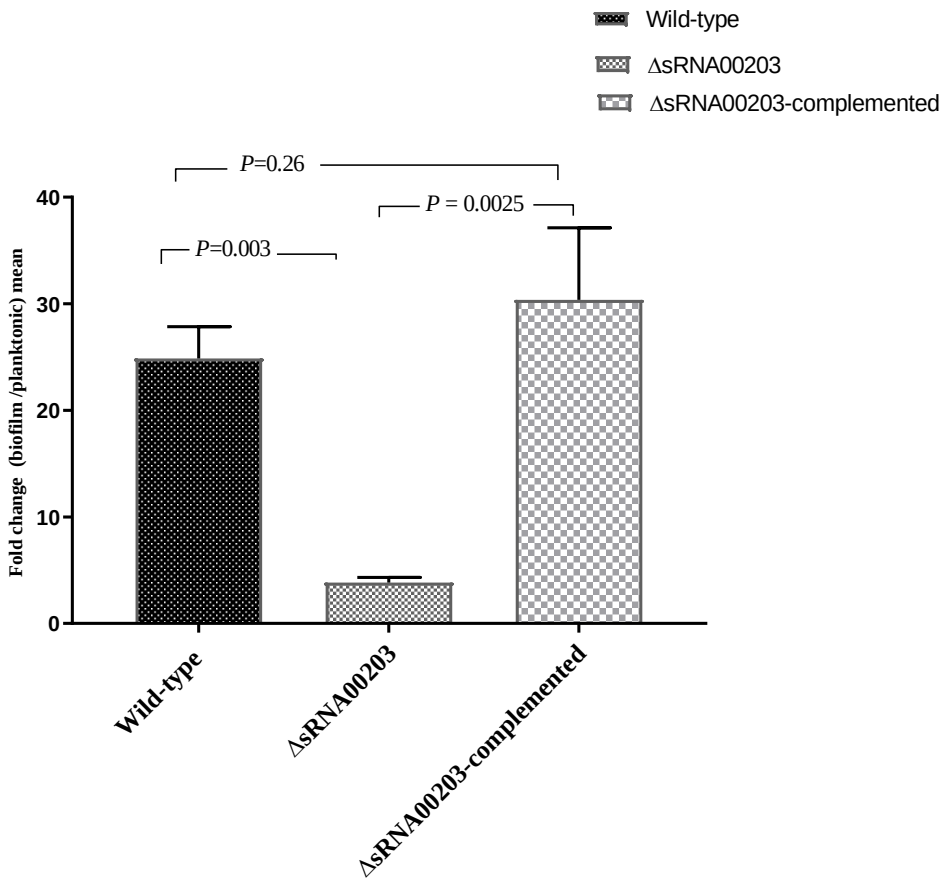


Figure 5-12. Expression levels of novel00738 in biofilm cells of the wild type, sRNA00203-mutant and sRNA00203-complemented *A. baumannii* ST1894 strains.

The mean fold changes in the three biofilms relative to planktonic cells of the wild-type strain are shown. Three independent biological replicates were assessed for each group. The error bars represent standard deviations between different replicates. Analysis of variance (ANOVA) and *post-hoc* tests were performed to analyse the statistical differences between the three groups. A p value < 0.05 was regarded as statistically significant.

5.4.8.3 Impact of sRNA00203 on the expression of the novel00626 gene

sRNA00203-mutant strains exhibited a significant reduction in the expression of the novel00626 gene compared with the wild-type (p = 0038) and sRNA00203-complemented strains (p = 0.0011). The novel00626 gene is primarily involved in metabolic pathways such as LPS biosynthesis and cationic antimicrobial peptide resistance. When sRNA00203 was knocked out, the expression of novel00626 was 2.2-fold lower in biofilm cells than in planktonic cells, as demonstrated in Figure 5-13. The expression of novel00626 was 26.8-fold and 8.2-fold higher in biofilm cells than in planktonic cells of the wild-type and sRNA00203-complemented strains, respectively.

5.4.8.4 Impact of sRNA00203 on the expression of the *secA* gene

Expression of the gene encoding the preprotein translocase subunit SecA was 49.2-fold, 2.6-fold and 47.1-fold higher in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains, respectively, compared with the corresponding planktonic cells. In other words, the expression of *secA* decreased by 94% when sRNA00203 was deleted from the wild-type *A. baumannii* strain (p = 0.007; Figure 5-14).

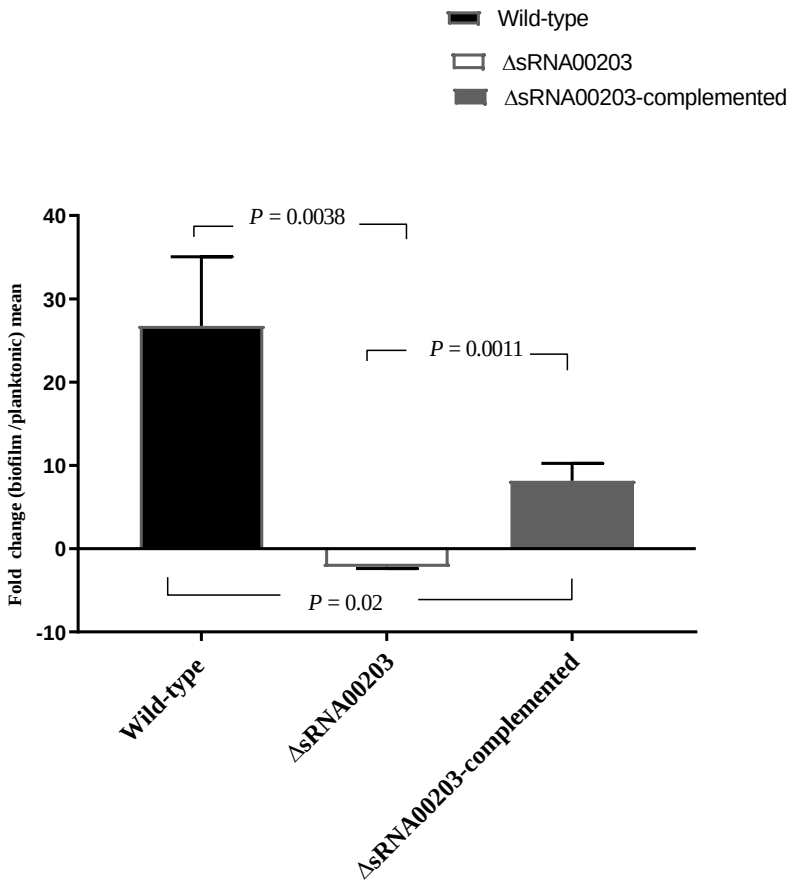


Figure 5-13. Expression levels of novel00626 in the biofilm cells of wild-type, ΔsRNA00203-mutant and ΔsRNA00203-complemented strains of *A. baumannii* ST1894.

The mean fold changes in the three biofilms relative to the planktonic cells of the wild-type strain are shown. Three independent biological replicates were assessed for each group. The error bars represent standard deviations between different replicates. Analysis of variance (ANOVA) and *post-hoc* tests were performed to analyse the statistical differences between the three groups. A p value < 0.05 was regarded as statistically significant.

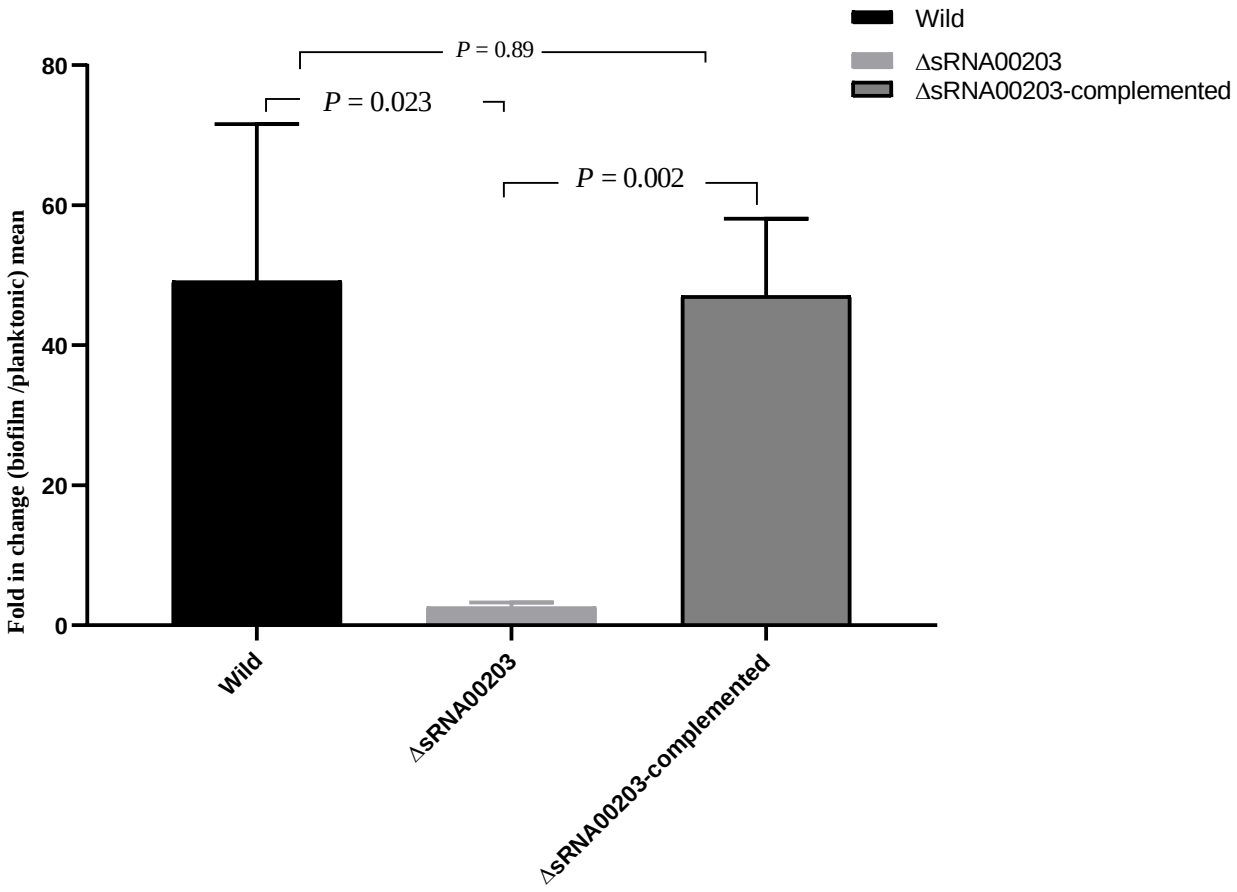


Figure 5-14. Expression levels of the preprotein translocase subunit SecA in the biofilm cells of wild-type, ΔsRNA00203-mutant and ΔsRNA00203-complemented strains of *A. baumannii* ST1894.

The mean fold changes in the three biofilms relative to planktonic cells of the wild-type strain are shown. Three independent biological replicates were assessed for each group. The error bars represent standard deviations between the different replicates. Analysis of variance (ANOVA) and *post-hoc* tests were performed to analyse the statistical differences between the three groups. A p value < 0.05 was regarded as statistically significant.

5.4.8.5 Impact of sRNA00203 on the expression of the CRP transcriptional regulator gene

The expression of the gene encoding the CRP transcriptional regulator was 44.7-fold, 2.9-fold and 18.8-fold higher in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains, respectively, than in the corresponding planktonic cells. The sRNA00203 knockout strain, therefore, showed a substantial decrease in the expression of this gene compared with the wild-type (p = 0.04) and sRNA00203-complemented strains (p = 0.012; Figure 5-15).

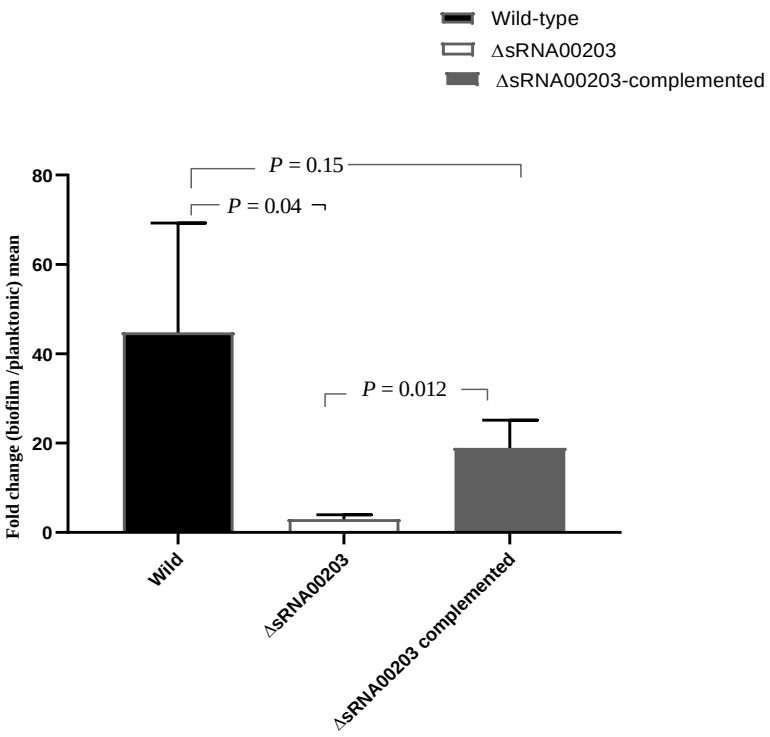


Figure 5-15. Expression levels of the gene encoding the CRP transcriptional regulator in the biofilm cells of wild-type, ΔsRNA00203-mutant and ΔsRNA00203-complemented strains of *A. baumannii* ST1894.

The mean fold changes in the three biofilms relative to planktonic cells of the wild-type strain are shown. Three independent biological replicates were assessed for each group. The error bars represent standard deviations between the different replicates. Analysis of variance (ANOVA) and *post-hoc* tests were performed to analyse the statistical differences between the three groups. A p value < 0.05 was regarded as statistically significant.

5.5 Discussion

In bacteria, sRNAs are essential regulators of the expression of genes, including those involved in biofilm formation. sRNAs enable biofilm cells to recognise environmental signals, transfer information in the form of metabolic changes and significantly affect physiological behaviours (Bak et al., 2015). Using total RNA-seq data from the biofilm cells of *A. baumannii* ST1894, we identified the novel sRNA00203. We found that sRNA00203 is highly upregulated in the biofilm state compared with the planktonic state and that its expression is further induced in biofilm cells by treatment with sub-inhibitory concentrations of imipenem.

We further found that the deletion of sRNA002003 significantly reduced the biofilm mass by 85%, compared with the wild-type *A. baumannii* ST1894 strain. Complementation of the mutant with the sRNA00203 gene restored its biofilm formation capacity to a level similar that of the wild-type strain. Álvarez-Fraga et al. (2017) reported the role of sRNA13573 in regulating attachment to and biofilm formation on human A549 cells. However, the authors were unable to demonstrate the importance of sRNA in the development of biofilm-specific antibiotic resistance. Our study was designed to investigate the role of sRNA00203 in the development of biofilm-specific antibiotic resistance and assess its regulatory role in the expression of genes associated with biofilm formation and antibiotic resistance.

In this study, we identified *A. baumannii* ST1894 as a hyper biofilm-producing strain with tolerance to ciprofloxacin, imipenem and colistin. We aimed to assess the importance of sRNA00203 in the development of antibiotic susceptibility in biofilm cells, particularly against ciprofloxacin, imipenem and colistin. Our results showed that the MBICs for ciprofloxacin and imipenem in the sRNA00203-mutant were

respectively 128-fold and 1,024-fold lower than those in the wild-type *A. baumannii* ST1894 strain. sRNA00203 complementation restored the MBICs for ciprofloxacin and imipenem to values similar to those in the wild-type strains. This result implies that deleting sRNA00203 makes biofilm cells more sensitive to ciprofloxacin and imipenem and confirms that the inhibition of this sRNA could enhance the efficacy of both antibiotics in the treatment of biofilm-specific *A. baumannii* infections. These findings also indicate that sRNA00203 is involved in regulating the expression of genes responsible for ciprofloxacin and imipenem resistance in *A. baumannii* biofilms.

We further found that deletion of the gene encoding sRNA00203 reduced the MBIC for colistin by half, compared with those in the wild-type and sRNA00203-complemented strains. However, this reduction in the MBIC did not change the susceptibility profile of the sRNA00203-mutant strain or render its biofilm cells as resistant to colistin as the biofilm cells of the wild-type and sRNA00203-complemented strains. The decrease in the MBIC for colistin might reflect the effects of sRNA00203 on the expression of genes associated with biofilm formation rather than genes involved in colistin resistance per se.

We evaluated the role of sRNA00203 in the viability of biofilm cells after treatment with ciprofloxacin, imipenem and colistin. The MBECs for these three antibiotics were evaluated to determine the antibiotic concentration required to eradicate biofilm cells after sRNA00203 deletion. We observed that the MBEC for imipenem in the sRNA00203-mutant strain was approximately 128-fold lower than that in the wild-type strain, whereas the MBEC in the sRNA00203-complemented strain was restored to a level comparable to that in the wild-type strain. After deleting sRNA00203 in *A. baumannii* ST1894, although the MBEC for imipenem decreased substantially, the

MBECs for colistin and ciprofloxacin did not significantly change. These findings suggest that sRNA00203 is responsible for regulating the expression of genes involved in the development of antibiotic tolerance in biofilms exposed to imipenem. Thus, when sRNA00203 is blocked, imipenem can effectively eradicate *Acinetobacter* biofilm cells. Interestingly, the sequence of sRNA00203 is conserved across all 465 *Acinetobacter* sequences deposited in the NCBI database, suggesting that sRNA00203 could be used as a therapeutic target for *Acinetobacter* biofilm-specific infections, regardless of whether the strain is MDR or sensitive in its planktonic state.

