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GENOMIC EPIDEMIOLOGY AND RESISTOME OF *ACINETOBACTER CALCOACETICUS-BAUMANNII* COMPLEX IN THE HOST AND NON-HOSPITAL ENVIRONMENT

WEE SOON KEONG

LEE KONG CHIAN SCHOOL OF MEDICINE

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WEE SOON KEONG

Lee Kong Chian School of Medicine

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in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2022

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Date

Assoc Prof Yap Peng Huat Eric

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal in which I am listed as an author.

Chapter 2 is published as Wee, S. K., & Yap, E. P. H. (2021). GALAXY workflow for bacterial next-generation sequencing de novo assembly and annotation. *Current Protocols*, 1, e242. doi: 10.1002/cpz1.242

The contributions of the co-authors are as follows:

- I co-designed the study with A/Prof Eric Yap and performed the development of GALAXY workflow protocol.
- I prepared the manuscript draft. The manuscript was revised by A/Prof Eric Yap.

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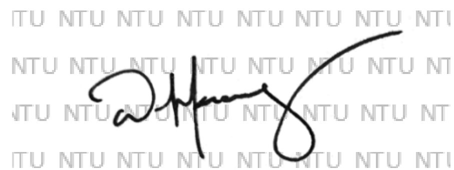

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List of Abbreviations

Acb complex	*Acinetobacter calcoaceticus-baumannii* complex
AMR	Antimicrobial resistance
ANI	Average nucleotide identity
ARG	Antimicrobial resistance genes
AST	Antibiotics susceptibility testing
CAI	Community-acquired infections
CDS	Coding sequences
CRAB	Carbapenem resistant *Acinetobacter baumannii*
dDDH	Digital DNA-DNA hybridisation
DNA	Deoxyribonucleic acid
GC	Guanine-cytosine
IC	International clone
IS	Insertion sequence
MicroBIGG-E	Microbial Browser for Identification of Genetic and Genomic Elements
MRG	Metal resistance genes
MLST	Multi locus sequence typing
MGE	Mobile genetic elements
NCBI	National Center for Biotechnology Information
OXA	Oxacillinase
PCR	Polymerase Chain Reaction
PD	Pathogen Detection
SNP	Single nucleotide polymorphism
ST	Sequence type
TA	Toxin-Antitoxin
WGS	Whole genome sequencing
WHO	World Health Organisation

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Journal Papers

- **Wee, S.K.**, & Yap, E.P.H. (2021). GALAXY workflow for bacterial next-generation sequencing de novo assembly and annotation. Current Protocols, 1, e242. doi: 10.1002/cpz1.242

Conference Presentations

- **Wee, S.K.** Resistome of clinical and environmental *Acinetobacter baumannii* in Singapore and beyond. November 10, 2021. LKCMedicine Scientific Seminar: Singapore.
- **Wee, S.K.** GALAXY Workflow for Bacterial Next Generation Sequencing De Novo Assembly and Annotation. October 21, 2021. Acing Research Topics – Interschool Seminar Series: Singapore.
- **Wee, S.K.** Antimicrobial resistance in the environment: Early lessons on global health challenges in ASEAN. November 26, 2020. ASEAN Online Conference for Young Scientists.
- **Wee, S.K.** Genomic epidemiology of non-hospital environmental *Acinetobacter baumannii*. August 19, 2020. LKCMedicine Scientific Seminar: Singapore.
- **Wee, S.K**. Molecular epidemiology of *Acinetobacter baumannii* from the non-hospital environment. May 7, 2020. AcinetoVibes 2020: International Virtual Conference.
- **Wee, S.K.**, Sivalingam, S.P., Mustaffa, S.B., Loh, C.P. and Yap, E.P.H. Resistome and genomic epidemiology of *Acinetobacter* spp. and *A. baumannii* isolated from the natural non-hospital environment. September 6, 2019. The 12th International Symposium on the Biology of Acinetobacter: Frankfurt, Germany.
- **Wee, S.K.** Whole genome sequencing of a novel environmental *Acinetobacter baumannii* that is innately carbapenem-resistant. January 23, 2019. LKCMedicine Research Retreat: Singapore.

Poster Presentations

- **Wee, S.K.**, Yap, E.P.H. The Urban Environment as a Hidden Reservoir for *Acinetobacter baumannii* with Clinically Relevant Sequence Types and Resistome. June 20-24, 2021. World Microbe Forum 2021: Virtual Conference.
- **Wee, S.K.**, Yap, E.P.H. The Urban Environment as a Hidden Reservoir for *Acinetobacter baumannii* with Clinically Relevant Sequence Types and Resistome. May 5-6, 2021. AcinetoVibes 2021: Virtual Conference.
- **Wee, S.K.**, Sivalingam, S.P., Yap, E.P.H. Environmental ecology of *Acinetobacter baumannii* and *Acinetobacter* spp. isolated from the natural non-hospital environment. August 12-14, 2020. Microbial Ecology and Evolution 2020: MEEvirtual.
- **Wee, S.K.**, Sivalingam, S.P., Mustaffa, S.B., Loh, C.P. & Yap, E.P.H. Whole Genome Sequencing of a Novel Environmental *Acinetobacter baumannii* that is Innately Carbapenem-Resistant. June 20-24, 2019. ASM Microbe 2019: San Francisco, USA.
- **Wee, S.K.**, & Yap, E.P.H. Isolation of carbapenem resistant *Acinetobacter baumannii* from the environment. November 13-14, 2018. Antimicrobial Resistance in the Asia-Pacific and its impact on Singapore Symposium: Singapore.

Summary

Acinetobacter baumannii is a nosocomial pathogen that has arisen relatively recently over the past two decades and is increasingly multi-drug resistant. It can also cause community-acquired infections (CAI). However, little is known about the evolution and source of these clinical clones and their resistance genes. We hypothesised that the local natural environment contains a diverse reservoir of *A. baumannii* that share genomes, resistomes and plasmidomes with pathogenic strains that cause clinical disease through CAI. This thesis aims to characterize *A. baumannii* and their resistome in the natural non-hospital environment and determine their genetic relatedness with clinical strains. The study also aims to investigate the potential mechanisms behind the transmission and evolution of antimicrobial resistance genes (ARG) in the environment.

Briefly, field sampling of soil, water and urban environment was conducted to isolate and characterize environmental *A. baumannii* isolates. Whole genome sequencing of both environmental and clinical *Acinetobacter calcoaceticus-baumannii* (Acb) complex isolates was performed to determine their sequence types, ARG and phylogenetic relationship. Publicly available genomes (n = 15,262) were analysed to better understand the genomic epidemiology, plasmidome and resistome of *A. baumannii*.

We have characterized local clinical (n = 117) and environmental (n = 97) *A. baumannii* isolates and found evidence of shared sequence types, such as CAI sequence type (Pasteur ST10), and acquired shared ARG in the host and non-hospital environment. International Clones 1 and 2 were the main circulating clades found in the local clinical setting with *bla*$_{\text{OXA-23}}$ as the main driver for carbapenem resistance. Carbapenem resistance in an environmental *A. baumannii* isolate was mediated by a plasmid encoding the beta-lactamase oxacillinase gene, *bla*$_{\text{OXA-72}}$, which was also present in other clinical *A. baumannii* isolates. *bla*$_{\text{OXA-58}}$ was found in a megaplasmid of a local clinical *A. baumannii* isolate with other *bla*$_{\text{OXA-58-like}}$ variants of found sharing similar IS elements flanks in other plasmids. Plasmids and mobile genetic elements were likely involved in the inter-species dissemination and evolution of ARG in Acb complex across different countries. The analysis of *A. seifertii* pangenome further supported the role of plasmids in transmitting ARG as well as expanding its accessory

gene pool. The isolation of Acb complex, including ST10 *A. baumannii*, from the non-hospital environments suggests that such sources could be an overlooked reservoir for CAI infections.

These findings contribute to the understanding of the ecology and evolution of *A. baumannii* in the environment, the resistome of environmental and clinical genomes, and the possible role of the plasmidome of *Acinetobacter* species in inter-species and inter-niche transmission. Our observations highlight the importance of adopting a One Health approach to understand the genomic epidemiology and transmission of *A. baumannii* and its resistome.

Chapter 1. Introduction

1.1. Literature Review

1.1.1. *Acinetobacter baumannii* as a pathogen

Acinetobacter was first isolated from soil and described in 1911 by a Dutch microbiologist named Martinus Willem Beijerinck (Beijerinck, 1911). Initially, the organism was named *Micrococcus calcoaceticus* before undergoing several taxonomic re-assignments (Peleg et al., 2008). In 1954, the genus *Acinetobacter* (termed from Greek "akinetos"; non-motile), was first proposed to differentiate non-motile microorganisms in *Achromobacter* genus (Brisou and Prevot, 1954). Later in 1968, a more comprehensive study was published to conclude that strains from different taxa should be characterized as *Acinetobacter* genus (Baumann et al., 1968). Three years later, the proposed genus *Acinetobacter* was officially acknowledged and recognised (Lessel, 1971). Today, there are 73 validly described names for the *Acinetobacter* genus published under the List of Prokaryotic Names with Standing in Nomenclature (LBSN) accessed on 5 January 2022 (Parte, 2018). While most of the described species are non-pathogenic, *Acinetobacter baumannii* is the most clinically relevant species that is clustered phylogenetically with its close neighbours.