This study initially aimed to assess the underlying mechanisms by which sRNA00203 regulates biofilm formation and antibiotic resistance. The total RNA-seq data showed 1,592 DEGs, of which 5 were highly differentially expressed in biofilms. Of these, only the role of sRNA00203 was examined in detail. sRNA00203 also has binding sites complementary to the mRNAs of the genes *pgaB* , novel00738, novel00626, *secA* and the CRP transcriptional regulator gene. The deletion of sRNA00203 decreased the expression of *pgaB* in the biofilm cells relative to planktonic cells by 12.6-fold, 1.42-fold and 9.4-fold in the wild-type, sRNA00203-mutant and sRNA00203-complemented strains, respectively, indicating that deletion of this sRNA greatly suppresses the expression *of pgaB* , which is predominantly involved in biofilm matrix synthesis. However, we were unable to establish the exact mechanism by which this occurred.

When sRNA00203 was deleted, the reduced expression of *pgaB* likely decreased biofilm matrix synthesis, which in turn impaired the development of mature biofilms as shown in Figure 5-16. Decreased biofilm matrix synthesis can also increase the speed of penetration of antimicrobial agents into the deeper layers of the biofilm (Branda et al., 2005; Mah, 2012). A previous study reported that deletion of the *pga* locus led to a

loss of the strong biofilm formation phenotype of *A. baumannii* (Choi et al., 2009). This observation is identical to ours, wherein the deletion of sRNA00203 decreased *pgaB* expression and biofilm formation. Most remarkably, we were able to demonstrate for the first time the impact of sRNAs on the expression of genes involved in biofilm formation.

We further found that the deletion of the gene encoding sRNA00203 substantially reduced the expression of novel00738 compared with the levels in the wild-type and sRNA00203-complemented strains. The expression of this gene was also restored when the mutant strain was complemented by sRNA00203. Accordingly, sRNA00203 may also regulate the expression of the novel00738 gene, which is thought to be related to the RND family drug transporters and potentially confers resistance to β-lactams. The deletion of sRNA00203 reduced the expression of novel00738, which may have increased the sensitivity of biofilm cells to β-lactams and cationic antimicrobial peptides, as demonstrated by the MBICs in wild-type and sRNA00203-mutant strains. The deletion of the gene encoding sRNA00203 also decreased the expression of genes involved in the efflux pathway in *A. baumannii*, resulting in the hypersensitivity of biofilm cells to imipenem as illustrated in Figure 5-16. This finding is significant and clinically relevant. As observed in an earlier study, novel00738 is functionally identical to *acrB*. The reduced expression of novel00738 could affect the expression of the *tolC* gene, which is believed to reduce the tolerance of bacterial cells to antibiotics. This in turn might reduce the ratio of persister cells to normal viable cells (Pu et al., 2017).

The effects of both deleting and restoring sRNA00203 strongly point to its role in the development of imipenem tolerance during biofilm formation. Although the direct mechanism of action of sRNA00203 has not been demonstrated experimentally, we

have shown that sRNA00203 positively regulates the tolerance of biofilm cells to imipenem.

sRNA00203-mutant strains displayed a substantial decrease in the expression of the novel00626 gene relative to the levels in the wild-type and sRNA-complemented *A. baumannii* strains. The novel00626 gene is mainly involved in metabolic pathways, especially LPS biosynthesis and cationic antimicrobial peptide resistance.

The sRNA00203-mutant strain also showed reduced expression of UDP-N acetyl glucosamine O-acyltransferase (novel00626), leading to reductions in LPS and biofilm matrix synthesis (Figure 5-16). This finding indicates that the expression of sRNA00203 positively regulates the expression of novel00626. Previous studies have shown that expression of the *lpxA* gene allows the pathogen to survive desiccation by virtue of the outer membrane structure; strains that mutant for LOS do not survive desiccation (Boll et al., 2015). The deletion of sRNA00203, which reduces the expression of novel00626, may also prevent the pathogen from surviving extended periods of desiccation.

Our analysis further demonstrated a significant decrease in the expression of genes encoding the SecA and CRP transcriptional regulators when sRNA00203 is deleted. These two genes regulate the production of QS molecules; the deletion of sRNA00203 may therefore indirectly decrease biofilm formation and biofilm-specific antibiotic tolerance as summarized in Figure 5-16. Further investigations are required to establish the complete regulatory mechanism by which these genes and sRNA00203 act.

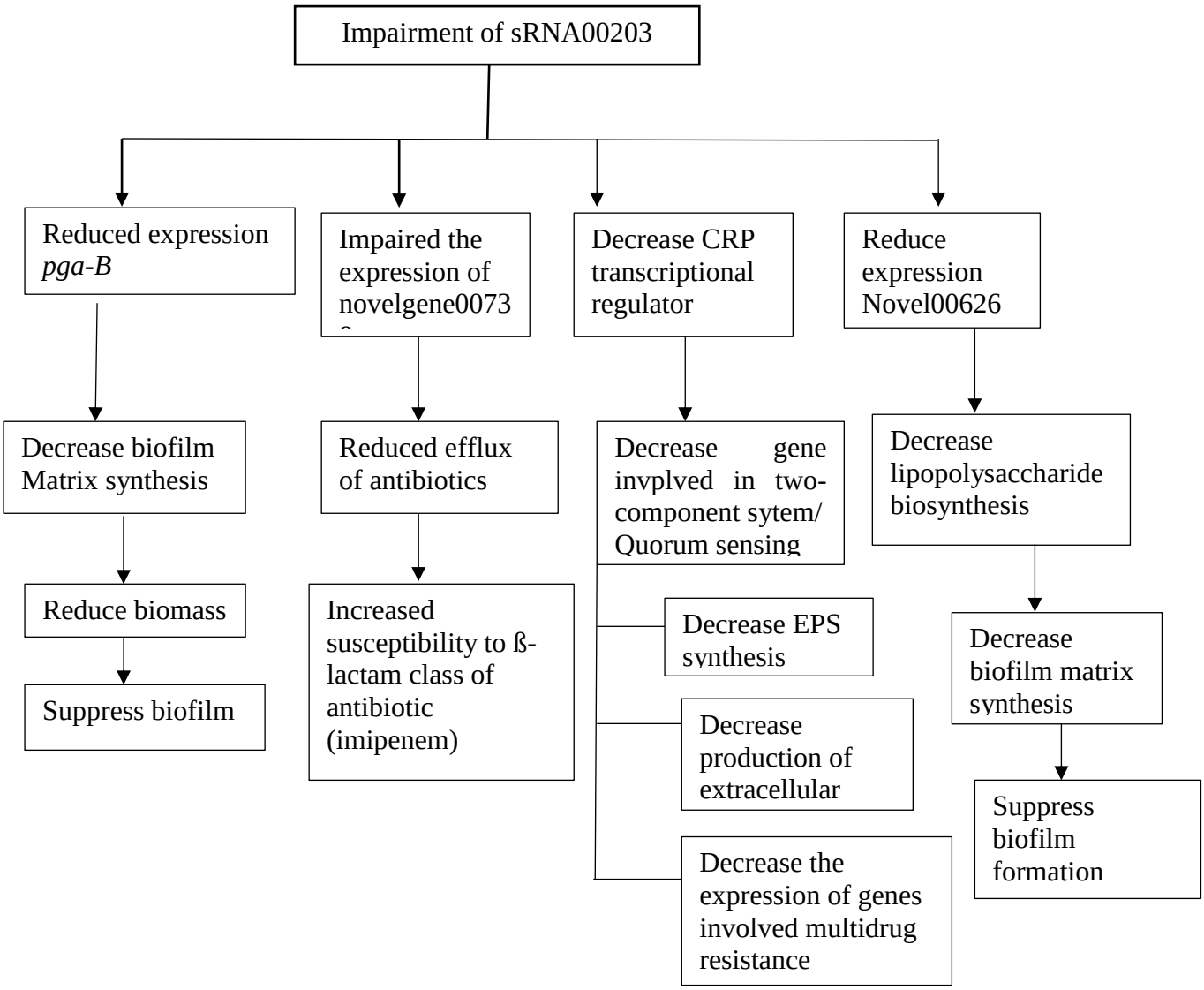


Figure 5-16. Hypothetical mechanisms of sRNA00203 in the regulation of biofilm formation and biofilm-specific antibiotic genes.

In summary, this study revealed that the deletion of sRNA00203-encoding genes significantly decreases the biofilm formation capabilities of *A. baumannii.* sRNA00203 inactivation also increases the sensitivity of biofilm cells to ciprofloxacin and imipenem and decreases the expression of genes involved in biofilm matrix synthesis, efflux pumps, LPS biosynthesis, *SecA* and the CRP transcriptional regulator. sRNA00203 deletion may therefore affect the expression of genes involved in biofilm formation and the development of biofilm-specific resistance to antibiotics. To the best of our knowledge, this study is the first to show a link between the expression of sRNA00203 and the development of biofilm-specific antibiotic resistance in *A. baumannii*. Sequence-specific inhibition of sRNA00203 offers a new strategy for the treatment of biofilm-specific infections or the resensitisation of biofilm cells to imipenem and ciprofloxacin.

6 CONCLUDING REMARKS AND FUTURE DIRECTIONS

6.1 Concluding remarks

A. baumannii is an emerging pathogen that has evolved from an organism of questionable pathogenicity to a top priority pathogen according to the World Health Organization (WHO, 2017). This pathogen has spread rapidly across the globe owing to its capacity to acquire antibiotic resistance and form biofilm (Penesyan et al., 2019).

The formation of biofilms enables *A. baumannii* to survive adverse growth conditions, thus enhancing its persistence in hospital environments and host epithelial cells. During the transition from the planktonic to the biofilm state, bacterial cells undergo morphological and physiological changes as a result of the expression of virulence and antibiotic resistance genes.

Biofilm-specific antibiotic resistance and tolerance play an important role in the pathogenesis of chronic tissue and medical device-related infections. Over the past decade, most research efforts have focused on the development of antibiotic resistance in the planktonic phase of *A. baumannii*. To gain a thorough understanding of the genetic mechanisms underlying the development of biofilm-specific antibiotic resistance, the genetic factors responsible for reduced antibiotic susceptibility in the biofilm cells of *A. baumannii* were explored in this thesis research.

When we studied the relationship between biofilm formation capability and antibiotic susceptibility, we found that non-MDR strains were strong biofilm formers that exhibited a strong reduction in susceptibility during biofilm growth. We further found that although biofilm growth induced reversible antibiotic tolerance in non-MDR strains, it induced a higher level of irreversible resistance in the XDR strain and even

converted it from colistin-sensitive to colistin-resistant. These results have clinical implications; once a non-MDR strain forms a biofilm, it cannot be eradicated and will soon become a source of infection and contamination.

CLSM images of the non-MDR *A. baumannii* ST1894 strain also reveal that biofilm cells treated with high concentrations of colistin, ciprofloxacin and imipenem in our study contained 0.5–3.7% viable cells. These persisters could be responsible for the recalcitrance of chronic infections and the evolution of *A. baumannii* into antibiotic-resistant strains in clinical settings.

Total RNA-seq was performed to identify the transcriptional responses of untreated biofilm cells and those treated with sub-inhibitory concentrations of imipenem and colistin. These treatments respectively revealed the differential expression of 106 and 368 genes specific to biofilms. The biofilm-specific DEGs induced by imipenem and colistin were associated with biofilm matrix synthesis, outer membrane transport protein, fimbrial proteins, RND multidrug efflux pumps and the QS-mediator AHL synthase. These findings demonstrate that exposure to sub-inhibitory antibiotic concentrations can enhance biofilm production by modulating QS regulatory pathways and can reduce antibiotic susceptibility by enhancing the elimination of drugs through efflux pumps. Such transcriptional responses improve survival and facilitate the evolution of non-MDR *A. baumannii* strains into MDR strains.

The expression levels of genes involved in metabolic processes such as the citric acid cycle, iron uptake, DNA replication and translation were significantly reduced in biofilm cells compared with planktonic cells. The reduced expression of genes linked to these various metabolic pathways during biofilm formation might slow metabolism.

Subsequently, metabolically quiescent cells embedded in the biofilm can contribute to the recalcitrance of biofilms to high concentrations of bactericidal antibiotics.

In addition to protein-coding transcripts, 5 of the 138 sRNAs identified in this study were differentially expressed in biofilm cells relative to planktonic cells. Of these five sRNAs, sRNA00203 was the most highly upregulated in biofilm cells. sRNA00203 also contains binding sites complementary to the mRNAs of genes encoding the global CRP transcriptional regulator, the preprotein translocase subunit (*secA*), and components of biofilm matrix production (*pgaB*), cell wall synthesis (novel00626) and drug efflux (novel00738), thereby increases the expression of these genes. These results were in line with the observations of increased biofilm formation and reduced susceptibility of biofilm cell to ciprofloxacin and imipenem, indicating that sRNA00203 positively regulates the expression of genes associated with biofilm formation and biofilm-specific antibiotic resistance. The sequence-specific inhibition of sRNA00203 offers a new strategy for the treatment of biofilm-specific infections by resensitising biofilm cells to imipenem and ciprofloxacin. To the best of our knowledge, this study is the first of its kind to show the link between sRNA00203, biofilm formation and biofilm-specific antibiotic resistance in *A. baumannii*.

6.2 Future directions

Additional research is required to verify the mechanism by which the differentially expressed novel genes and sRNAs regulate the development of biofilm-specific antibiotic resistance.

6.2.1 Role of the novel00738 and novel00626 genes in the development of biofilm-specific antibiotic resistance

The expression of novel00738, which putatively encodes a multidrug efflux pump, was found to be upregulated in biofilm cells treated with sub-inhibitory concentrations of imipenem. We also observed that novel00626, which putatively encodes UDP-N acetylglucosamine O-acyltransferase and appears to play a role similar to that of the *lpxA* gene, was highly upregulated in biofilm cells. The expression of this gene also significantly increased when biofilm cells were treated with sub-inhibitory concentrations of colistin. Additional studies that use site-directed mutagenesis will be required to understand the roles of novel00738 and novel00626 genes in the development of antibiotic susceptibility and biofilm formation in *A. baumannii*.

6.2.2 Verification of interactions between sRNAs and mRNAs

sRNAs are key regulators of bacterial gene expression. Our findings highlight the role of sRNA00203 in regulating the expression of *pgaB* , novel00738, novel00626, *secA*, and the CRP transcriptional regulator gene. Although we also predicted interactions between sRNA00203 and the mRNAs of these genes, experimental evidence for these interactions is lacking and must be demonstrated to confirm the regulatory mechanisms by which sRNA00203 exerts its effects.

REFERENCES

Abbo, A., Carmeli, Y., Navon-Venezia, S., Siegman-Igra, Y., & Schwaber, M. J. (2007). Impact of multidrug-resistant *Acinetobacter baumannii* on clinical outcomes. *European Journal of Clinical Microbiology and Infectious Diseases*, 26(11), 793–800.

Abbott, I., Cerqueira, G. M., Bhuiyan, S., & Peleg, A. Y. (2013). Carbapenem resistance in *Acinetobacter baumannii*: Laboratory challenges, mechanistic insights, and therapeutic strategies. In *Expert Review of Anti-Infective Therapy*,11, 395–409.

Abdallah, M., Olafisoye, O., Cortes, C., Urban, C., Landman, D., & Quale, J. (2015). Activity of eravacycline against Enterobacteriaceae and *Acinetobacter baumannii*, including multidrug-resistant isolates, from New York City. *Antimicrobial Agents and Chemotherapy*, 59(3), 1802–1805.

Abdi-Ali, A., Hendiani, S., Mohammadi, P., & Gharavi, S. (2014). Assessment of biofilm formation and resistance to imipenem and ciprofloxacin among clinical isolates of *Acinetobacter baumannii* in Tehran. In *Jundishapur Journal of Microbiology*, 7(1), 1-4

Abel Zur Wiesch, P., Abel, S., Gkotzis, S., Ocampo, P., Engelstädter, J., Hinkley, T., Magnus, C., Waldor, M. K., Udekwu, K., & Cohen, T. (2015). Classic reaction kinetics can explain complex patterns of antibiotic action. *Science Translational Medicine*, 7(287), 1-9

Adams, M. D., Nickel, G. C., Bajaksouzian, S., Lavender, H., Murthy, A. R., Jacobs, M. R., & Bonomo, R. A. (2009). Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the *PmrAB* two-component system. *Antimicrobial Agents and Chemotherapy*, 53(9), 3628–3634.

Ahmed, W., Hafeez, M. A., & Mahmood, S. (2018). Identification and functional characterization of bacterial small non-coding RNAs and their target: A review. In *Gene Reports*, 10, 167–176.

Aiba, H. (2007). Mechanism of RNA silencing by Hfq-binding small RNAs. In *Current Opinion in Microbiology*, 10(2),134–139.

Alav, I., Sutton, J. M., & Rahman, K. M. (2018). Role of bacterial efflux pumps in biofilm formation. *Journal of Antimicrobial Chemotherapy*, 73(8), 2003–2020.

Almasaudi, S. B. (2018). Acinetobacter spp. as nosocomial pathogens: Epidemiology and resistance features. In *Saudi Journal of Biological Sciences*, 25(3), 586–596.