Acinetobacter baumannii, a gram-negative opportunistic pathogen, is part of the *Acinetobacter calcoaceticus-baumannii* (Acb) complex. Other members of the complex include, *A. calcoaceticus*, *A. pittii*, *A. nosocomialis, A. lactucae* and *A. seifertii* (Cosgaya et al., 2016; Nemec et al., 2015). *A. dijkshoorniae* has been shown to be a later synonym of *A. lactucae (Dunlap and Rooney, 2018). A. baumannii* has been found to asymptomatically colonise healthy individuals on their skin flora (Berlau et al., 1999). However, for immunocompromised patients, trauma and burn patients, the bacteria can infect the patients through exposed skin, respiratory tracts and soft tissues, causing severe manifestations ranging from wound infections, urinary tract infections, pneumonia, endocarditis to septicaemia (Peleg et al., 2008). It can enter the body through open wounds, mechanical ventilators and intravascular catheters (Antunes et al., 2014). These nosocomial infections, such as ventilator-associated pneumonia and bloodstream infections, can be difficult to treat. They cause a

healthcare burden, prolonging the length of hospital stays and increasing healthcare costs (Peleg et al., 2008). Furthermore, the emergence of multi-drug resistance in *A. baumannii* significantly increases the difficulty and complexity of treatment and infection control (Hawkey et al., 2017; Lee et al., 2017; Schultz et al., 2016).

Though *Acinetobacter* derived its name from being non-motile, it has been shown that some *A. baumannii* and *A. nosocomialis* isolates have pili-dependent motility, more specifically, the surface-associated motility and twitching motility (Harding et al., 2018). Motility can be triggered under iron-limiting condition and in response to light (Eijkelkamp et al., 2011; Mussi et al., 2010). The motility and ability to form biofilm, together with the ability to survive desiccation, are crucial factors for *A. baumannii* to persist as a hospital-acquired pathogen. These factors make it difficult to fully eradicate the microorganism as they give them an advantage to persist and form biofilms on medical equipment and catheter devices. Other virulence factors involved in the pathogenesis of *A. baumannii* include capsular polysaccharide, surface adhesions, glycoconjugates and secretion systems (Harding et al., 2018).

Despite being a hospital-acquired pathogen, there have been incidences of community-acquired infections (CAI) by *A. baumannii* (Dexter et al., 2015; Falagas et al., 2007). Majority of community-acquired *A. baumannii* infections occur in countries with tropical or subtropical climates (Dexter et al., 2015). Locally, there are limited reports on community-acquired *A. baumannii* infections with a report observing that CAI pneumonia due to *A. baumannii* can have high mortality with 5 out of 8 deaths between 2007 and 2008 period (Ong et al., 2009). Community-acquired *A. baumannii* isolates have also been described to be generally sensitive to antibiotics. The risk factors of CAI associated include chronic lung disease, heavy drinking, heavy smoking and poor host immune system (Dexter et al., 2015; Eugenin, 2013). Increased onset of community-acquired *A. baumannii* infections was associated with warmer weather seasonality and temperature fluctuation based on a single-centre study in Korea (Kim et al., 2018). However, no correlation between the infections and weather seasonality was observed for hospital-acquired cases. The high incidence of early colonisation in local burn patients and seasonal differences observed in regional community-acquired pneumonia infections caused by *A. baumannii* suggest the possibility of extra-hospital

reservoirs. Therefore, there is a need to understand the non-hospital environmental reservoirs of *A. baumannii*.

1.1.2. Antibiotic Resistome

The concept of antibiotic resistome was first introduced about fifteen years ago (Wright, 2007). The resistome comprises all antimicrobial resistance genes (ARGs), including precursor genes and cryptic resistance genes that are present in bacterial chromosomes but not expressed. These ARGs can be found intrinsically in the bacterial chromosomes as part of their core genomes or acquired from an external source through mechanisms such as horizontal gene transfer. The acquired resistance genes would contribute to the accessory genomes of the bacteria. As a result, there is a natural variation in resistance levels towards antibiotics in different bacteria, with some being intrinsically resistant to a range of antibiotics (Wright, 2010).

The discovery of antimicrobial resistance genes in pristine environments, such as in a bacterium isolated from a deep cave or in a deep-frozen arctic core, supports the hypothesis that antimicrobial resistance genes are naturally occurring in the natural environment (McCann et al., 2019; Pawlowski et al., 2016). These genes can be part of a defence mechanism against antibiotics producers in the ecosystem or part of a metabolism pathway that uses antibiotic molecules as a nutrient source (Davies and Davies, 2010). Antibiotic producers could also disseminate ARG to bacterial pathogens through a series of steps involving conjugative transfer, homologous recombination, and natural transformation (Jiang et al., 2017). Conjugation is a process where DNA transfer occurs between a donor and recipient cell through direct cell-to-cell contact, such as the use of a conjugative pilus to transfer plasmids between cells (Arnold et al., 2021). Homologous recombination is the exchange of DNA sequences between similar DNA molecules that share identical regions. DNA from the environment can subsequently be taken up by the cells through natural transformation. Together, these recombination processes allow the dissemination of ARG between bacterial cells.

We hypothesise that antimicrobial resistance genes originate from the natural environment and are gradually selected for in the clinical environment. The selection pressure could have occurred through many steps, such as antibiotics use in

agriculture and aquaculture environment, colonisation in animals and humans, before eventually being selected for in the clinical setting through prolonged antibiotics usage (Larsson and Flach, 2021). In the absence of antimicrobial use, these genes can also be co-selected or hitchhiked onto other mechanisms, such as toxin-antitoxin system in the plasmid or by metal resistance genes, to persist in bacterial genomes.