Álvarez-Fraga, L., Rumbo-Feal, S., Pérez, A., Gómez, M. J., Gayoso, C., Vallejo, J. A., Ohneck, E. J., Valle, J., Actis, L. A., Beceiro, A., Bou, G., & Poza, M. (2017). Global assessment of small RNAs reveals a non-coding transcript involved in biofilm formation and attachment in *Acinetobacter baumannii* ATCC 17978. *PLoS ONE*, 12(8), 1-24.

Amato, S. M., & Brynildsen, M. P. (2015). Persister heterogeneity arising from a single metabolic stress. *Current Biology*, 25(16), 2090–2098.

Amin, I. M., Richmond, G. E., Sen, P., Koh, T. H., Piddock, L. J., & Chua, K. L. (2013). A method for generating marker-less gene deletions in multidrug-resistant *Acinetobacter baumannii*. *BMC Microbiology*, 13**,** 158

Anbazhagan, D., Mansor, M., Yan, G. O. S., Yusof, M. Y. M., Hassan, H., & Sekaran, S. D. (2012). Detection of quorum sensing signal molecules and identification of an autoinducer synthase gene among biofilm forming clinical isolates of Acinetobacter spp. *PLoS ONE*, 7(7),1-12.

Anderl, J. N., Franklin, M. J., & Stewart, P. S. (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and

ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, 44(7), 1818–1824.

Anghel, I., Chifiriuc, M. C., & Anghel, G. A. (2011). Pathogenic features and therapeutical implications of biofilm development ability in microbial strains isolated from rhinologic chronic infections. *Farmacia*, 59(6), 770–783.

Anstey, N. M., Currie, B. J., & Withnall, K. M. (1992). Community-acquired acinetobacter pneumonia in the Northern Territory of Australia. *Clinical Infectious Diseases*, 14(1), 83–91.

Antonelli, M., De Pascale, G., Ranieri, V. M., Pelaia, P., Tufano, R., Piazza, O., Zangrillo, A., Ferrario, A., De Gaetano, A., Guaglianone, E., & Donelli, G. (2012). Comparison of triple-lumen central venous catheters impregnated with silver nanoparticles (AgTive®) vs conventional catheters in intensive care unit patients. *Journal of Hospital Infection*, 82(2), 101–107.

Antunes, L. C. M., Ferreira, R. B. R., Buckner, M. M. C., & Finlay, B. B. (2010). Quorum sensing in bacterial virulence. In *Microbiology*,156(8), 2271–2282.

Antunes, L. C. S., Visca, P., & Towner, K. J. (2014). *Acinetobacter baumannii*: evolution of a global pathogen. In *Pathogens and Disease*, 71(3), 292–301.

Asif, M., Alvi, I. A., & Ur Rehman, S. (2018). Insight into *Acinetobacter baumannii*: Pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. In *Infection and Drug Resistance*,11, 1249–1260.

Asplund, M. B., Coelho, C., Cordero, R. J. B., & Martinez, L. R. (2013). Alcohol impairs J774.16 macrophage-like cell antimicrobial functions in *Acinetobacter baumannii* infection. *Virulence*, 4(6). 467-471

Azimi, L., Talebi, M., Pourshafie, M.-R., Owlia, P., & Rastegar Lari, A. (2015). Characterization of carbapenemases in extensively drug resistance *Acinetobacter baumannii* in a burn care center in Iran. *International Journal of Molecular and*

Cellular Medicine, 4(1), 46–53.

Babapour, E., Haddadi, A., Mirnejad, R., Angaji, S. A., & Amirmozafari, N. (2016). Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. *Asian Pacific Journal of Tropical Biomedicine*, 6(6), 528–533.

Bae, J., Oh, E., & Jeon, B. (2014). Enhanced transmission of antibiotic resistance in *Campylobacter jejuni* biofilms by natural transformation. *Antimicrobial Agents and Chemotherapy*, 58(12), 7573–7575.

Bagge, N., Ciofu, O., Skovgaard, L. T., & Høiby, N. (2000). Rapid development invitro and in vivo of resistance to ceftazidime in biofilm-growing *Pseudomonas aeruginosa* due to chromosomal β-lactamase. *Apmis*, 108(9), 589–600.

Bagge, N., Hentzer, M., Andersen, J. B., Ciofu, O., Givskov, M., & Høiby, N. (2004). Dynamics and spatial distribution of β-Lactamase expression in *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy*, 48(4), 1168–1174.

Bak, G., Lee, J., Suk, S., Kim, D., Lee, J. Y., Kim, K. S., Choi, B. S., & Lee, Y. (2015). Identification of novel sRNAs involved in biofilm formation, motility, and fimbriae formation in *Escherichia coli*. *Scientific Reports*, 5,1-17.

Ballouz, T., Aridi, J., Afif, C., Irani, J., Lakis, C., Nasreddine, R., & Azar, E. (2017). Risk factors, clinical presentation, and outcome of *Acinetobacter baumannii* bacteremia. *Frontiers in Cellular and Infection Microbiology*, 7,1-7.

Bartual, S. G., Seifert, H., Hippler, C., Luzon, M. A. D., Wisplinghoff, H., & Rodríguez-Valera, F. (2005). Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. *Journal of Clinical Microbiology*, 43(9), 4382–4390.

Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M.,

Dhanji, H., Chart, H., Bou, G., Livermore, D. M., & Woodford, N. (2011). Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the *pmrAB* two-component regulatory system. *Antimicrobial Agents and Chemotherapy*, 55(7), 3370–3379.

Bejerano-Sagie, M., & Xavier, K. B. (2007). The role of small RNAs in quorum sensing. In *Current Opinion in Microbiology*, 10(2),189–198.

Bergogne-Bérézin, E., & Towner, K. J. (1996). Acinetobacter spp. as nosocomial pathogens: Microbiological, clinical, and epidemiological features. In *Clinical Microbiology Reviews*, 9(2), 148–165.

Bernier, S. P., Lebeaux, D., DeFrancesco, A. S., Valomon, A., Soubigou, G., Coppée, J. Y., Ghigo, J. M., & Beloin, C. (2013). Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone Ofloxacin. *PLoS Genetics*, 9(1), 1-12.

Bhando, T., Dubey, V., & Pathania, R. (2019). Biofilms in antimicrobial activity and drug resistance. In *Bacterial Adaptation to Co-resistance*, 109–139.

Bhattacharya, M., Toth, M., Antunes, N. T., Smith, C. A., & Vakulenko, S. B. (2014). Structure of the extended-spectrum class C β-lactamase ADC-1 from *Acinetobacter baumannii*. *Acta Crystallographica Section D: Biological Crystallography*, *70*(3), 760–771.

Bobrovskyy, M., Vanderpool, C. K., & Richards, G. R. (2015). Small RNAs regulate primary and secondary metabolism in Gram-negative bacteria. In metabolism and bacterial pathogenesis, 3(3),59–94.

Bogachev, M. I., Volkov, V. Y., Markelov, O. A., Trizna, E. Y., Baydamshina, D. R., Melnikov, V., Murtazina, R. R., Zelenikhin, P. V., Sharafutdinov, I. S., & Kayumov, A. R. (2018). Fast and simple tool for the quantification of biofilm-

embedded cells sub-populations from fluorescent microscopic images. *PLoS ONE*, 13(5), 1-24.

Boles, B. R., & Singh, P. K. (2008). Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proceedings of the National Academy of Sciences of the United States of America*, 105(34), 12503–12508.

Boll, J. M., Crofts, A. A., Peters, K., Cattoir, V., Vollmer, W., Davies, B. W., & Trent, M. S. (2016). A penicillin-binding protein inhibits the selection of colistin-resistant, lipooligosaccharide-deficient *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America*, 113(41), 6228-6237

Bonomo, R. A., & Szabo, D. (2006). Mechanisms of multidrug resistance in Acinetobacter species and *Pseudomonas aeruginosa*. *Clinical Infectious Diseases*, 43(2), 49-53.

Bosshard, P. P., Zbinden, R., Abels, S., Böddinghaus, B., Altwegg, M., & Böttger, E. C. (2006). 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory. *Journal of Clinical Microbiology*, 44(4), 1359–1366.

Bouvier, M., Sharma, C. M., Mika, F., Nierhaus, K. H., & Vogel, J. (2008). Small RNA binding to 5′ mRNA coding region inhibits translational initiation. *Molecular Cell*, *32*(6), 827–837.

Bowler, L. L., Zhanel, G. G., Ball, T. B., & Saward, L. L. (2012). Mature *Pseudomonas aeruginosa* biofilms prevail compared to young biofilms in the presence of ceftazidime. *Antimicrobial Agents and Chemotherapy*, 56(9), 4976–4979.

Branda, S. S., Vik, Å., Friedman, L., & Kolter, R. (2005). Biofilms: The matrix

revisited. In *Trends in Microbiology*, 13(1), 20–26.

Brelles-Mario, G., & Bedmar, E. J. (2001). Detection, purification,and characterisation of quorum-sensing signal molecules in plant-associated bacteria. *Journal of Biotechnology*, 91 (2–3), 197–209.

Bronsard, J., Pascreau, G., Sassi, M., Mauro, T., Augagneur, Y., & Felden, B. (2017). sRNA and cis-Antisense sRNA identification in *Staphylococcus aureus* highlight an unusual sRNA gene cluster with one encoding a secreted peptide. *Scientific Reports*, 7(1),1-15.

Brooun, A., Liu, S., & Lewis, K. (2000). A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy*, *44*(3), 640–646.

Brossard, K. A., & Campagnari, A. A. (2012). The *Acinetobacter baumannii* biofilm-associated protein plays a role in adherence to human epithelial cells. *Infection and Immunity*, 80(1), 228–233.

Brown, M. R. W., Allison, D. G., & Gilbert, P. (1988). The resistance of bacterial biofilms to antibiotics a growth-rate related effect? *Journal of Antimicrobial Chemotherapy*, 22(6), 777–780.

Brown, P. C., Borowska, E., Schwartz, T., & Horn, H. (2019). Impact of the particulate matter from wastewater discharge on the abundance of antibiotic resistance genes and facultative pathogenic bacteria in downstream river sediments. *Science of the Total Environment*, 649, 1171-1178

Burmølle, M., Webb, J. S., Rao, D., Hansen, L. H., Sørensen, S. J., & Kjelleberg, S. (2006). Enhanced biofilm formation and increased resistance to antimicrobial

agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Applied and Environmental Microbiology*, 72(6), 3916–3923.

Busch, A., Richter, A. S., & Backofen, R. (2008). IntaRNA: Efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. *Bioinformatics*, *24*(24), 2849–2856.

Carruthers, M. D., Nicholson, P. A., Tracy, E. N., & Munson, R. S. (2013). *Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition. *PLoS ONE*, 8(3), 1-7.

Carter, J. D., Cutolo, E. P., Behnke, R. H., & Adelman, H. M. (1999). Pulmonary infiltrates elderly in a man. *Hospital Practice*, 34(8), 21–24.

Catel-Ferreira, M., Nehmé, R., Molle, V., Aranda, J., Bouffartigues, E., Chevalier, S., Bou, G., Jouenne, T., & Dé, E. (2012). Deciphering the function of the outer membrane protein *OprD* homologue of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 56(7), 3826–3832.

Cayô, R., Rodríguez, M. C., Espinal, P., Fernández-Cuenca, F., Ocampo-Sosa, A. A., Pascual, Á., Ayala, J. A., Vila, J., & Martínez-Martínez, L. (2011). Analysis of genes encoding penicillin-binding proteins in clinical isolates of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 55(12), 5907–5913.

Cerceo, E., Deitelzweig, S. B., Sherman, B. M., & Amin, A. N. (2016). Multidrug-resistant Gram-negative bacterial infections in the hospital setting: overview, implications for clinical practice, and emerging treatment options. *Microbial Drug Resistance*, 22(5), 412–431.

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D., & Buret, A. (1999). The Calgary biofilm device: New technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology*, 37(6),

1771–1776.

Cerqueira, G. M., & Peleg, A. Y. (2011). Insights into *Acinetobacter baumannii* pathogenicity. In *IUBMB Life*, 63(12), 1055–1060.

Chambers, J. R., & Sauer, K. (2013). Small RNAs and their role in biofilm formation. In *Trends in Microbiology*, 21(1), 39–49.

Chang, W. N., Lu, C. H., Huang, C. R., & Chuang, Y. C. (2000). Community-acquired *Acinetobacter meningitis* in adults. *Infection*, 28(6), 395–397.

Chen, M. Z., Hsueh, P. R., Lee, L. N., Yu, C. J., Yang, P. C., & Luh, K. T. (2001). Severe community-acquired pneumonia due to *Acinetobacter baumannii*. *Chest*, 120(4), 1072–1077.

Chen, T. L., Lee, Y. T., Kuo, S. C., Yang, S. P., Fung, C. P., & Lee, S. D. (2014). Rapid identification of *Acinetobacter baumannii*, *Acinetobacter nosocomialis,* and *Acinetobacter pittii* with a multiplex PCR assay. *Journal of Medical Microbiology*, 63(9), 1154–1159.

Cheng, V. C. C., Wong, S. C., Chen, J. H. K., So, S. Y. C., Wong, S. C. Y., Ho, P. L., & Yuen, K. Y. (2018). Control of multidrug-resistant *Acinetobacter baumannii* in Hong Kong: Role of environmental surveillance in communal areas after a hospital outbreak. *American Journal of Infection Control*, 46(1), 60–66.

Choi, A. H. K., Slamti, L., Avci, F. Y., Pier, G. B., & Maira-Litrán, T. (2009). The *pgaABCD* locus of *Acinetobacter baumannii* encodes the production of poly-β-1-6-N-acetylglucosamine, which is critical for biofilm formation. *Journal of Bacteriology*, 191(19), 5953–5963.

Choi, C. H., Lee, E. Y., Lee, Y. C., Park, T. I., Kim, H. J., Hyun, S. H., Kim, S. A., Lee, S. K., & Lee, J. C. (2005). Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cellular*

Microbiology, 7(8), 1127–1138.

Chopra, S., Torres-Ortiz, M., Hokama, L., Madrid, P., Tanga, M., Mortelmans, K., Kodukula, K., & Galande, A. K. (2010). Repurposing FDA-approved drugs to combat drug-resistant *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy*, 65(12), 2598–2601.

Cisneros, José M., Reyes, M. J., Pachón, J., Becerril, B., Caballero, F. J., García-Garmendía, J. L., Ortiz, C., & Cobacho, A. R. (1996). Bacteremia due to *Acinetobacter baumannii*: Epidemiology, clinical findings, and prognostic features. *Clinical Infectious Diseases*, 22(6), 1026–1032.

Conlon, B. P., Rowe, S. E., & Lewis, K. (2015). Persister cells in biofilm-associated infections. *Advances in Experimental Medicine and Biology*, 831, 1–9.

Cook, L., Chatterjee, A., Barnes, A., Yarwood, J., Hu, W. S., & Dunny, G. (2011). Biofilm growth alters the regulation of conjugation by a bacterial pheromone. *Molecular Microbiology*, 81(6), 1499–1510.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: A common cause of persistent infections. In *Science*, 284 (5418), 1318–1322.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annual Review of Microbiology*, 49, 711–745.

Coyne, S., Courvalin, P., & Périchon, B. (2011). Efflux-mediated antibiotic resistance in Acinetobacter spp. In *Antimicrobial Agents and Chemotherapy*, 55(3), 947–953.

Coyne, S., Rosenfeld, N., Lambert, T., Courvalin, P., & Périchon, B. (2010). Overexpression of resistance-nodulation-cell division pump *AdeFGH* confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 54 (10), 4389–4393.

Daddi Oubekka, S., Briandet, R., Fontaine-Aupart, M. P., & Steenkeste, K. (2012). Correlative time-resolved fluorescence microscopy to assess antibiotic diffusion-reaction in biofilms. *Antimicrobial Agents and Chemotherapy*, 56(6), 3349–3358.

Damier-Piolle, L., Magnet, S., Brémont, S., Lambert, T., & Courvalin, P. (2008). *AdeIJK*, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 52(2), 557–562.

Davies, D. (2003). Understanding biofilm resistance to antibacterial agents. In *Nature Reviews Drug Discovery*, 292, 114–122.

Davis, J. S., McMillan, M., Swaminathan, A., Kelly, J. A., Piera, K. E., Baird, R. W., Currie, B. J., & Anstey, N. M. (2014). A 16-year prospective study of community -onset bacteremic acinetobacter pneumonia: Low mortality with appropriate initial empirical antibiotic protocols. *Chest*, 146(4), 1038–1045.

De la Fuente-Núñez, C., Reffuveille, F., Fernández, L., & Hancock, R. E. W. (2013). Bacterial biofilm development as a multicellular adaptation: Antibiotic resistance and new therapeutic strategies. In *Current Opinion in Microbiology*,16(5), 580–589.

De la Fuente-Núñez, C., Reffuveille, F., Haney, E. F., Straus, S. K., & Hancock, R. E. W. (2014). Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathogens*, 10(5), 1-11.

De Oliveira, D. M. P., Forde, B. M., Kidd, T. J., Harris, P. N. A., Schembri, M. A., Beatson, S. A., Paterson, D. L., & Walker, M. J. (2020). Antimicrobial resistance in ESKAPE pathogens. *Clinical Microbiology Reviews*, 33(3), 6-15

Decré, D. (2012). *Acinetobacter baumannii* et résistance aux antibiotiques: Un modèle d'adaptation. *Revue Francophone Des Laboratoires*, 441, 43–52.

Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: Multidrug-resistant *Acinetobacter baumannii*. In *Nature Reviews Microbiology* 5(12), 939–951.

Donelli, G., Francolini, I., Romoli, D., Guaglianone, E., Piozzi, A., Ragunath, C., & Kaplan, J. B. (2007). Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes. *Antimicrobial Agents and Chemotherapy*, 51(8), 2733–2740.

Dönhöfer, A., Franckenberg, S., Wickles, S., Berninghausen, O., Beckmann, R., & Wilson, D. N. (2012). Structural basis for *TetM*-mediated tetracycline resistance. *Proceedings of the National Academy of Sciences of the United States of America*, 109(42), 16900-16905

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. In *Clinical Microbiology Reviews*,15(2), 167–193.

Dou, Y., Song, F., Guo, F., Zhou, Z., Zhu, C., Xiang, J., & Huan, J. (2017). *Acinetobacter baumannii* quorum-sensing signalling molecule induces the expression of drug-resistance genes. *Molecular Medicine Reports*, 15(6), 4061-4068.

Driffield, K., Miller, K., Bostock, J. M., O'neill, A. J., & Chopra, I. (2008). Increased mutability of *Pseudomonas aeruginosa* in biofilms. *Journal of Antimicrobial Chemotherapy*, 61(5), 1053–1056.

Ehlers, L. J., & Bouwer, E. J. (1999). RP4 plasmid transfer among species of Pseudomonas in a biofilm reactor. *Water Science and Technology*, 39(7), 163-171

Ellis, M. J., Trussler, R. S., & Haniford, D. B. (2015). A cis-encoded sRNA, Hfq and mRNA secondary structure act independently to suppress IS200 transposition. *Nucleic Acids Research*, 43(13), 6511–6527.

Erridge, C., Moncayo-Nieto, O. L., Morgan, R., Young, M., & Poxton, I. R. (2007). *Acinetobacter baumannii* lipopolysaccharides are potent stimulators of human monocyte activation via Toll-like receptor 4 signalling. *Journal of Medical Microbiology*, 56(2), 165–171.

Espinal, P., Martí, S., & Vila, J. (2012). Effect of biofilm formation on the survival of *Acinetobacter baumannii* on dry surfaces. *Journal of Hospital Infection*, 80(1), 56–60.

Esterly, J. S., Richardson, C. L., Eltoukhy, N. S., Qi, C., & Scheetz, M. H. (2011). Genetic mechanisms of antimicrobial resistance of *Acinetobacter baumannii*. In *Annals of Pharmacotherapy*, 45(2), 218–228).

Evans, B. A., & Amyes, S. G. B. (2014). OXA β-lactamases. *Clinical Microbiology Reviews*, 27(2), 241–263.

Evans, D. J., Allison, D. G., Brown, M. R. W., & Gilbert, P. (1991). Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: Effect of specific growth rate. *Journal of Antimicrobial Chemotherapy*, 27(2), 177-184.

Fabbri, S., Johnston, D. A., Rmaile, A., Gottenbos, B., De Jager, M., Aspiras, M., Starke, M. E., Ward, M. T., & Stoodley, P. (2016). *Streptococcus mutans* biofilm transient viscoelastic fluid behavior during high-velocity microsprays. *Journal of the Mechanical Behavior of Biomedical Materials*, 59, 197–206.

Falagas, M. E., Karveli, E. A., Kelesidis, I., & Kelesidis, T. (2007). Community-acquired Acinetobacter infections. In *European Journal of Clinical Microbiology and Infectious Diseases*, 26(12), 857–868).

Falagas, Matthew E., & Karageorgopoulos, D. E. (2008). Pandrug resistance (PDR), extensive drug resistance (XDR), and multidrug resistance (MDR) among Gram-

negative bacilli: Need for international harmonization in terminology. In *Clinical Infectious Diseases*,46(7), 1121–1122.

Falagas, Matthew E., & Kasiakou, S. K. (2005). Colistin: The revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. In *Clinical Infectious Diseases*, 40(9), 1333–1341.

Fallah, F., Noori, M., Hashemi, A., Goudarzi, H., Karimi, A., Erfanimanesh, S., & Alimehr, S. (2014). Prevalence of *bla* $_{NDM}$, *bla* $_{PER}$, *bla* $_{VEB}$, *bla* $_{IMP}$, and *bla* $_{VIM}$ Genes among *Acinetobacter baumannii* isolated from two hospitals of Tehran, Iran . *Scientifica*, 1–6.

Farshadzadeh, Z., Taheri, B., Rahimi, S., Shoja, S., Pourhajibagher, M., Haghighi, M. A., & Bahador, A. (2018). Growth rate and biofilm formation ability of clinical and laboratory-evolved colistin-resistant strains of *Acinetobacter baumannii*. *Frontiers in Microbiology*, 9, 1-10.

Fernández, L., Breidenstein, E. B. M., & Hancock, R. E. W. (2011). Creeping baselines and adaptive resistance to antibiotics. *Drug Resistance Updates*, 14(1), 1–21.

Fishbain, J., & Peleg, A. Y. (2010). Treatment of Acinetobacter infections. In *Clinical Infectious Diseases*, 51(1), 79–84.

Fleming, D., & Rumbaugh, K. (2017). Approaches to dispersing medical biofilms. *Microorganisms*, 5(2), 15.

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. In *Nature Reviews Microbiology*, 8(9), 623–633).

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An emergent form of bacterial life. In *Nature Reviews Microbiology*,14(9), 563–575.

Fournier, P. E., & Richet, H. (2006). The epidemiology and control of *Acinetobacter*

baumannii in health care facilities. *Clinical Infectious Diseases*, 42(5), 692–699.

Francolini, I., Piozzi, A., & Donelli, G. (2014). Efficacy evaluation of antimicrobial drug-releasing polymer matrices. *Methods in Molecular Biology*, 1147, 215–225.

Fujikura, Y., Yuki, A., Hamamoto, T., Kawana, A., Ohkusu, K., & Matsumoto, T. (2016). Bloodstream infections caused by *Acinetobacter baumannii* group in Japan : Epidemiological and clinical investigation. *Journal of Infection and Chemotherapy*, 22(6), 366–371.

Gaddy, J. A., & Actis, L. A. (2009). Regulation of *Acinetobacter baumannii* biofilm formation. *Future Microbiology*, 4(3), 273–278.

Gaddy, J. A., Tomaras, A. P., & Actis, L. A. (2009). The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infection and Immunity*, 77(8), 3150–3160.

Gandhi, J. A., Ekhar, V. V., Asplund, M. B., Abdulkareem, A. F., Ahmadi, M., Coelho, C., & Martinez, L. R. (2014). Alcohol enhances *Acinetobacter baumannii*-associated pneumonia and systemic dissemination by impairing neutrophil antimicrobial activity in a murine model of infection. *PLoS ONE*, 9(4), 1-9.

Garnacho-Montero, J., Ortiz-Leyba, C., Jiménez-Jiménez, F. J., Barrero-Almodóvar, A. E., García-Garmendia, J. L., Bernabeu-Wittell, M., Gallego-Lara, S. L., & Madrazo-Osuna, J. (2003). Treatment of multidrug-resistant *Acinetobacter baumannii* ventilator-associated pneumonia (VAP) with intravenous colistin: A comparison with imipenem-susceptible VAP. *Clinical Infectious Diseases*, 36(9), 1111–1118.

Garnacho, J., Sole-Violan, J., Sa-Borges, M., Diaz, E., & Rello, J. (2003). Clinical impact of pneumonia caused by *Acinetobacter baumannii* in intubated patients: A

matched cohort study. *Critical Care Medicine*, 31(10), 2478–2482.

Ghasemi, E., Ghalavand, Z., Goudarzi, H., Yeganeh, F., Hashemi, A., Dabiri, H., Mirsamadi, E. S., & Foroumand, M. (2018). Phenotypic and genotypic investigation of biofilm formation in clinical and environmental isolates of *Acinetobacter baumannii*. *Archives of Clinical Infectious Diseases*, 13(4), 1158-1166

Ghigo, J. M. (2001). Natural conjugative plasmids induce bacterial biofilm development. *Nature*, 412(6845), 442-445

Gillis, R. J., White, K. G., Choi, K. H., Wagner, V. E., Schweizer, H. P., & Iglewski, B. H. (2005). Molecular basis of azithromycin-resistant *Pseudomonas aeruginosa* biofilms. *Antimicrobial Agents and Chemotherapy*, 49(9), 3858–3867.

Giwercman, B., Jensen, E. T., Hoiby, N., Kharazmi, A., & Costerton, J. W. (1991). Induction of β-lactamase production in *Pseudomonas aeruginosa* biofilm. *Antimicrobial Agents and Chemotherapy*, 35 (5), 1008–1010.

Gonzalez-Villoria, A. M., & Valverde-Garduno, V. (2016). Antibiotic-resistant *Acinetobacter baumannii* increasing success remains a challenge as a nosocomial pathogen. *Journal of Pathogens*, 2016, 1–10.

Gordon, N. C., & Wareham, D. W. (2010). Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. In *International Journal of Antimicrobial Agents*,35(3), 219–226).

Greene, C., Vadlamudi, G., Newton, D., Foxman, B., & Xi, C. (2016). The influence of biofilm formation and multidrug resistance on environmental survival of clinical and environmental isolates of *Acinetobacter baumannii*. *American Journal of Infection Control*, 44(5), e65–e71.

Guardabassi, L., Dijkshoorn, L., Collard, J. M., Olsen, J. E., & Dalsgaard, A. (2000).

Distribution and invitro transfer of tetracycline resistance determinants in clinical and aquatic Acinetobacter strains. *Journal of Medical Microbiology*, 49(10), 929–936.

Guha, S., Udupa, S., Ahmed, W., & Nagaraja, V. (2018). Rewired downregulation of DNA gyrase impacts cell division, expression of topology modulators, and transcription in *Mycobacterium smegmatis*. *Journal of Molecular Biology*, 430(24), 4986–5001

Guillier, M., & Gottesman, S. (2006). Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. *Molecular Microbiology*, 59(1), 231–247.

Hall, C. W., & Mah, T. F. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. In *FEMS Microbiology Reviews*, 41(3), 276–301.

Harding, C. M., Hennon, S. W., & Feldman, M. F. (2018). Uncovering the mechanisms of *Acinetobacter baumannii* virulence. In *Nature Reviews Microbiology*,16(2), 91–102.

Harding, C. M., Tracy, E. N., Carruthers, M. D., Rather, P. N., Actis, L. A., & Munson, R. S. (2013). *Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility. *MBio*, 4(4), 442-445.

Harris, L. G., & Richards, R. G. (2006). Staphylococci and implant surfaces: a review. *Injury*, 37*(2)*, 3-14

Hausner, M., & Wuertz, S. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied and Environmental Microbiology*, 65(8), 3710-3713

He, X., Lu, F., Yuan, F., Jiang, D., Zhao, P., Zhu, J., Cheng, H., Cao, J., & Lu, G. (2015). Biofilm formation caused by clinical *Acinetobacter baumannii* isolates is associated with overexpression of the *AdeFGH* efflux pump. *Antimicrobial Agents and Chemotherapy*, 59(8), 4817–4825.

Henry, R., Crane, B., Powell, D., Lucas, D. D., Li, Z., Aranda, J., Harrison, P., Nation, R. L., Adler, B., Harper, M., Boyce, J. D., & Li, J. (2014). The transcriptomic response of *Acinetobacter baumannii* to colistin and doripenem alone and in combination in an invitro pharmacokinetics/pharmacodynamics model. *Journal of Antimicrobial Chemotherapy*, 70(5), 1303–1313.

Higgins, P. G., Lehmann, M., Wisplinghoff, H., & Seifert, H. (2010). *gyrB* multiplex PCR to differentiate between *Acinetobacter calcoaceticus* and *Acinetobacter genomic species 3*. *Journal of Clinical Microbiology*, 48(12), 4592–4594.

Ho, C. M., Ho, M. W., Chi, C. Y., Lin, C. Der, Lin, C. W., Tseng, S. P., Teng, L. J., Chang, H. Y., Chang, H. L., Chang, Y. F., Lin, H. H., Tien, N., Lu, J. J., & Lai, C. H. (2013). Repeated colonization by multi-drug-resistant Acinetobacter calcoaceticus- Acinetobacter baumannii complex and changes in antimicrobial susceptibilities in surgical intensive care units. *Surgical Infections*, 14(1), 43–48.

Hofacker, I. L., & Stadler, P. F. (2006). Memory efficient folding algorithms for circular RNA secondary structures. *Bioinformatics*, *22*(10), 1172–1176.

Høiby, N., Bjarnsholt, T., Moser, C., Bassi, G. L., Coenye, T., Donelli, G., Hall-Stoodley, L., Holá, V., Imbert, C., Kirketerp-Møller, K., Lebeaux, D., Oliver, A., Ullmann, A. J., Williams, C., ESCMID Study Group for Biofilms (ESGB), &

Consulting External Expert Werner Zimmerli. (2015). ESCMID* guideline for the diagnosis and treatment of biofilm infections 2014. *Clinical Microbiology and Infection*, 21, 1–25.

Høiby, Niels, Bjarnsholt, T., Givskov, M., Molin, S., & Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. In *International Journal of Antimicrobial Agents*, 35 (4) , 322–332.

Hori, K., & Matsumoto, S. (2010). Bacterial adhesion: From mechanism to control. In *Biochemical Engineering Journal*, 48(3), 424–434.

Howard, A., O'Donoghue, M., Feeney, A., & Sleator, R. D. (2012). *Acinetobacter baumannii* An emerging opportunistic pathogen. *Virulence*, 3(3), 5.

Hoyle, B. D., & Costerton, J. W. (1991). Bacterial resistance to antibiotics: The role of biofilms. *Progress in Drug Research*, 37, 91–105.

Hsein, C. C., Yu, F. W., Dijkshoorn, L., Vaneechoutte, M., Chung, T. T., & Tsung, C. C. (2005). Species-level identification of isolates of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex by sequence analysis of the 16S-23S rRNA gene spacer region. *Journal of Clinical Microbiology*, 43(4), 1632–1639.

Hua, X., Chen, Q., Li, X., & Yu, Y. (2014). Global transcriptional response of *Acinetobacter baumannii* to a subinhibitory concentration of tigecycline. *International Journal of Antimicrobial Agents*, 44(4), 337–344.

Hujer, K. M., Hujer, A. M., Endimiani, A., Thomson, J. M., Adams, M. D., Goglin, K., Rather, P. N., Pennella, T. T. D., Massire, C., Eshoo, M. W., Sampath, R., Blyn, L. B., Ecker, D. J., & Bonomo, R. A. (2009). Rapid determination of quinolone resistance in Acinetobacter spp. *Journal of Clinical Microbiology*, 47(5), 1436–1442.

Huslage, K., Rutala, W. A., Sickbert-Bennett, E., & Weber, D. J. (2010). A quantitative approach to defining "High-Touch" surfaces in hospitals. *Infection Control & Hospital Epidemiology*, 31(8), 850–853.

Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., Reiz, B., Cordwell, S. J., Whittal, R., Schild, S., & Feldman, M. F. (2012). Identification of a general O-linked protein glycosylation system in *Acinetobacter baumannii* and its role in virulence and biofilm formation. *PLoS Pathogens*, 8(6), 1-12.

Jacobs, A. C., Hood, I., Boyd, K. L., Olson, P. D., Morrison, J. M., Carson, S., Sayood, K., Iwen, P. C., Skaar, E. P., & Dunman, P. M. (2010). Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infection and Immunity*, 78(5), 1952–1962.

Jacobs, A. C., Sayood, K., Olmsted, S. B., Blanchard, C. E., Hinrichs, S., Russell, D., & Dunman, P. M. (2012). Characterization of the *Acinetobacter baumannii* growth phase-dependent and serum responsive transcriptomes. *FEMS Immunology and Medical Microbiology*, 64(3), 403–412.

Jahn, C. E., Charkowski, A. O., & Willis, D. K. (2008). Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. *Journal of Microbiological Methods*, *75*(2), 318–324.

Jakubovics, N. S., Shields, R. C., Rajarajan, N., & Burgess, J. G. (2013). Life after death: The critical role of extracellular DNA in microbial biofilms. *Letters in Applied Microbiology*, 57(6), 467–475.

Jamal, S., Al Atrouni, A., Rafei, R., Dabboussi, F., Hamze, M., & Osman, M. (2018). Molecular mechanisms of antimicrobial resistance in *Acinetobacter baumannii*, with a special focus on its epidemiology in Lebanon. In *Journal of Global Antimicrobial Resistance*, 15, 154–163.

Jefferson, K. K., Goldmann, D. A., & Pier, G. B. (2005). Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrobial Agents and Chemotherapy,* 49(6), 2467–2473.

Jiménez-Mejías, M., Pichardo-Guerrero, C., Márquez-Rivas, F., Martín-Lozano, D., Prados, T., & Pachón, J. (2002). Cerebrospinal fluid penetration and pharmacokinetic/pharmacodynamic parameters of intravenously administered colistin in a case of multidrug-resistant *Acinetobacter baumannii* meningitis. *European Journal of Clinical Microbiology and Infectious Diseases*, 21(3), 212–214.

Jung, J. Y., Park, M. S., Kim, S. E., Park, B. H., Son, J. Y., Kim, E. Y., Lim, J. E., Lee, S. K., Lee, S. H., Lee, K. J., Kang, Y. A., Kim, S. K., Chang, J., & Kim, Y. S. (2010). Risk factors for multi-drug resistant *Acinetobacter baumannii* bacteremia in patients with colonization in the intensive care unit. *BMC Infectious Diseases*, 10, 1-11.