To understand the evolution and origins of antimicrobial resistance (AMR), it is important to adopt the One Health approach and characterise the resistome of microorganisms in the natural environment (Walsh, 2018). The One Health is a multidisciplinary and multisectoral approach to understand that human health is closely related to the interactions with environmental and animal health. This extends to the understanding that infectious diseases, such as bacteria, and their antimicrobial resistance genes, could be transmitted between various environment and eventually leading to nosocomial dissemination (Figure 1). The environment can be a potential reservoir and source for antimicrobial resistance genes (McEwen and Collignon, 2018).

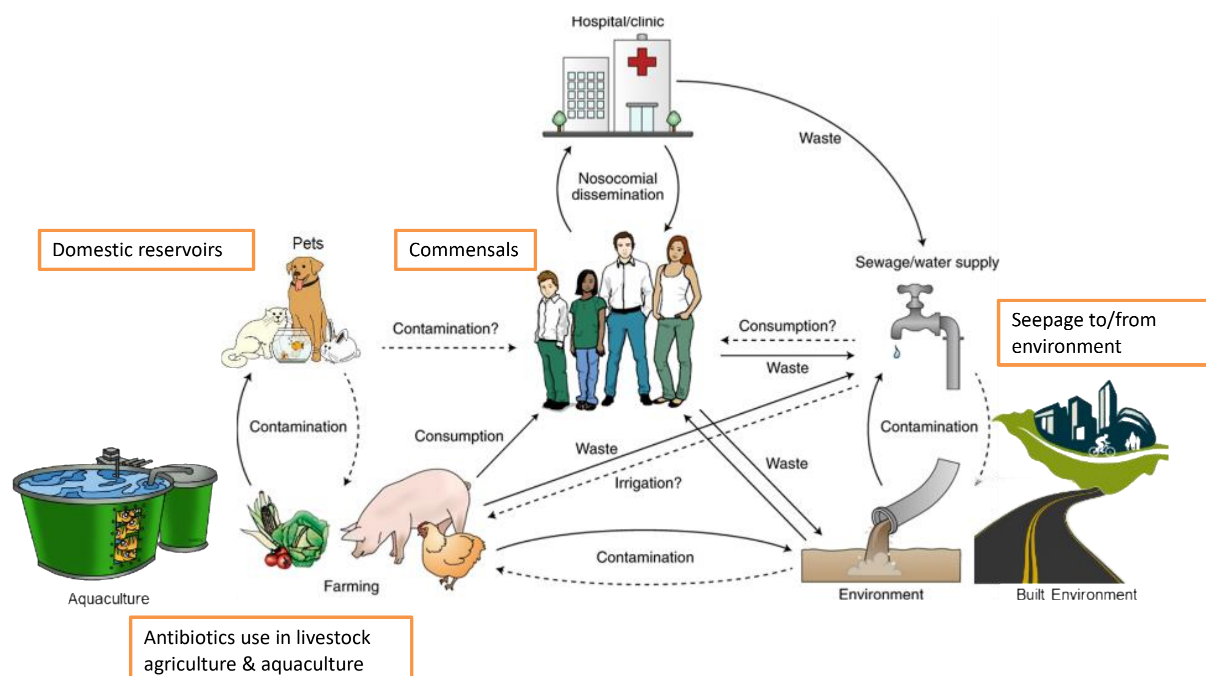

Figure 1. One Health approach to understand the interaction and transmission of antimicrobial resistance in humans, animals, and environmental reservoir. Adapted by permission from Springer Nature: Nature Microbiology, A one-health approach to antimicrobial resistance (Walsh, 2018).

1.1.3. Intrinsic resistance in *A. baumannii*

A. baumannii has been reported to have high level of intrinsic resistance against ampicillin, aminopenicillin, first and second-generation cephalosporins and chloramphenicol (Bergogne-Bérézin and Towner, 1996; Dijkshoorn et al., 2007; Seifert et al., 1993). Phenotypically, Acb complex members are intrinsically resistant to ampicillin, amoxicillin, amoxicillin-clavulanate, aztreonam, ertapenem, trimethoprim, chloramphenicol and fosfomycin (CLSI, 2018).