Kamolvit, W., Higgins, P. G., Paterson, D. L., & Seifert, H. (2014). Multiplex PCR to detect the genes encoding naturally occurring oxacillinases in Acinetobacter spp. *Journal of Antimicrobial Chemotherapy*, 69(4), 959–963.

Kamoshida, G., Akaji, T., Takemoto, N., Suzuki, Y., Sato, Y., Kai, D., Hibino, T., Yamaguchi, D., Kikuchi-Ueda, T., Nishida, S., Unno, Y., Tansho-Nagakawa, S., Ubagai, T., Miyoshi-Akiyama, T., Oda, M., & Ono, Y. (2020). Lipopolysaccharide-deficient *Acinetobacter baumannii* due to colistin resistance is killed by neutrophil-produced lysozyme. *Frontiers in Microbiology*, 11, 1-13.

Kanafani, Z. A. (2014). Acinetobacter infection: Treatment and prevention. *UptoDate*.

Kaplan, J. B. (2010). Biofilm dispersal: Mechanisms, clinical implications, and potential therapeutic uses. In *Journal of Dental Research*, 89(3), 205–218.

Kaplan, J. B. (2011). Antibiotic-induced biofilm formation. In *International Journal of Artificial Organs* , 34 (9), 737–751.

Karygianni, L., Ruf, S., Follo, M., Hellwig, E., Bucher, M., Anderson, A. C., Vach, K., & Al-Ahmad, A. (2014). Novel broad-spectrum antimicrobial photoinactivation of in situ oral biofilms by visible light plus water-filtered infrared A. *Applied and Environmental Microbiology*, 80(23), 7324–7336.

Katsikogianni, M., Missirlis, Y. F., Harris, L., & Douglas, J. (2004). Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. In *European Cells and Materials*, 8, 37–57.

Keren, I., Shah, D., Spoering, A., Kaldalu, N., & Lewis, K. (2004). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *Journal of Bacteriology*, 186(24), 8172–8180.

Kim, C. H., Jeong, Y. J., Lee, J., Jeon, S. J., Park, S. R., Kang, M. J., Park, J. H., & Park, J. H. (2013). Essential role of toll-like receptor 4 in *Acinetobacter baumannii*-induced immune responses in immune cells. *Microbial Pathogenesis*, 54(1), 20–25.

Knapp, S., Wieland, C. W., Florquin, S., Pantophlet, R., Dijkshoorn, L., Tshimbalanga, N., Akira, S., & Van Der Poll, T. (2006). Differential roles of CD14 and toll-like receptors 4 and 2 in murine Acinetobacter pneumonia. *American Journal of Respiratory and Critical Care Medicine*, 173(1), 122–129.

Koo, H., Allan, R. N., Howlin, R. P., Stoodley, P., & Hall-Stoodley, L. (2017). Targeting microbial biofilms: Current and prospective therapeutic strategies. In *Nature Reviews Microbiology*,15(12), 740–755.

Kramer, A., Schwebke, I., & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. In *BMC Infectious Diseases*, 6(1), 1-8.

Kröger, C., Kary, S. C., Schauer, K., & Cameron, A. D. S. (2017). Genetic regulation of virulence and antibiotic resistance in *Acinetobacter baumannii.* In *Genes*, 8(1), 12

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), 1547–1549.

Kumon, H., Tomochika, K. I., Matunaga, T., Ohmori, H., & Ogawa, M. (1994). A sandwich cup method for the penetration assay of antimicrobial agents through pseudomonas exopolysaccharides. *Microbiology and Immunology*, 38(8), 615–619.

Kuo, L. C., Lai, C. C., Liao, C. H., Hsu, C. K., Chang, Y. L., Chang, C. Y., & Hsueh, P. R. (2007). Multidrug-resistant *Acinetobacter baumannii* bacteraemia: Clinical features, antimicrobial therapy, and outcome. *Clinical Microbiology and Infection*, 13(2), 196–198.

La Scola, B., Gundi, V. A. K. B., Khamis, A., & Raoult, D. (2006). Sequencing of the *rpoB* gene and flanking spacers for molecular identification of Acinetobacter species. *Journal of Clinical Microbiology*, 44(3), 827–832.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with bowtie 2. *Nature Methods*, 9(4), 357–359.

Langmead, B., Trapnell, C., Pop, M., & Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology*, 10(3), 1-10.

Laureti, L., Matic, I., & Gutierrez, A. (2013). Bacterial responses and genome instability induced by subinhibitory concentrations of antibiotics. In *Antibiotics*, 2(1), 100–114.

Lebeaux, D., Ghigo, J.-M., & Beloin, C. (2014). Biofilm-related infections: Bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiology and Molecular Biology Reviews*, 78(3), 510–543.

Lee, C. R., Lee, J. H., Park, M., Park, K. S., Bae, I. K., Kim, Y. B., Cha, C. J., Jeong, B. C., & Lee, S. H. (2017). Biology of *Acinetobacter baumannii*: Pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. *Frontiers in Cellular and Infection Microbiology*, 7,1-22.

Lee, H. W., Koh, Y. M., Kim, J., Lee, J. C., Lee, Y. C., Seol, S. Y., & Cho, D. T. (2008). Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. *Clinical Microbiology and Infection*, 14(1), 49–54.

Lee, N. Y., Wang, C. L., Chuang, Y. C., Yu, W. L., Lee, H. C., Chang, C. M., Wang, L. R., & Ko, W. C. (2007). Combination carbapenem-sulbactam therapy for critically ill patients with multidrug-resistant *Acinetobacter baumannii* bacteremia: Four case reports and an invitro combination synergy study. *Pharmacotherapy*, 27(11), 1506–1511.

Lees-Miller, R. G., Iwashkiw, J. A., Scott, N. E., Seper, A., Vinogradov, E., Schild, S., & Feldman, M. F. (2013). A common pathway for O-linked protein-glycosylation and synthesis of capsule in *Acinetobacter baumannii*. *Molecular Microbiology*, 89(5), 816–830.

Lenz, D. H., Miller, M. B., Zhu, J., Kulkarnl, R. V., & Bassler, B. L. (2005). *CsrA* and

three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Molecular Microbiology*, 58(4), 1186–1202.

Lesho, E., Wortmann, G., Moran, K., & Craft, D. (2005). Fatal *Acinetobacter baumannii* infection with discordant carbapenem susceptibility. In *Clinical Infectious Diseases*, 41(5), 758–759.

Leung, W. S., Chu, C. M., Tsang, K. Y., Lo, F. H., Lo, K. F., & Ho, P. L. (2006). Fulminant community-acquired *Acinetobacter baumannii* pneumonia as a distinct clinical syndrome. *Chest*, 129(1), 102–109.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., & Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science*, *355*(6327), 826–830.

Levin, B. R., & Rozen, D. E. (2006). Non-inherited antibiotic resistance. In *Nature Reviews Microbiology*, 4(7), 556–562).

Lewis, K. (2001). Riddle of biofilm resistance. In *Antimicrobial Agents and Chemotherapy*,45(4), 999–1007.

Lewis, K. (2008). Multidrug tolerance of biofilms and persister cells. In *Current Topics in Microbiology and Immunology*, 322, 107–131.

Lewis, Kim. (2007). Persister cells, dormancy, and infectious disease. In *Nature Reviews Microbiology*, 5, 48–56.

Li, L., Mendis, N., Trigui, H., Oliver, J. D., & Faucher, S. P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. In *Frontiers in Microbiology*,5, 1–1.

Li, Z., & Jiang, H. dong. (2009). The effect of biofilms on the production of beta-lactamases in *Pseudomonas aeruginosa*. *Chinese Journal of Tuberculosis and Respiratory Diseases*, *32*(8), 613–616.

Lin, D., Chen, K., Guo, J., Ye, L., Li, R., Chan, E. W. C., & Chen, S. (2020). Contribution of biofilm formation genetic locus, *pgaABCD*, to antibiotic resistance development in gut microbiome. *Gut Microbes*, 12(1), 1–12.

Lin, M. F., Lin, Y. Y., & Lan, C. Y. (2017). Contribution of *EmrAB* efflux pumps to colistin resistance in *Acinetobacter baumannii*. *Journal of Microbiology*, 55(2), 130–136.

Linden, P. K., & Paterson, D. L. (2006). Parenteral and inhaled colistin for treatment of ventilator-associated pneumonia. *Clinical Infectious Diseases*, 43(2), 89-93.

Liou, M. L., Soo, P. C., Ling, S. R., Kuo, H. Y., Tang, C. Y., & Chang, K. C. (2014). The sensor kinase *BfmS* mediates virulence in *Acinetobacter baumannii*. *Journal of Microbiology, Immunology, and Infection*, 47(4), 275–281.

Liu, B. H., Yu, H., Tu, K., Li, C., Li, Y. X., & Li, Y. Y. (2010). DCGL: An R package for identifying differentially coexpressed genes and links from gene expression microarray data. *Bioinformatics*, 26(20), 2637–2638.

Liu, Q., Li, W., Du, X., Li, W., Zhong, T., Tang, Y., Feng, Y., Tao, C., & Xie, Y. (2015). Risk and prognostic factors for multidrug-resistant *Acinetobacter baumannii* complex bacteremia: A retrospective study in a tertiary hospital of West China. *PLoS ONE*, 10(6), 1-13.

Loehfelm, T. W., Luke, N. R., & Campagnari, A. A. (2008). Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *Journal of Bacteriology*, 190(3), 1036–1044.

López, D., Vlamakis, H., & Kolter, R. (2010). Biofilms. In *Cold Spring Harbor perspectives in biology*,2(7), 1-9.

Lv, W., Zhang, X., Hou, M., Han, D., Li, Y., & Xiong, W. (2019). Draft genome sequence of an *OXA-23*, *OXA-66*, *ADC-25* and *TEM-1D* co-producing

Acinetobacter baumannii ST195 isolated from a patient with neonatal pneumonia in China. In *Journal of Global Antimicrobial Resistance*,16, 1–3.

Madsen, J. S., Burmølle, M., Hansen, L. H., & Sørensen, S. J. (2012). The interconnection between biofilm formation and horizontal gene transfer. In *FEMS Immunology and Medical Microbiology*, 65(2), 183–195.

Magnet, S., Courvalin, P., & Lambert, T. (2001). Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrobial Agents and Chemotherapy*, 45(12), 3375–3380.

Mah, T. F. (2012). Biofilm-specific antibiotic resistance. In *Future Microbiology*,7(9), 1061–1072).

Mah, T. F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. In *Trends in Microbiology*, 9(1), 34–39.

Maki, K., Uno, K., Morita, T., & Aiba, H. (2008). RNA, but not protein partners, is directly responsible for translational silencing by a bacterial Hfq-binding small RNA. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(30), 10332–10337.

Marques, C. (2015). Isolation of persister cells from biofilm and planktonic populations of *Pseudomonas aeruginosa*. *Bio-Protocol*, *5*(18), e1590

McClure, R., Balasubramanian, D., Sun, Y., Bobrovskyy, M., Sumby, P., Genco, C. A., Vanderpool, C. K., & Tjaden, B. (2013). Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Research*, 41(14), 1-16.

McConnell, M. J., Actis, L., & Pachón, J. (2013). *Acinetobacter baumannii*: Human infections, factors contributing to pathogenesis and animal models. In *FEMS Microbiology Reviews*, 37(2), 130–155.

Mehta, P., Goyal, S., & Wingreen, N. S. (2008). A quantitative comparison of sRNA-based and protein-based gene regulation. *Molecular Systems Biology, 4,* 221.

Merino, S., & Tomás, J. M. (2015). Bacterial capsules and evasion of immune responses. In *eLS*,1-10.

Methé, B. A., Nelson, K. E., Pop, M., Creasy, H. H., Giglio, M. G., Huttenhower, C., Gevers, D., Petrosino, J. F., Abubucker, S., Badger, J. H., Chinwalla, A. T., Earl, A. M., Fitzgerald, M. G., Fulton, R. S., Hallsworth-Pepin, K., Lobos, E. A., Madupu, R., Magrini, V., Martin, J. C., White, O. (2012). A framework for human microbiome research. *Nature*, 486(7402), 215–221.

Michalopoulos, A., Kasiakou, S. K., Rosmarakis, E. S., & Falagas, M. E. (2005). Cure of multidrug-resistant *Acinetobacter baumannii* bacteraemia with continuous intravenous infusion of colistin. *Scandinavian Journal of Infectious Diseases*, 37(2), 142–145.

Michalopoulos, A. S., Tsiodras, S., Rellos, K., Mentzelopoulos, S., & Falagas, M. E. (2005). Colistin treatment in patients with ICU-acquired infections caused by multiresistant Gram-negative bacteria: The renaissance of an old antibiotic. *Clinical Microbiology and Infection*, 11(2), 115–121.

Michiels, J. E., Van den Bergh, B., Verstraeten, N., & Michiels, J. (2016). Molecular mechanisms and clinical implications of bacterial persistence. *Drug Resistance Updates*, 29, 76–89.

Mihailescu, R., Tafin, U. F., Corvec, S., Oliva, A., Betrisey, B., Borens, O., & Trampuza, A. (2014). High activity of fosfomycin and rifampin against methicillin-resistant *Staphylococcus aureus* biofilm in vitro and in an experimental foreign-body infection model. *Antimicrobial Agents and Chemotherapy*, 58(5), 2547–2553.

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D. F., Vinogradov, E., Seemann, T., Henry, R., Crane, B., St. Michael, F., Cox, A. D., Adler, B., Nation, R. L., Li, J., & Boyce, J. D. (2010). Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial Agents and Chemotherapy*, 54(12), 4971–4977.

Moskowitz, S. M., Foster, J. M., Emerson, J., & Burns, J. L. (2004). Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *Journal of Clinical Microbiology*, 42(5), 1915–1922.

Mugnier, P. D., Poirel, L., Naas, T., & Nordmann, P. (2010). Worldwide dissemination of the $bla_{OXA\text{-}23}$ carbapenemase gene of *Acinetobacter baumannii*. *Emerging Infectious Diseases*, 16(1), 35–40.

Mulcahy, H., Charron-Mazenod, L., & Lewenza, S. (2008). Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathogens*, *4*(11), e1000213.

Mussi, M. A., Limansky, A. S., & Viale, A. M. (2005). Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumannii*: Natural insertional inactivation of a gene encoding a member of a novel family of β-barrel outer membrane proteins. *Antimicrobial Agents and Chemotherapy*, 49(4), 1432–1440.

Navidifar, T., Amin, M., & Rashno, M. (2019). Effects of sub-inhibitory concentrations of meropenem and tigecycline on the expression of genes regulating pili, efflux pumps and virulence factors involved in biofilm formation by *Acinetobacter baumannii*. *Infection and Drug Resistance*, 12, 1099–1111.

Navon-Venezia, S., Leavitt, A., & Carmeli, Y. (2007). High tigecycline resistance in multidrug-resistant *Acinetobacter baumannii*. *Journal of Antimicrobial*

Chemotherapy, 59(4), 772–774.

Nickel, J. C., Ruseska, I., Wright, J. B., & Costerton, J. W. (1985). Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrobial Agents and Chemotherapy*, 27(4), 619–624.

Nikaido, H. (1998). Multiple antibiotic resistance and efflux. *Current Opinion in Microbiology*, 1(5), 516–523.

Niu, C., Clemmer, K. M., Bonomo, R. A., & Rather, P. N. (2008). Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii. *Journal of Bacteriology*, *190*(9), 3386–3392.

Noori, M., Karimi, A., Fallah, F., Hashemi, A., Alimehr, S., Goudarzi, H., & Aghamohammad, S. (2014). High prevalence of metallo-beta-lactamase producing *Acinetobacter baumannii* isolated from two hospitals of Tehran, Iran. *Archives of Pediatric Infectious Diseases*, 2(3), 1-4.

Novović, K., Mihajlović, S., Dinić, M., Malešević, M., Miljković, M., Kojić, M., & Jovčić, B. (2018). Acinetobacter spp. porin *Omp33-36*: Classification and transcriptional response to carbapenems and host cells. *PLoS ONE*, 13(8), 1-17.

Núñez, M. L., Martínez-Toldos, M. C., Bru, M., Simarro, E., Segovia, M., & Ruiz, J. (1998). Appearance of resistance to meropenem during the treatment of a patient with meningitis by Acinetobacter. *Scandinavian Journal of Infectious Diseases*, 30(4), 421–423.

Nwugo, C. C., Gaddy, J. A., Zimbler, D. L., & Actis, L. A. (2011). Deciphering the iron response in *Acinetobacter baumannii*: A proteomics approach. *Journal of Proteomics*, 74(1), 44-58

Olsen, I. (2015). Biofilm-specific antibiotic tolerance and resistance. In *European Journal of Clinical Microbiology and Infectious Diseases*,34 (5), 877–886.

Ong, C. W. M., Lye, D. C. B., Khoo, K. L., Chua, G. S. W., Yeoh, S. F., Leo, Y. S., Tambyah, P. A., & Chua, A. C. (2009). Severe community-acquired *Acinetobacter baumannii* pneumonia: An emerging highly lethal infectious disease in the Asia-Pacific. *Respirology*, 14(8), 1200–1205.

Ophir, T., & Gutnick, D. L. (1994). A role for exopolysaccharides in the protection of microorganisms from desiccation. In *Applied and Environmental Microbiology*, 60 (2), 740–745.

Orman, M. A., & Brynildsen, M. P. (2015). Inhibition of stationary phase respiration impairs persister formation in *E. coli*. *Nature Communications*, 6(1), 1-13.