The high level of intrinsic resistance in *A. baumannii* can be attributed to several known possible intrinsic resistance genes, such as the beta-lactamase *bla*$_{ADC}$, and *bla*$_{OXA-51}$, that are part of the core genomes of *A. baumannii*. There are other antibiotic resistance determinants such as efflux pumps and outer membrane porins that could play a role in conferring antimicrobial resistance. Mutations in *adeRS* two-component regulatory system of *adeABC* efflux pump could lead to an overexpression of the pump resulting in decreased susceptibility to various antibiotics (Coyne et al., 2011; Marchand et al., 2004). Outer membrane porins, *Omp* and *CarO,* play a role in virulence and antibiotics resistance (Uppalapati et al., 2020).The non-specific slow porin, *OmpA* has been described to limit the outer membrane permeability, decreasing the uptake of antibiotics into the bacterial cell (Iyer et al., 2018). In terms of intrinsic beta-lactamases, it has been demonstrated previously that an *Acinetobacter*-derived cephalosporinase, *bla*$_{ADC}$, confers resistance to cephalosporins. Another variant of the extended-spectrum *AmpC* beta-lactamase, *bla*$_{ADC-56}$ has been shown to confer resistance to cefepime (Tian et al., 2011). Insertion sequence (IS) elements could increase the levels of expression of these intrinsic resistance genes (Héritier et al., 2006). The ISs are short transposable elements that encodes transposase (*tnp*) gene and are flanked by inverted terminal repeats (Siguier et al., 2015). The overexpression of *bla*$_{ADC-30}$ with an upstream IS*Aba1* element has been shown to confer resistance to sulbactam (Kuo et al., 2015). Similarly, the presence of IS*Aba1* upstream of the promoter region of intrinsic chromosomally encoded beta-lactamase *bla*$_{OXA-51-like}$ could increase the expression of the beta-lactamase gene (Turton et al., 2006). The overexpression of certain OXA-51-like variants, such as OXA-82, could lead to carbapenem resistance (Zander et al., 2012a). That said, though the presence of IS*Aba1* upstream of *bla*$_{OXA-}$

$_{51\text{-like}}$ would increase expression, it does not necessarily result in carbapenem resistance as it would depend on the OXA variant (Nigro and Hall, 2018). The role of IS*Aba1* has also been associated with evolution of beta-lactamases, particularly to increase its expression level and in transposition (Mugnier et al., 2009; Pournaras et al., 2014). Therefore it is important to look for the presence of IS elements upstream of ARGs when interpreting results to determine the genetic factors of antimicrobial resistance.

Recently, aminoglycoside nucleotidyltransferases, *ant(3")-IIa*, has been proposed by Zhang et al. (2017) to be a possible intrinsic gene. It was found in 73% of *A. baumannii* genomes in the same chromosomal locus (Zhang et al., 2017). The study demonstrated that *A. baumannii* could become more sensitive to streptomycin when *ant(3”)-IIa* gene was deleted. This finding presented the possibility of having therapeutic agents to target the intrinsic resistance mechanism to allow existing antibiotics to be used for treatment. It also highlighted the importance of understanding the intrinsic resistance genes present in the bacteria.

1.1.4. Emergence of carbapenem resistant *A. baumannii*

The global emergence of antimicrobial resistance (AMR) has been predicted to be the world's leading cause of death by 2050 (Tagliabue and Rappuoli, 2018). Carbapenem-resistant *Acinetobacter baumannii* (CRAB) has been listed by the World Health Organisation (WHO) to be one of the critical priority pathogens for new drug development (Tacconelli et al., 2018). In hospitals, *A. baumannii* typically cause nosocomial infections such as ventilator associated pneumonia and bloodstream infections. For the management of patients infected with CRAB, physicians are required to prescribe last-line drugs.

Locally, CRAB is a major concern too. Some 62% of *A. baumannii* isolates in Singapore's public hospitals were carbapenem resistant in 2010, and neighbouring countries such as Malaysia and Philippines reported rates up to 55% (Hsu et al., 2017). A recent point prevalence study conducted in Singapore revealed that 71.9% of *Acinetobacter* spp. collected between July 2015 to February 2016 from private and public acute hospitals were carbapenem non-susceptible (Cai et al., 2017).

Carbapenem resistance in *A. baumannii* is mediated mainly by OXA-type Class D beta-lactamases (Evans and Amyes, 2014). Broadly, there are four big groups, namely the OXA-51-like, OXA-23-like, OXA-40-like (formerly named as OXA-24-like) and OXA-58-like, circulating in *Acinetobacter* population (Peleg et al., 2008). Two other subclasses OXA-143-like and OXA-235-like have also been found in *A. baumannii* (Higgins et al., 2013; Higgins et al., 2009). These OXA-type beta lactamases have been described to be the main ARG conferring carbapenem resistance in Singapore (Hsu et al., 2017; Koh et al., 2012). *bla*$_{\text{OXA-23}}$-like gene was found to be the main driver of carbapenem resistance together with *bla*$_{\text{OXA-58}}$-like, *bla*$_{\text{OXA-40}}$-like and *bla*$_{\text{OXA-143}}$-like in Singapore (Koh et al., 2012).

The presence of mobile genetic elements (MGE) can lead to an increase in the expression of intrinsic *bla*$_{\text{OXA-51-like}}$ gene to confer phenotypic resistance (Nigro and Hall, 2016b). These IS elements have also been found in different transposons to mobilise the *bla*$_{\text{OXA}}$ genes. One such example is *bla*$_{\text{OXA-23}}$ that has been described to be commonly found with the presence of IS*Aba1* upstream, as well as in transposons (Nigro and Hall, 2016b). Instead of being associated often with IS elements, the *bla*$_{\text{OXA-}}$

$_{40\text{-like}}$ genes were often flanked by XerC/XerD recombinase sites which provided homologous target sites for recombination to take place (Merino et al., 2010). These OXA beta-lactamases may be chromosomally encoded or plasmid borne with different families being associated with plasmids of different replicase (*rep*) gene group (Evans and Amyes, 2014). In another carbapenemase family, *bla*$_{\text{OXA-58-like}}$ group contained three variants (Figure 2) with one of the *bla*$_{\text{OXA-58-like}}$ variant, *bla*$_{\text{OXA-96}}$, found in *A. baumannii* DU16891/96 isolate from Singapore General Hospital in Singapore (Koh et al., 2007). Insertion sequence (IS) elements were found to be responsible for the increased expression and mobilisation of these beta-lactamases (Evans and Amyes, 2014).