Pachón-Ibánẽz, M. E., Jiménez-Mejías, M. E., Pichardo, C., Llanos, A. C., & Pachón, J. (2004). Activity of tigecycline (GAR-936) against *Acinetobacter baumannii* strains, including those resistant to imipenem. *Antimicrobial Agents and Chemotherapy*, 48(11), 4479–4481.

Pamp, S. J., Gjermansen, M., Johansen, H. K., & Tolker-Nielsen, T. (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells and depends on the *pmr* and *mexAB-oprM* genes. *Molecular Microbiology*, 68(1), 223–240.

Papenfort, K., & Vogel, J. (2010). Regulatory RNA in bacterial pathogens. In *Cell Host and Microbe*, 8(1), 116–127.

Park, S. C., Park, Y., & Hahm, K. S. (2011). The role of antimicrobial peptides in preventing multidrug-resistant bacterial infections and biofilm formation. In *International Journal of Molecular Sciences*,12(9), 5971–5992).

Parte, A. C. (2018). LPSN - List of prokaryotic names with standing in nomenclature (Bacterio.net), 20 years on. In *International Journal of Systematic and Evolutionary Microbiology*, 68(6), 1825–1829.

Paton, R., Miles, R. S., Hood, J., Amyes, S. G. B., Miles, R. S., & Amyes, S. G. B. (1993). ARI 1:β-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*. *International Journal of Antimicrobial Agents*, 2(2), 81–87.

Paulsen, I. T. (2003). Multidrug efflux pumps and resistance: Regulation and evolution. In *Current Opinion in Microbiology*, 6 (5), 446–451.

Peleg, A. Y., Seifert, H., & Paterson, D. L. (2008). *Acinetobacter baumannii*: Emergence of a successful pathogen. In *Clinical Microbiology Reviews*, 21(3), 538–582.

Penesyan, A., Gillings, M., & Paulsen, I. T. (2015). Antibiotic discovery: Combatting bacterial resistance in cells and in biofilm communities. In *Molecules*, 20(4), 5286–5298.

Penesyan, A., Nagy, S. S., Kjelleberg, S., Gillings, M. R., & Paulsen, I. T. (2019). Rapid microevolution of biofilm cells in response to antibiotics. *Npj Biofilms and Microbiomes*, 5(1), 1-34.

Percival, S. L., Vuotto, C., Donelli, G., & Lipsky, B. A. (2015). Biofilms and wounds: An identification algorithm and potential treatment options. *Advances in Wound Care*, 4(7), 389–397.

Perez-Llarena, F., & Bou, G. (2009). β-lactamase inhibitors: The story so far. *Current Medicinal Chemistry*, 16(28), 3740–3765.

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., & Bonomo, R. A. (2007). Global challenge of multidrug-resistant *Acinetobacter baumanni*. In *Antimicrobial Agents and Chemotherapy*, 51(10), 3471–3484.

Petrova, O. E., & Sauer, K. (2016). Escaping the biofilm in more than one way: Desorption, detachment,or dispersion. In *Current Opinion in Microbiology*, 30, 67–78.

Pi, B., Yu, D., Hua, X., Ruan, Z., & Yu, Y. (2017). Genomic and transcriptome analysis of triclosan response of a multidrug-resistant *Acinetobacter baumannii* strain, MDR-ZJ06. *Archives of Microbiology*, 199(2), 223–230.

Piepenbrink, K. H., Lillehoj, E., Harding, C. M., Labonte, J. W., Zuo, X., Rapp, C. A., Munson, R. S., Goldblum, S. E., Feldman, M. F., Gray, J. J., & Sundberg, E. J. (2016). Structural diversity in the type IV pili of multidrug-resistant acinetobacter. *Journal of Biological Chemistry*, 291(44), 22924–22935.

Pintado, V., San Miguel, L. G., Grill, F., Mejía, B., Cobo, J., Fortún, J., Martín-Dávila, P., & Moreno, S. (2008). Intravenous colistin sulphomethate sodium for therapy of infections due to multidrug-resistant Gram-negative bacteria. *Journal of Infection*, 56(3), 185–190.

Piperaki, E. T., Tzouvelekis, L. S., Miriagou, V., & Daikos, G. L. (2019). Carbapenem-resistant *Acinetobacter baumannii*: in pursuit of an effective treatment. In *Clinical Microbiology and Infection*, 25(8), 951–957.

Poirel, L., & Nordmann, P. (2006). Carbapenem resistance in *Acinetobacter baumannii*: Mechanisms and epidemiology. In *Clinical Microbiology and Infection*, 12(9), 826–836.

Poole, K., Krebes, K., McNally, C., & Neshat, S. (1993). Multiple antibiotic resistance in *Pseudomonas aeruginosa*: Evidence for involvement of an efflux operon. *Journal of Bacteriology*, 175(22), 7363–7372.

Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. In *Annals of Medicine*, 39(3), 162–176.

Potron, A., Poirel, L., & Nordmann, P. (2015). Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology. In *International Journal of Antimicrobial Agents*, 45(6), 568–585.

Pourhajibagher, M., Mokhtaran, M., Esmaeili, D., & Bahador, A. (2016). Assessment of biofilm formation among *Acinetobacterbaumannii* strains isolated from burned patients. *Der Pharmacia Lettre*, 8(8), 108–112.

Pu, Y., Ke, Y., & Bai, F. (2017). Active efflux in dormant bacterial cells – New insights into antibiotic persistence. *Drug Resistance Updates*, 30, 7–14.

Qi, L., Li, H., Zhang, C., Liang, B., Li, J., Wang, L., Du, X., Liu, X., Qiu, S., & Song, H. (2016). Relationship between antibiotic resistance, biofilm formation, and biofilm-specific resistance in *Acinetobacter baumannii. Frontiers in Microbiology*, *7*, *483.*

Qin, H., Lo, N. W. S., Loo, J. F. C., Lin, X., Yim, A. K. Y., Tsui, S. K. W., Lau, T. C. K., Ip, M., & Chan, T. F. (2018). Comparative transcriptomics of multidrug-resistant *Acinetobacter baumannii* in response to antibiotic treatments. *Scientific Reports*, 8(1), 1-10.

Qu, J., Du, Y., Yu, R., & Lü, X. (2016). The first outbreak caused by *Acinetobacter baumannii* ST208 and ST195 in China. *BioMed Research International*, 2016, 1-6.

Rajamohan, G., Srinivasan, V. B., & Gebreyes, W. A. (2009). Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. *Journal of Antimicrobial Chemotherapy*, 65(2), 228–232.

Rao, R., Karthika, R., Singh, S., Shashikala, P., Kanungo, R., Jayachandran, S., & Prashanth, K. (2008). Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acinetobacter baumannii*. *Indian Journal of Medical Microbiology*, 26(4), 333–337.

Reid, G. E., Grim, S. A., Aldeza, C. A., Janda, W. M., & Clark, N. M. (2007). Rapid development of *Acinetobacter baumannii* resistance to tigecycline. *Pharmacotherapy*, 27(8), 1198–1201.

Ribera, A., Ruiz, J., & Vila, J. (2003). Presence of the *tet M* determinant in a clinical isolate of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 47(7), 2310–2312.

Richards, G. R., & Vanderpool, C. K. (2011). Molecular call and response: The physiology of bacterial small RNAs. In *Biochimica et Biophysica Acta Gene Regulatory Mechanisms*,1809 (10), 525–531).

Roberts, M. C. (1996). Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. In *FEMS Microbiology Reviews*,19(1), 1–24.

Rodrigues Perez, L. R. (2015). *Acinetobacter baumannii* displays inverse relationship between meropenem resistance and biofilm production. *Journal of Chemotherapy*, 27(1), 13–15.

Rodríguez-Baño, J., Martí, S., Soto, S., Fernández-Cuenca, F., Cisneros, J. M., Pachón, J., Pascual, A., Martínez-Martínez, L., Mcqueary, C., Actis, L. A., & Vila, J. (2008). Biofilm formation in *Acinetobacter baumannii*: Associated features and clinical implications. *Clinical Microbiology and Infection*, 14(3), 276–278.

Rodríguez-Martínez, J. M., Ballesta, S., & Pascual, Á. (2007). Activity and penetration of fosfomycin, ciprofloxacin, amoxicillin/clavulanic acid and co-trimoxazole in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms. In *International Journal of Antimicrobial Agents*, 30(4), 366–368.

Romby, P., Vandenesch, F., & Wagner, E. G. H. (2006). The role of RNAs in the

regulation of virulence-gene expression. In *Current Opinion in Microbiology*,9(2), 229–236.

Ronish, L. A., Lillehoj, E., Fields, J. K., Sundberg, E. J., & Piepenbrink, K. H. (2019). The structure of PilA from *Acinetobacter baumannii* AB5075 suggests a mechanism for functional specialization in Acinetobacter type IV pili. *Journal of Biological Chemistry*, 294(1), 218–230.

Rosenfeld, N., Bouchier, C., Courvalin, P., & Périchon, B. (2012). Expression of the resistance-nodulation-cell division pump *AdeIJK* in *Acinetobacter baumannii* is regulated by *AdeN*, a *TetR*-type regulator. *Antimicrobial Agents and Chemotherapy*, 56(5), 2504–2510.

Roy, R., Tiwari, M., Donelli, G., & Tiwari, V. (2018). Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. In *Virulence*, 9(1), 522–554.

Rumbo-Feal, S., Gómez, M. J., Gayoso, C., Álvarez-Fraga, L., Cabral, M. P., Aransay, A. M., Rodríguez-Ezpeleta, N., Fullaondo, A., Valle, J., Tomás, M., Bou, G., & Poza, M. (2013). Whole transcriptome analysis of *Acinetobacter baumannii* assessed by RNA-sequencing reveals different mRNA expression profiles in biofilm compared to planktonic cells. *PLoS ONE*, 8(8), 1-15.

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., Schultz, L. W., Umland, T. C., & Campagnari, A. A. (2010). The K1 capsular polysaccharide of *Acinetobacter baumannii* strain 307-0294 is a major virulence factor. *Infection and Immunity*, 78(9), 3993–4000.

Ryu, S. H., Yu, C. M., Suh, S. O., Kim, J. H., Ko, Y. J., Park, J. H., & Cho, J. H. (2012). A case of community-acquired acinetobacter meningitis. *Infection and Chemotherapy*, 44 (6), 473–476.

Sahu, P. K., Iyer, P. S., Oak, A. M., Pardesi, K. R., & Chopade, B. A. (2012). Characterization of eDNA from the clinical strain *Acinetobacter baumannii* AIIMS 7 and its role in biofilm formation. *The Scientific World Journal*, *2012*, 1-9.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Santos-Lopez, A., Marshall, C. W., Scribner, M. R., Snyder, D. J., & Cooper, V. S. (2019). Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. *ELife*, 8, 1-23.

Sauer, K. (2003). The genomics and proteomics of biofilm formation. In *Genome Biology*,4(6), 1-4.

Savage, V. J., Chopra, I., & O'Neill, A. J. (2013). *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrobial Agents and Chemotherapy*, 57(4), 1968–1970.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, 3(6), 1101–1108.

Scott, P., Deye, G., Srinivasan, A., Murray, C., Moran, K., Hulten, E., Fishbain, J., Craft, D., Riddell, S., Lindler, L., Mancuso, J., Milstrey, E., Bautista, C. T., Patel, J., Ewell, A., Hamilton, T., Gaddy, C., Tenney, M., Christopher, G., Petruccelli, B. (2007). An outbreak of multidrug-resistant Acinetobacter baumannii-calcoaceticus complex infection in the US military health care system associated with military operations in Iraq. *Clinical Infectious Diseases*, 44(12), 1577–1584.

Sengstock, D. M., Thyagarajan, R., Apalara, J., Mira, A., Chopra, T., & Kaye, K. S. (2010). Multidrug-resistant *Acinetobacter baumannii*: An emerging pathogen

among older adults in community hospitals and nursing homes. *Clinical Infectious Diseases,* 50(12), 1611–1616.

Sharma, D., Misba, L., & Khan, A. U. (2019). Antibiotics versus biofilm: An emerging battleground in microbial communities. In *Antimicrobial Resistance and Infection Control*, 8(1), 1-10.

Sharma, R., Arya, S., Patil, S. D., Sharma, A., Jain, P. K., Navani, N. K., & Pathania, R. (2014). Identification of novel regulatory small RNAs in *Acinetobacter baumannii*. *PLoS ONE*, *9*(4), e93833

Sharon Goh, H. M., Beatson, S. A., Totsika, M., Moriel, D. G., Phan, M. D., Szubert, J., Runnegar, N., Sidjabat, H. E., Paterson, D. L., Nimmo, G. R., Lipman, J., & Schembri, M. A. (2013). Molecular analysis of the *Acinetobacter baumannii* biofilm-associated protein. *Applied and Environmental Microbiology*, 79(21), 6535–6543.

Shenkutie, A. M., Yao, M. Z., Siu, G. K. H., Wong, B. K. C., & Leung, P. H. M. (2020). Biofilm-induced antibiotic resistance in clinical *Acinetobacter baumannii* isolates. *Antibiotics*, 9(11), 1–15.

Singh, H., Thangaraj, P., & Chakrabarti, A. (2013). *Acinetobacter baumannii*: A brief account of mechanisms of multidrug resistance and current and future therapeutic management. In *Journal of Clinical and Diagnostic Research*,7(11), 2602–2605.

Singh, R., Ray, P., Das, A., & Sharma, M. (2010). Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Journal of Antimicrobial Chemotherapy*, 65(9), 1955–1958.

Singhai, M., Malik, A., Shahid, M., Malik, M. A., & Goyal, R. (2012). A study on device-related infections with special reference to biofilm production and

antibiotic resistance. *Journal of Global Infectious Diseases*, 4(4), 193–198.

Sittka, A., Lucchini, S., Papenfort, K., Sharma, C. M., Rolle, K., Binnewies, T. T., Hinton, J. C. D., & Vogel, J. (2008). Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genetics*, 4(8), 1-18.

Smolyakov, R., Borer, A., Riesenberg, K., Schlaeffer, F., Alkan, M., Porath, A., Rimar, D., Almog, Y., & Gilad, J. (2003). Nosocomial multi-drug resistant *Acinetobacter baumannii* bloodstream infection: Risk factors and outcome with ampicillin-sulbactam treatment. *Journal of Hospital Infection*, 54(1), 32–38.

Soper, T., Mandin, P., Majdalani, N., Gottesman, S., & Woodson, S. A. (2010). Positive regulation by small RNAs and the role of Hfq. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(21), 9602–9607.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, *40*(2), 175–179.

Stewart, P. S., & Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. In *Lancet*, 358 (9276),135–138.

Stewart, P. S., & Franklin, M. J. (2008). Physiological heterogeneity in biofilms. In *Nature Reviews Microbiology*,6(3), 199–210.

Storz, G., Vogel, J., & Wassarman, K. M. (2011). Regulation by small RNAs in bacteria: expanding frontiers. In *Molecular Cell*, 43(6), 880–891.

Sugawara, E., & Nikaido, H. (2014). Properties of *AdeABC* and *AdeIJK* efflux systems of *Acinetobacter baumannii* compared with those of the *AcrAB-TolC* system of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*, 58(12), 7250–7257.

Tatman-Otkun, M., Gürcan, Ş., Özer, B., & Shokrylanbaran, N. (2004). Annual trends

in antibiotic resistance of nosocomial *Acinetobacter baumannii* strains and the effect of synergistic antibiotic combinations. *New Microbiologica,* 27(1), 21–28.

Tayabali, A. F., Nguyen, K. C., Shwed, P. S., Crosthwait, J., Coleman, G., & Seligy, V. L. (2012). Comparison of the virulence potential of Acinetobacter strains from clinical and environmental sources. *PLoS ONE,* 7(5), 1-9.

Thomson, J. M., & Bonomo, R. A. (2005). The threat of antibiotic resistance in Gram-negative pathogenic bacteria: β-lactams in peril! In *Current Opinion in Microbiology,* 8(5), 518–524.

Thummeepak, R., Kongthai, P., Leungtongkam, U., & Sitthisak, S. (2016). Distribution of virulence genes involved in biofilm formation in multi-drug resistant *Acinetobacter baumannii* clinical isolates. *International Microbiology,* 19(2), 121–129.

Tjaden, B., Goodwin, S. S., Opdyke, J. A., Guillier, M., Fu, D. X., Gottesman, S., & Storz, G. (2006). Target prediction for small, noncoding RNAs in bacteria. *Nucleic Acids Research, 34*(9), 2791–2802.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., & Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: Involvement of a novel chaperone-usher pili assembly system. *Microbiology*, 149(12), 3473–3484.

Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A., & Actis, L. A. (2008). Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology,* 154(11), 3398–3409.

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., Van Baren, M. J., Salzberg, S. L., Wold, B. J., & Pachter, L. (2010). Transcript assembly and

quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology*, 28(5), 511–515.

Turton, J. F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E., & Pitt, T. L. (2006). Identification of *Acinetobacter baumannii* by detection of the *bla* $_{OXA-51}$-*l*ike carbapenemase gene intrinsic to this species. *Journal of Clinical Microbiology*, 44(8), 2974–2976.

Urish, K. L., DeMuth, P. W., Craft, D. W., Haider, H., & Davis, C. M. (2014). Pulse lavage is inadequate at removal of biofilm from the surface of total knee arthroplasty materials. *Journal of Arthroplasty*, 29(6), 1128–1132.

Valentin-Hansen, P., Johansen, J., & Rasmussen, A. A. (2007). Small RNAs controlling outer membrane porins. In *Current Opinion in Microbiology*,10 (2), 152–155.

Van Acker, H., Van Dijck, P., & Coenye, T. (2014). Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. In *Trends in Microbiology*, 22(6), 326–333.

Vasudevan, R. (2014). Biofilms: Microbial cities of scientific significance. *Journal of Microbiology & Experimentation*, 1(3), 84-98.

Viegas, S. C., Silva, I. J., Saramago, M., Domingues, S., & Arraiano, C. M. (2011). Regulation of the small regulatory RNA MicA by ribonuclease III: A target-dependent pathway. *Nucleic Acids Research*, *39*(7), 2918–2930.

Vila, J., Martí, S., & Sánchez-Céspedes, J. (2007). Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. In *Journal of Antimicrobial Chemotherapy*, 59(6), 1210–1215.

Vogel, J., & Wagner, E. G. H. (2007). Target identification of small noncoding RNAs in bacteria. In *Current Opinion in Microbiology*,10 (3), 262–270.

Vu, B., Chen, M., Crawford, R. J., & Ivanova, E. P. (2009). Bacterial extracellular

polysaccharides involved in biofilm formation. In *Molecules*,14 (7), 2535–2554.

Vuotto, C., & Donelli, G. (2019). Novel treatment strategies for biofilm-based infections. In *Drugs*,79(15), 1635–1655.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. In *Nature*,406 (6797), 775–781.

Wang, Y. C., Huang, T. W., Yang, Y. S., Kuo, S. C., Chen, C. T., Liu, C. P., Liu, Y. M., Chen, T. L., Chang, F. Y., Wu, S. H., How, C. K., & Lee, Y. T. (2018). Biofilm formation is not associated with worse outcome in *Acinetobacter baumannii* bacteraemic pneumonia. *Scientific Reports*, 8(1), 1-9.

Waters, L. S., & Storz, G. (2009). Regulatory RNAs in bacteria. In *Cell*, 136 (4), 615–628.

Weber, B. S., Ly, P. M., Irwin, J. N., Pukatzki, S., & Feldman, M. F. (2015). A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proceedings of the National Academy of Sciences of the United States of America*, 112(30), 9442–9447.

Wayne, P. (2019). Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 29th ed. CLSI supplement M100. In *Clinical and Laboratory Standards Institute,* 45-46.

Weiner, L. M., Webb, A. K., Limbago, B., Dudeck, M. A., Patel, J., Kallen, A. J., Edwards, J. R., & Sievert, D. M. (2016). Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the national healthcare safety network at the centers for disease control and prevention, *Infection Control and Hospital Epidemiology*, 37(11), 1288–1301.

Weiss, A., Broach, W. H., Lee, M. C., & Shaw, L. N. (2016). Towards the complete small RNome of *Acinetobacter baumannii*. *Microbial Genomics*, *2*(3), 1–18.

Whiteley, G. S., Knight, J. L., Derry, C. W., Jensen, S. O., Vickery, K., & Gosbell, I. B. (2015). A pilot study into locating the bad bugs in a busy intensive care unit. *American Journal of Infection Control*, *43* (12), 1270–1275.

WHO. (2017). WHO priority pathogens list for R&D of new antibioticse. Http://Www.Who.Int/En/News-Room/Detail/27-02-2017-Who-Publishes-List-of-Bacteria-for-Which-New-Antibiotics-Are-Urgently-Needed.

Wieczorek, D., Delauriere, L., & Schagat, T. (2012). Methods of RNA quality assessment. *Promega Corporation*, 1–14.

Wieland, K., Chhatwal, P., & Vonberg, R. P. (2018). Nosocomial outbreaks caused by *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: Results of a systematic review. *American Journal of Infection Control*, 46(6), 643–648.

Wilharm, G., Piesker, J., Laue, M., & Skiebe, E. (2013). DNA Uptake by the nosocomial pathogen *Acinetobacter baumannii* occurs during movement along wet surfaces. *Journal of Bacteriology*, 195(18), 4146–4153.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., & Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases*, 39(3), 309–317.

Wisplinghoff, H., Perbix, W., & Seifert, H. (1999). Risk factors for nosocomial bloodstream infections due to *Acinetobacter baumannii*: A case-control study of adult burn patients. *Clinical Infectious Diseases*, 28(1), 59–66.

Wong, D., Nielsen, T. B., Bonomo, R. A., Pantapalangkoor, P., Luna, B., & Spellberg,

B. (2017). Clinical and pathophysiological overview of Acinetobacter infections: A century of challenges. *Clinical Microbiology Reviews*, 30(1), 409–447.

Wood, G. C., Hanes, S. O., Croce, M. A., Fabian, T. C., & Boucher, B. A. (2002). Comparison of ampicillin-sulbactam and imipenem-cilastatin for the treatment of Acinetobacter ventilator-associated pneumonia. *Clinical Infectious Diseases*, 34(11), 1425–1430.

Wu, N., He, L., Cui, P., Wang, W., Yuan, Y., Liu, S., Xu, T., Zhang, S., Wu, J., Zhang, W., & Zhang, Y. (2015). Ranking of persister genes in the same *Escherichia coli* genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. *Frontiers in Microbiology*, *6*, 1-9.

Xie, R., Zhang, X. D., Zhao, Q., Peng, B., & Zheng, J. (2018). Analysis of global prevalence of antibiotic resistance in *Acinetobacter baumannii* infections disclosed a faster increase in OECD countries. *Emerging Microbes and Infections*, *7*(1), 1-10

Yang, C. H., Su, P. W., Moi, S. H., & Chuang, L. Y. (2019). Biofilm formation in *Acinetobacter baumannii*: Genotype-phenotype correlation. In *Molecules*, 24(10), 1-12.

Yoon, E. J., Courvalin, P., & Grillot-Courvalin, C. (2013). RND-type efflux pumps in multidrug-resistant clinical isolates of *Acinetobacter baumannii*: Major role for *AdeABC* overexpression and aders mutations. *Antimicrobial Agents and Chemotherapy*, 57(7), 2989–2995.

Young, M. D., Wakefield, M. J., Smyth, G. K., & Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biology*, 11(2), 1-12.

Zhou, Q., Wang, M., Zhong, X., Liu, P., Xie, X., Wangxiao, J., & Sun, Y. (2019). Dissemination of resistance genes in duck/fish polyculture ponds in Guangdong Province: correlations between Cu and Zn and antibiotic resistance genes. *Environmental Science and Pollution Research*, 26(8), 8182–8193

APPENDICES

Appendix 1: The sequence of differentially expressed novel genes that were induced or repressed by imipenem or colistin treatment. The raw sequences were deposited in the NCBI Sequence Read Archive (SRA) under submission ID (SUB9038432) and biosample accession number (SAMN17808362).

>Novel00171
ATTTTTTGCAAGATCAAGACATATAAACGGATAGAAATATCTATCTGTAAGAAGGAGTATGCATGAGCG
TATCGGAAATCCGACCGATTGCCATTGAGGACGAACTCAAGCATTCATATTTAGATTACGCGATGAGTG
TAATTGTATCTCGTGCATTGCCGGATGTGAGAGACGGTCTTAAACCTGTTCACCGTCGTGTGCTTTATG
CCATGCACGAATTGGGCAATGACTATAACAAAGCCTACAAGAAATCTGCTCGTGTCGTTGGGGACGTAA
TCGGTAAATATCACCCGCATGGTGACTCAGCTGTTTATGAAACCATTGTTCGTATGGCTCAAGACTTTA
GCTTACGTTATTTATTGGTTGATGGTCAGGGTAACTTCGGTTCGATCGATGGTGATAGCGCTGCGGCAA
TGCGTTATACCGAAGTCCGTATGACTAAGCTGGCACATGAGCTTCTTGCAGATTTAGAAAAAGACACAG
TTGACTGGGAAGATAACTACGACGGTTCGGAACGTATCCCTGAAGTACTTCCGACACGTGTTCCAAACT
TGTTAATTAACGGTGCTGCTGGTATTGCTGTAGGTATGGCAACTAACATGGCACCACACAACATGACAG
AAGTTGTGAATGCATGTTTGGCTTATGCTGACAATCCGAATATCTCGATTGAAGGATTGATGGAATACA
TTACTGGTCCTGACTTCCCTACAGGCGGTATTATTTACGGTAAATCAGGTATTGTTGATGCCTACCGTA
CCGGTAAAGGTCGTTTACACATTCGTGGTAAATACCATTTCGAAGAAGATGAAAAGACAGGTCGTACAA
CCATCGTCTTTACTGAAATTCCATATCAAGTAAACAAAGCAAGAGTTATTGAACGTATTGCCGAGTTAG
TAAAAGAGAAAAAGCTTGAAGGTATTTCAGAACTTCGTGATGAGTCTGATAAAGAAGGTATGCGTATTG
CAATTGACTTGAAACGCGGTGAAAACGCAGAAGTCGTTGTAAATAACTTATTCTTAAATACCCAGCTTG
AAAACTCATTCAGCATCAACATGGTTTGTCTAGACAATGGACAACCAAAATTGATGAATCTAAAAGATA
TTATTGCGGCATTTATTCGTCACCGCCAAGAAGTTGTGACACGCCGTACCATGTTCGAATTACGTAAAG
CACGTGAACGTGGTCATATCTTGGAAGGCTTGACAGTTGCCTTAGCCAATATTGATGAAATTATTGAAA
CCATCAAAACTTCTGCAAACCCTGCTGAAGCGCGTGAGCGTTTACTTGCGGGTGAGTGGGCAGGTGGTG
GCGTTGTTGCACTACTTGAAAAAGCTGGTGCAATTTCTGTTCGCCCAGATGAAATTGAAGGTGAAGATC
CAAATCGTCCATTTGGTTTAAGTGATTCAATTTACCGTCTGTCACCAACACAAGTAGGCGCAATTTTAG
AATTACGTTTACACCGTTTAACTGGTCTTGAACAAGACAAGTTACATGCGGAATATACTGAAATTTTAG
GTCAAATTGCTGAACTTACTGCAATTTTAAATGACTTTAACTTGTTAATGGGTG

>Novel00490
AGGGAATCGTCTTCTGTCCGGCTTCTTACAAGACACAAACATAAAGATCGCCACTCGGCAACAGAAATT
GTTTTTTCACATTTCTCTTCGAAACAGTTAATTAGACTTCTGAATTGTTATTGTGTAAATACAATTGCG
ATACTCTTTTTTGTATGCGAGCGATTTTTCTTCTTTTTAAACCACACTCTACTCTTTCCTAATTCTGAC
CCTTATGAACTTAACTGAACTCAAGAAAAAACCAATCGGCGAACTAATTAAAATTGCTGAATTTATGGG
CCTTGAAGGTATGGCTCGTAACCGAAAGCAAGATATTATCTTTGCCATCTTGAAACGCCATGCGATGAA
TGGCGAAGAAATTTTTGGTGACGGCGTTCTTGAAATTCTCTCTGATGGTTTTGGTTTTTTGCGCTCTGC
CGCAGGTTCGTATTTAGCTGGTCCGGATGATATTTATGTGAGTCCTTCACAAATCCGACGCTTTAACTT
GCGTACAGGTGACACCATCACAGGTACCATTCGTCCACCAAAAGAAGGTGAGCGTTATTTTGCTTTGCT
CAAAGTTAATCAAATTAACTATGACACACCAGAAAATTCACGTAATAAAATCTTATTTGAAAACTTAAC
TCCACTTTTCCCGACCGAACAACTGGTTATGGAACTTGGTAATGGTACAACAGAAGACTTGACCGCGCG
TGTAGTCGATTTAGTTGCACCAATCGGTAAAGGTCAACGTTCTATTATTGTCGCTCCGCCAAAAGCGGG
TAAAACAATGTTACTTCAAAACATTGCTCAATCTATTGTGAGAAACAATCCGGAAGTTTTCCTTATTGT
TTTATTAATTGATGAACGCCCAGAAGAAGTAACTGAGATGGAGCGTACCGTACGCGGTGAAGTCGTTGC
GTCAACATTTGATGAAGCACCAGCACGTCACGTACAAGTTGCAGAAATGGTCATTGAAAAAGCAAAACG
TCTTGTTGAGCACAAAAAGGACGTCGTGATTTTACTTGACTCCATTACACGTTTAGCCCGTGCATACAA
CACCGTAATTCCTTCATCAGGTAAAGTATTAACTGGTGGTGTGGATGCACATGCTTTAGAACGTCCAAA
ACGTTTCTTCGGTGCTGCTCGTAATATCGAAGAAGGTGGTTCTTTAACAATCATCTCTACTGCTCTTAT
TGAGACTGGCAGTAAAATGGATGATGTAATTTACGAAGAGTTTAAAGGTACAGGTAACCAAGAAATTAC
ACTTGATCGCCGTATTGCTGAAAAACGCGTCTTCCCTGCCATGAATATTAAGAAATCTGGCACACGTCG
TGAAGAACGTTTAATGGATGAAGATAAATTACGTAAAGTTTGGATTCTCCGTAAACTTCTTCATCCTAT
GGATGAGTTGGCGGC

>Novel00584
GATGGTTACACCAGTTTACTGGATTGACCACAGCCCGCCCAGACCAACTTATTTTCAAGGAGTGCAAGA
GTGGCAAACTCTGCTCAAGCTAAAAAACGTGCGCGCCAAAACGTTAAAGCGCGTAAACACAACGCAAGC
TTGCGTTCTATGGTTCGTACTTACATCAAACGTACTTTAAGTGCAATCGCTGGTGGTGATTATGCTGTT
GCTACAGAAGCTTACAAAAAAGCTGTTCCTGTAATCGACCGCATGGCTGATAAAGGCATCATCCACAAA
AATAAAGCTGCTCGTCATAAGAGC

>Novel00626
AATCAACTTTTAATTTGCGTAAATAGAATATTATGACCGAGTCTACTACACCTAAATTTGCCATCCCTG
AATTACCAATGCAGATTCAAACGATTCGTCAATATTTGCCGCATCGTTACCCATTCTTATTGGTTGATC
GTGTGACTGAAGTTACTGACAATAGTATTGTTGGTTATAAAAATGTTTCTATCAATGAAGAGTTCCTAC
AAGGGCATTTCCCAGAATATCCAATTATGCCTGGTGTTCTCATTGTTGAAGCATTAGCTCAAGTTTCAG
GCGTTCTTGGTTTTATTATGAACAATGAAACGCCAAAACCAGGTTCATTATTCCTGTTTGCTGGTGCAG
AAAGAGTTAGATTTAAAAAACAAGTAGTTGCAGGTGATCAACTTGTATTAAAATCTGAGTTGGTAATGC
AGAAGCGCGGTATCTACAAATACAATTGTACAGCTAGCGTTGATGGCATTGTAGCAGCAACCGCTGAGA
TTATGATTTCACACCAAAAAACAGAGCAGGCATGAGCAATCACGATTTAATCCATTCTACCGCCATTAT
TGATCCATCTGCAGTGATTGCTTCAGATGTTCAAATCGGACCTTATTGTATTATTGGTCCTCAAGTGAC
TATTGGTGCTGGTACTAAATTACATTCTCATGTGGTTGTAGGTGGTTTTACCAGAATTGGCCAAAATAA
CGAAATCTTTCAATTTGCAAGTGTTGGCGAAGTTTGCCAAGACCTCAAATATAAAGGTGAAGAAACGTG
GCTTGAAATTGGTAACAATAATCTAATTCGCGAACATTGCAGCTTACATAGAGGTACGGTGCAAGATAA
TGCATTAACCAAGATAGGTAGTCATAACCTATTAATGGTAAATACACATATTGCACATGATTGTATCGT
AGGTGACTATAATATCTTTGCTAATAATGTAGGTGTCGCTGGACATGTACATATTGGTGATCACGTTAT
TGTGGGTGGTAATTCTGGAATTCATCAATTCTGTAAGATCGATTCTTATAGCATGATTGGTGGAGCTTC
TTTGATCCTTAAAGATGTTCCAGCCTATGTGATGGCGTCTGGTAACCCTGCACATGCGTTTGGTATAAA
TATTGAAGGTATGCGAAGAAAAGGTTGGTCTAAAAATACAATTCAAGGCTTAAGAGAAGCTTATAAATT
GATATTTAAATCTGGATTAACTTCTGTTCAAGCTATTGACCAAATTAAAAGTGAAATTTTACCTTCAGT
TCCAGAAGCTCAACTCTTGATTGATTCTCTTGAACAATCAGAGCGTGGAATTGTGCGCTAATCAAGA