Figure 2. Maximum likelihood amino acid tree of the OXA-type β-lactamases found in *Acinetobacter* species (Evans and Amyes, 2014). Republished with permission of American Society for Microbiology, from OXA β-lactamases, Evans B.A. and Amyes S.G.B., 2(27), 2014; permission conveyed through Copyright Clearance Center, Inc.

1.1.5. Acquired resistance in *A. baumannii*

Over the years, there have been increasing reports of resistance against other classes of antibiotics used for treatment, such as against third-generation cephalosporins, carbapenems, fluoroquinolones, and colistin (Dijkshoorn et al., 2007). There are other resistance mechanisms contributing to multi-drug resistance phenotype in *A. baumannii*. Strategies may include increased expression of enzymes, such as beta-lactamases to cleave antibiotics, increased expression of efflux pumps, acquisition of enzymes that modify drug target sites and decreased outer membrane protein permeability (Peleg et al., 2008). These resistance determinants usually exist as genomic resistance islands (GRIs) and can be acquired from plasmids too. Being naturally competent, *A. baumannii* also has high levels of mobile genetic elements, such as transposons and insertion elements, that may contribute towards its genomic diversity (Touchon et al., 2014).

The emergence of multi-drug resistance (MDR) and extensively-drug (XDR) resistance worldwide is worrying. GRIs play an important role in conferring MDR status and genome plasticity to *A. baumannii*. Large GRIs have been documented to have evolved and disseminated in International Clone 1 and 2 (Blackwell et al., 2015; Hamidian and Hall, 2018). Notably some of the earliest extensively drug resistant (XDR) isolates with a particular genomic resistance island, GRI3, were first isolated in Singapore in an International Clone 2 (Pasteur ST2) clinical isolate in 2004 (Blackwell et al., 2017). The general structure of the GRIs includes having several different antimicrobial resistance genes flanked by transposons and IS elements which are responsible for their mobilisation and further rearrangement (Blackwell et al., 2015).

Dissemination of antimicrobial resistance genes

Horizontal gene transfer plays an important role in the dissemination of ARG in *Acinetobacter* species. This can be mediated through transformation, and conjugation and transduction (Arnold et al., 2022; Lerminiaux and Cameron, 2019; von Wintersdorff et al., 2016). *A. baumannii* has been described to be naturally competent, allowing the uptake of foreign DNA through transformation (Domingues et al., 2019). ARG could be horizontally transferred via plasmids using various mechanisms. Plasmid conjugation through type VI secretion system (T6SS) has also been shown

to propagate drug resistance (Di Venanzio et al., 2019). *A. baumannii* plasmids were described to have stable structure and have limited number of plasmid lineages (Salgado-Camargo et al., 2020). These plasmids seemed to bear replication systems that have varying host ranges from being only able to replicate in *Acinetobacter* species to replicating in other families and classes of bacteria (Salgado-Camargo et al., 2020). This highlights the potential role of non-*Acinetobacter* species contributing to the plasmidome of *Acinetobacter.* Similar to the term "resistome" referring to all ARG present, the term "plasmidome" refers to all the plasmids present. Furthermore, some of these plasmid lineages harbour ARG and pose as a risk of cross species transmission (Brovedan et al., 2020; Lean and Yeo, 2017). It has also been proposed that permafrost environmental *Acinetobacter* plasmids could have possibly been the origins of plasmids in clinical strains (Mindlin et al., 2020). The plasmids of ancient *A. lwoffii* and *A. pseudolwoffii* obtained through permafrost sediments shared similarity with modern *Acinetobacter* plasmids. It suggests that the backbone structure of these ancient plasmids largely remained the same, with these plasmids gradually evolving into the modern plasmids observed in the clinical environment. Plasmid typing tools would allow the detection and categorisation of plasmids to better study the relatedness between the ancient and modern plasmids. However, there is a lack of plasmid typing tools for *Acinetobacter* plasmids, further complicating the analysis of ARG and their context in plasmids (Salgado-Camargo et al., 2020).