Novel00738
GAATAAGCAAAGGTGTGTTTATGCCCTATCTATTGCTTTGTATTGGTTGCGTTTTTTTAGGATTAGGTG
TTCTTGGTCTTTTTGTACCAAGTCTTCAATCTTTGGACTTGCTCACCGTTCAAACATTGAGTCACCATC
GTTTAGATTATCTTAATACTATAACCACCTTTCTTGCACGCGTAGGCGGCATGCCTTTTGTATGTTTTT
TATCCTTTCTAGTATGTATATATTTAGCATGGTATAAAAAATATATTACTGTTATTTTCATTAGCTTGG
GGGTTATTGGCAGTATCACCATGGGTTGGCTGCTCAAGTGGTGTGTTAACCGGCCTAGACCTCCTGAGG
CATATCATATTGTTGAAAGTTACGGTGCATCGTTCCCAAGTGCACATAGTGTTTATGCATCAACACTGG
CTTGTCTGGCAATGATTATGTTATGCCATAAGCACAACATTAACTCTCCTTATATTGTTTTGATCTCCT
GTCTTTGGTTTGTGTGTATGGGGCTTTCAAGAATATATGCAGGAGTTCATTTCCCAACAGACGTACTCG
CTGGTTGGGGCATTGGTTTTATTTGGATTGCACTGCTTTGGCTCTGGTTATTACAAACACAAAGTAGGT
TAAGTAGAAAACAAATATATTTTTAGATTTTATCTAAACGAGGTGGAACAATGATGTCGGCTAAGCTTT
GGGCACCAGCCCTTACTGCTTGCGCATTAGCAACAAGTATCGCGCTTGTTGGTTGTAGCAAAGGCTCCG
ATGAGAAACAGCAAGCTGCTGCTGCTCAGAAAATGCCGCCTGCAGAAGTAGGTGTTATTGTTGCTCAAC
CACAAAGTGTTGAACAAAGCGTTGAGCTTTCAGGCCGTACTTCAGCATATCAAATTTCTGAAGTTCGTC
CTCAGACAAGTGGCGTGATTTTAAAACGCTTATTTGCTGAAGGAAGCTATGTTCGTGAAGGTCAGGCGC
TTTATGAGCTCGACTCTAGAACGAACCGTGCAACGTTAGAAAATGCAAAAGCATCACTCCTACAACAAC
AGGCAAATCTAGCTTCACTACGTACCAAGTTAAATCGTTATAAACAACTTGTTTCTAGTAATGCTGTGT
CTAAACAGGAATATGATGACTTACTTGGTCAAGTCAATGTTGCAGAAGCACAAGTTGCAGCAGCTAAGG
CTCAAGTAACAAATGCAAATGTAGATCTTGGTTATTCTACAATTCGCTCTCCTATTTCTGGCCAATCTG
GTCGTTCTTCAGTAACGGCTGGTGCTTTGGTTACTGCAAACCAGACTGACCCGTTGGTAACGATTCAAC
AGTTAGATCCTATCTATGTTGATATTAATCAGTCTAGTGCTGAGTTATTGCGTTTACGTCAACAACTAA
GCAAAGGCAGTTTAAATAACAGTAACAACACGAAAGTAAAATTAAAGCTTGAAGATGGTTCTACCTATC
CAATCGAAGGGCAACTTGCTTTCTCTGACGCTTCTGTAAACCAAGATACAGGAACAATTACATTACGTG
CCGTATTCTCTAACCCGAATCATTTATTGCTTCCGGGTATGTATACCACTGCGCAAATTGTTCAGGGCG
TTGTTCCAAATGCTTACCTGATTCCTCAAGCTGCCATTACTCGTTTACCTACAGGGCAAGCTGTAGCGA
TGCTTGTTAATGCTAAAGGGGTTGTTGAGAGCCGTCCTGTTGAAACCTCTGGTGTTCAAGGACAAAATT
GGATTGTGACTAACGGCTTAAAAGCCGGCGATAAAGTCATTGTTGATGGTGTTGCCAAAGTTAAAGAAG
GGCAAGAAGTATCAGCAAAACCTTATCAAGCTCAACCAGCAAACTCTCAAGGTGCAGCACCAAATGCTG
CGAAACCGGCTCAATCAGGTAAACCTCAAGCAGAACAGAAAGCAGCTTCAAATGCATAAGGGGTAGATT
GAATGGCACAATTTTTTATTCATCGCCCCATATTTGCGTGGGTGATTGCATTAGTCATTATGTTGGCGG
GTATTCTTACGCTAACAAAAATGCCTATTGCACAATATCCAACGATTGCACCACCAACCGTAACGATTG
CTGCGACTTATCCTGGTGCATCGGCTGAAACAGTTGAAAATACTGTAACCCAGATCATTGAACAACAAA

TGAATGGTCTTGATGGCTTACGTTATATTTCATCTAACAGTGCTGGTAATGGTCAGGCATCTATTCAAT
TAAACTTTGAACAAGGTGTTGACCCTGATATTGCACAGGTTCAAGTTCAAAACAAATTGCAATCTGCAA
CTGCGCTTTTACCTGAAGATGTACAACGTCAAGGTGTAACAGTTACTAAATCTGGTGCGAGCTTCTTGC
AAGTTATTGCATTCTATTCACCAGATAACAACCTGTCAGACTCTGACATTAAAGACTACGTAAACTCGT
CAATTAAAGAACCGCTTAGCCGTGTTGCCGGTGTTGGTGAGGTACAGGTCTTCGGTGGCTCATACGCAA
TGCGTATCTGGCTTGATCCAGCTAAATTAACAAGCTACCAACTTACTCCTAGTGATATTGCAACTGCCT
TACAAGCGCAGAACTCGCAAGTTGCTGTAGGTCAGTTAGGTGGTGCTCCGGCTGTACAAGGTCAAGTTC
TTAACGCAACAGTAAATGCACAAAGCTTATTGCAGACTCCTGAACAGTTTAAAAATATCTTCTTAAAGA
ACACAGCATCAGGTGCTGAGGTTCGATTAAAAGATGTTGCTCGCGTAGAATTAGGTTCGGATAACTATC
AATTTGACTCGAAGTTTAACGGTAAACCGGCAGCTGGTCTTGCAATTAAAATTGCAACAGGTGCTAACG
CACTCGACACAGCCGAAGCAGTTGAACAACGTTTATCTGAACTACGTAAGAACTATCCAACAGGTCTTG
CAGATAAACTGGCTTATGACACGACTCCATTTATCCGTCTTTCAATTGAAAGTGTAGTACACACATTAA
TTGAAGCCGTGATTTTGGTATTCATTGTCATGTTCCTATTCTTACAAAACTGGCGTGCAACGATTATTC
CAACGCTTGCAGTTCCAGTAGTTGTATTAGGTACATTTGCGGTCATTAATATCTTTGGCTTCTCAATTA
ACACCTTAACCATGTTCGCTATGGTATTGGCAATCGGTCTTCTGGTCGACGACGCCATTGTTGTAGTCG
AAAACGTTGAACGTGTGATGAGTGAAGACCATACCGATCCGGTTACCGCCACTTCTCGCTCAATGCAGC
AGATTTCTGGTGCGTTAGTAGGTATTACCAGCGTATTGACAGCGGTATTCGTACCAATGGCTTTCTTTG
GTGGTACAACAGGTGTAATTTACCGCCAGTTCTCGATTACCCTTGTAACTGCAATGGTTCTGTCGTTAA
TTGTAGCGTTGACGTTCACACCGGCACTTTGTGCAACTATCTTGAAACAGCATGATCCTAATAAAGAAC
CAAGCAATAATATCTTTGCGCGTTTCTTTAGAAGCTTTAACAATGGTTTTGACCGCATGTCGCATAGCT
ACCAAAATGGTGTTAGCCGCATGCTTAAAGGCAAAATCTTCTCTGGCGTGCTCTATGCTGTTGTAGTTG
CCCTTTTAGTCTTCTTGTTCCAAAAACTCCCGTCTTCATTCTTACCAGAAGAAGATCAGGGTGTGGTCA
TGACACTTGTACAATTACCACCAAATGCAACGCTTGACCGTACCGGTAAAGTGATTGACACCATGACTA
ACTTCTTTATGAATGAAAAAGACACCGTGGAATCTATTTTCACTGTTTCTGGTTTCTCATTCACAGGTG
TTGGTCAAAACGCTGGTATTGGCTTCGTTAAGTTGAAAGACTGGAGCAAACGTACGACACCAGAAACTC
AAATTGGTTCATTGATTCAGCGTGGTATGGCATTAAATATGATCATTAAAGATGCATCATATGTTATGC
CGTTACAGCTTCCAGCAATGCCTGAACTTGGTGTAACTGCCGGATTTAACTTGCAGCTTAAAGATTCAA
GTGGTCAAGGCCATGAGAAACTGATCGCAGCTCGTAACACGATTTTAGGTTTGGCATCACAAGATAAAC
GTCTTGTAGGTGTGCGTCCAAATGGTCAGGAAGATACTCCTCAATATCAAATTAATGTAGATCAGGCTC
AAGCTGGTGCTATGGGCGTTAGTATTGCCGAAATCAACAATACAATGCGTATTGCATGGGGTGGCTCAT
ACATTAACGATTTCGTTGACCGTGGTCGTGTGAAAAAAGTTTATGTTCAAGGTGATGCGGGCAGCCGTA
TGATGCCTGAAGACTTAAACAAATGGTATGTACGTAATAACAAAGGTGAGATGGTTCCATTCTCGGCGT
TTGCTACAGGCGAATGGACGTATGGTTCTCCACGTCTCGAACGTTATAACGGTGTGTCATCAGTTAACA
TTCAAGGTACACCTGCACCTGGCGTGAGCTCTGGTGATGCCATGAAAGCAATGGAAAAAATTATTGGTA
AGTTGCCTTCTATGGGCTTACAAGGTTTCGACTATGAATGGACAGGCTTATCACTTGAAGAACGTGAGT
CTGGTGCTCAAGCGCCGTTCTTATACGCACTTTCATTGTTAATCGTATTCCTTTGCTTGGCTGCACTAT
ATGAAAGCTGGTCAATTCCGTTCTCGGTTTTACTTGTGGTACCACTTGGTGTCATTGGTGCAATCGTAT
TGACCTACTTGGGCATGATTATTAAAGGAGATCCAAATCTCTCAAATAACATTTACTTCCAAGTAGCGA
TTATTGCGGTTATCGGTCTTTCTGCAAAAAATGCGATCTTGATTGTTGAATTCGCAAAAGAATTGCAGG
AAAAAGGTGAAGATCTACTTGATGCAACCTTACATGCTGCAAAAATGCGTTTACGTCCAATTATCATGA
CCACCCTTGCCTTCGGTTTCGGTGTACTTCCACTTGCACTTTCAACAGGTGCCGGTGCAGGAAGTCAGC
ACTCTGTAGGCTTTGGTGTACTTGGTGGCGTACTCAGCGCGACGTTCTTAGGTATCTTCTTTATCCCTG
TATTCTATGTGTGGATTCGTAGTATCTTTAAGTACAAACCAAAAACCATAAACACTCAGGAGCATAAAT
CGTGATGCAAAAAGTATGGTCTATTTCAGGTCGTAGCATTGCGGTATCTGCACTTGCGCTTGCTTTGGC
AGCTTGTCAAAGCATGCGCGGCCCAGAACCAGTCGTGAAAACCGATATACCACAAAGCTATGCATATAA
CAGCGCTTCTGGTACGTCTATTGCTGAACAGGGTTATAAACAGTTCTTTGCTGACCCGCGTTTGCTTGA
AGTGATTGATTTGGCTCTTGCCAATAACCGTGACTTACGTACAGCAACGCTCAATATTGAACGTGCTCA
ACAGCAATATCAGATTACACAGAACAACCAGCTTCCAACAATCGGAGCAAGTGGTAGTGCAATTCGTCA
GGTTTCTCAAAGCCGTGATCCGAATAATCCCTACTCTACTTATCAAGTAGGTTTGGGTGTAACTGCTTA
TGAGCTAGATTTCTGGGGTCGTGTTCGTAGCCTCAAAGATGCTGCTTTAGATAGTTATCTTGCAACACA
AAGTGCTCGTGATTCGACTCAAATCAGTCTGATTAGCCAAGTTGCTCAAGCATGGTTAAATTATTCGTT
TGCAACAGCAAACTTAAGACTGGCAGAGCAAACGCTTAAAGCACAGTTAGATTCTTACAATCTCAACAA
AAAACGTTTTGATGTAGGTATTGACAGTGAAGTTCCATTACGTCAAGCACAGATTTCTGTAGAAACTGC
GCGTAATGATGTAGCGAACTACAAAACTCAAATTGCTCAAGCACAAAACTTGTTGAACTTGCTTGTAGG
CCAACCTGTTCCACAAAACTTGTTACCTACACAACCTGTAAAACGCATTGCTCAACAAAATGTGTTTAC
TGCCGGTTTACCAAGTGACTTGTTAAATAACCGTCCGGATGTAAAAGCTGCTGAATACAACTTAAGCGC
TGCGGGTGCGAATATCGGTGCTGCAAAAGCACGTTTATTCCCAACCATTAGCTTAACGGGTTCGGCTGG
TTATGCATCAACTGACTTAAGTGATCTATTTAAGTCTGGTGGTTTTGTATGGTCAGTTGGTCCAAGCTT
AGATTTACCAATCTTTGACTGGGGTACACGCCGTGCCAATGTAAAAATTTCTGAAACTGATCAGAAAAT
TGCATTGTCTGATTATGAAAAATCAGTTCAGTCGGCGTTCCGTGAAGTTAATGACGCGCTTGCAACTCG
TGCCAACATTGGTGAGCGTTTAACAGCACAACAACGTCTAGTAGAAGCGACTAACCGCAACTACACACT

TTCAAATGCCCGCTTCCGTGCTGGTATTGATAGTTACTTGACTGTTCTTGATGCGCAGCGTTCTTCATA
TGCAGCTGAACAAGGTTTGTTATTGCTTCAACAAGCAAACTTAAACAACCAAATCGAGTTATACAAAAC
TCTAGGTGGCGGTTTAAAAGCAAATACTTCAGATACAGTGGTACATCAAC

>Novel00822
AATAAGCCTAACCGATCCAATAATATATAAATTATGACTTTAAAAGGGAGTACATCATGAGTTTAGTTG
TACCTGCTGAACATCCTGAAACCGTACATAACGAAACGGATCGTGTCGAGCGCATTTTAGTGGTCGATG
ACGATGTGCGTTTACGTACCCTCTTGCAACGTTTTTTGGAAGATAAAGGCTTTGTTGTAAAAACTGCCC
ATGATGCCTCACAAATGGATCGTTTATTACAACGTGAGTTATTTTCACTTATCGTGCTCGACTTTATGT
TGCCCGTTGAAGATGGTTTAAGTATTTGTCGTCGTTTACGTCAGTCAAATATTGATACTCCAATTATCA
TGTTAACTGCACGTGGTAGTGACTCTGACCGTATTGCTGGTCTTGAAGCCGGCGCAGATGACTATTTAC
CAAAACCATTTAACCCAAATGAACTTTTAGCACGTATTCGCGCAGTATTGCGTCGTCAGGTACGTGAAG
TCCCGGGTGCGCCAAGCCAACAAGTTGAAGTGGTTAGCTTTGGTCCATGGTCTCTAGATTTATCTACAC
GTACACTGACTCGTGAGGGTCAAATCGTTACGTTGACTACAGGTGAATTTGCCGTATTAAAAGCTTTGG
TACAGCATCCACGTGAACCTTTAACACGCGACAAGCTCATGAACTTAGCTCGTGGCCGTGAATGGGGTG
CAATGGAACGTTCTATTGATGTTCAAGTGTCTCGTCTACGCCGCTTAATTGAAGATAACCCGGCACGCG
CACGTTACATTCAAACTGTATGGGGCGTAGGCTATGTGTTTGTTCCGGATGGTGCTGAATAAGCTTGAG
ATAAAATAGAGTGAGTTTAGAGCCAATACCACCGCAAACCTTTACCGACTACGTTGCGTACTCAGAGCG
TAAACGGACCCGATGGGAACGTTTTCTCGACAAGATTAAGC

Appendix 2: The sequence of differentially expressed small RNA between biofilm and planktonic cells of *A. baumannii* ST1894. The raw sequences were deposited in the NCBI Sequence Read Archive (SRA) under submission ID (SUB9038432) and biosample accession number (SAMN17808362).

>sRNA00203
CCGATATCAAGGAAGAGATCATAAGGAACTTGAGTAAGAACAGTTTCAAGAGG

>sRNA00207
CTGAACTTGTTGGTATTGAATAATGATTAAAAGGGCAGCAATTTGGAGTACGTAATTTGTACTCCATGC
AAAAAATTTGGCAGGCTTAAATTGTCAAAACTTCTGTCTCATAATCTTGTTCGAAAGTGTCATGAATGT
GCCAATAAGTTTTAAATATGTTCTGGCACCAAGAAGCTAAAGAATAGCTGGGGCATTGAATAACAGTTA
TTACTAAGGATTACGAATCGGAGATAATGGCATTATGGAAAAGATTTGGTTTGCAGAATACCAAAAGAC
AGGGA

>sRNA00226
TGTTACCTAGCTACGTTTTAGGAATAATAAAAGCAAAACAGTCCAACTTAGGTTTTTTTAATCTCAAAT
CTTTTTTGTGAGAGTGATGCCATGTTATTCAAAAAATTCGCTGTTGCTGCTGCTGTTGCTGCTACTTTA
GCTTTCGTTGGTTGTTCTAAGAAAGAAGAAGCTCCTGCTGCTGCTGCTGCATCTGAAGCTGTAGCTGCT
GCTTCTGAAGCTACTGCTGCTGCTTCTGAAGCTGCTGTAGCTGTTGACGCTGCTGCTTCTGAAACTGCT
GCTGCTGCTTCTGAAGCTACTACTGCTGCTTCTGAAGCTACTGCTGCTGCTTCTGCTGCACACTAATCT
ATACTCTAGATTAGCGAGAAAAGAG

>sRNA00272
TAGGATTAAAAATAAAATAACTTTACGACTGTTTTGCATTTCTATACATTTGTATTTTTAATCAAAAAA
TAAACTTATGAGTAAATCGGTGTTAGAAACCTAC

>sRNA00286
CTGTATTTACTCGGTATGCATGACAAAGATTTAAGCCGTGCTTAAATAATAGTGAACAATCATTAAG