MGE can be involved in the dissemination of ARG between bacteria and across different bacterial species (Evans and Amyes, 2014; Partridge et al., 2018). Insertion sequences and transposons are DNA elements that are able to mobilise themselves together with their associated genes to integrate themselves in the new locations randomly within the bacteria. This promotes the acquisition of ARG from plasmids to chromosome, or mobilisation of ARG from chromosome onto plasmids. It also partakes in gene duplication, gene deletion and other genome plasticity events. These can also be facilitated by homologous recombination given the multicopy nature of these MGE in a genome (Partridge, 2011). Apart from genome rearrangements, IS elements can play a role in the dissemination of beta-lactamase genes. Notably, the *bla*$_{OXA-23}$ carbapenemase gene has been traced to have originated from *A. radioresistens (Poirel et al., 2008)*. It was suggested that a plasmid has introduced *ISAba1* into *A. radioresistens* which then later mobilised the intrinsic resistance gene

from *A. radioresistens* into *A. baumannii*. It was noted that *ISAba1* was absent in *A. radioresistens* genomes with low level expression of the precursor gene. Subsequently, the dissemination of *bla*$_{OXA-23}$ into the different populations occurred through different strategies, such as via AbaR4 resistance island, and conjugative plasmids (Hamidian et al., 2019). Currently, *bla*$_{OXA-23}$ has been observed in different transposons, such as Tn*2006*, Tn*2008*, Tn*2008B* and Tn*2009*, in different genetic contexts (Nigro and Hall, 2016b). Transposons are a larger MGE that carry passenger genes in addition to transposase genes. Hence, MGE can also play a role in contributing to the diversity and plasticity genomic resistance islands (Blackwell et al., 2017; Blackwell et al., 2015; Hamidian and Hall, 2018).

Phage-mediated transduction of ARG has also been described for *bla*$_{NDM-1}$ gene to have taken place through a recombination event of a 66-kb region into the chromosome from a donor *A. baumannii* strain into a recipient *A. baumannii* strain (Krahn et al., 2016). The authors concluded that the activation of intact prophages in the chromosome may have been involved in the transduction event after ruling out possibilities of plasmid-mediated transfer, outer membrane vesicle mediated transfer and transformation. Horizontal gene transfer of plasmids as well as MGE contribute to the dissemination of ARG.

1.1.6. International clones of *A. baumannii*

Currently, there are two multi-locus sequence typing (MLST) schemes, namely Pasteur and Oxford, used to describe the population structure of *A. baumannii* (Bartual et al., 2005; Diancourt et al., 2010). Both schemes use a 7-gene based MLST approach with Pasteur scheme being more suitable for epidemiological analysis to identify clonal lineages at larger evolutionary distances as the genes used undergo less recombination (Gaiarsa et al., 2019). The Oxford scheme may be more discriminatory at shorter evolutionary distances when applied to closely related isolates as it considers the *gpi* gene, which is part of the capsular locus that has high recombination rates undergoing diversifying selection (Gaiarsa et al., 2019). Apart from MLST, there are also other higher resolution strain-typing phylogenetic analysis,

such as core genome MLST and SNP clustering, that can provide a deeper insight on how closely related the genomes are to one another (Maiden et al., 2013).

Pasteur scheme MLST sequence types (ST) have been used to describe the clonal relationship of *A. baumannii* isolates. The main circulating clonal complexes in the hospital were International Clone 1 (ST1) and International Clone 2 (ST2) (Diancourt et al., 2010). For *A. baumannii*, clonal complexes are a group of ST that shared a threshold level of allelic identity in six identical loci with at least one other ST in the group (Diancourt et al., 2010; Feil, 2004). Between each other ST in the clonal complexes, each ST is a single locus variant which differ in only one of the seven loci. Most of the clinical *A. baumannii* isolates were derived from these two clones and were multi-drug resistant. Clone 1 has been described to contain two lineages where the main lineage acquired antibiotics genomic resistance island AbaR through horizontal gene transfer event (Hamidian et al., 2019; Holt et al., 2016). Other sequence types, ST79 and ST25 together with ST2 account for around 71% *A. baumannii* genomes sequenced (Hamidian and Nigro, 2019).

Apart from these clones, there is also a diversity of non-clonal ST-types determined by MLST. These ST types do not have single locus variants and were not part of any clonal complexes. We hypothesised that these isolates might have originated from various environmental sources and eventually give rise to hospital- or community-acquired infections (Figure 3). While these clinical isolates were not associated with the different international clonal lineages, there might be an introduction of novel strains from unknown reservoirs into the clinical environment (Wilharm et al., 2017). There are evidences suggesting that the evolution of clinical lineages within a hospital is mediated by horizontal gene transfer events between reservoirs of *A. baumannii* from an extended care centre and those within the tertiary care hospital (Wright et al., 2014). There was limited geographic clustering of the isolates within the study hospital leading to the hypothesis that there may be more mixing of isolates or had more founder populations.

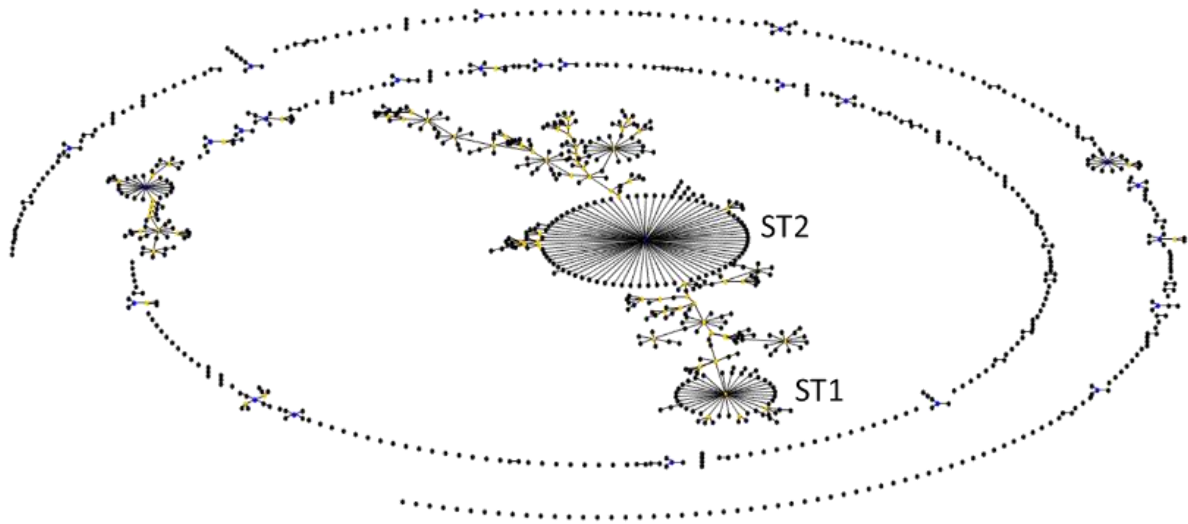

Figure 3. Visualisation of *A. baumannii* MLST (Pasteur scheme) using eBURST. Each dot is one ST and related ST are linked by lines. Two dominant global clones, ST1 and ST2, are evident. However, there are many ST that are singletons or not part of significant clusters demonstrating wide diversity of typed strains.

Community-acquired *A. baumannii* isolates from northern Australia were associated with ST10 as the most common dominant sequence type using the MLST Pasteur scheme (Meumann et al., 2019). These isolates were mainly not multi-drug resistant and do not harbour the AbaR genomic resistance island. ST10 isolates can be found in both community and nosocomial settings as well as in diverse geographic location. There is a possibility that the colonisation of ST10 in the community may have progressed into nosocomial infections after admission into the hospital (Meumann et al., 2019). In Vietnam, ST10 has also been described to be responsible for the colonisation of hospital-acquired ICU infections (Schultz et al., 2016). ST10 isolates can also acquire ARGs to become XDR isolates as seen in a US outbreak (Jones et al., 2015). Apart from this dominant ST, great diversity of STs have been observed for community-onset infections supporting the hypothesis that these isolates may originate from different ecological niches in diverse geographical locations.

The variations in chromosomally encoded $bla_{\text{OXA-51-like}}$ genes have also been proposed to be used for rapid characterisation of international clonal lineages 1 to 8 (Zander et al., 2012b). There was good association between $bla_{\text{OXA-51-like}}$ variants and rep-PCR based typing clonal lineages. Hence, there was potential to use $bla_{\text{OXA-51-like}}$ gene as a

single locus-based typing method to identify and type *A. baumannii* isolates (Pournaras et al., 2014). The discordance between *bla*$_{OXA-51}$ and expected clonal lineages may possibly reveal convergent evolution of *bla*$_{OXA-51}$ or other genetic events such as recombination. The DNA sequences of *bla*$_{OXA-51-like}$ variants may also reveal more clues on the evolution of *bla*$_{OXA-51-like}$ gene in the evolution of *A. baumannii*.

1.1.7. Natural environmental reservoirs of *A. baumannii*

The natural environmental reservoirs of *Acinetobacter baumannii* outside hospitals are poorly defined. The ability of *A. baumannii* to grow in a range of different temperature, pH and nutrient level conditions may have given it an advantage to adapt to different environmental reservoirs. Being able to persist on desiccated inanimate environment for up to 5 months, *A. baumannii* can spread within the hospital through contact surfaces, and medical equipment (Kramer et al., 2006). The ability of this obligate aerobe to grow at a higher temperature up to 44°C and in an acidic condition could be used to isolate *A. baumannii* from the environment (Baumann, 1968; Bouvet and Grimont, 1986). It has been reported to be found on soil contaminated with hydrocarbon petroleum, sludge and inanimate environmental surfaces such as on vegetables (Al Atrouni et al., 2016). The core genome of *A. baumannii* has been described to have an abundant of genes (35%) related to transport and metabolism genes (Peleg et al., 2012). These genes could be useful for its survival in environmental niches where complex or limited nutrients are available.

Soil environment

Conventionally, *A. baumannii* is often understood to be ubiquitous in the nature, and can be isolated easily from soil, water and animals (Towner, 2009). This has since been clarified as a common misconception in the understanding of *Acinetobacter* (Hrenovic et al., 2014; Towner, 2009). The misconception is likely from Bergey's Manual of Systematic Bacteriology which shared that members of *Acinetobacter* occur naturally in soil, water, sewage, and food (Garrity et al., 2006). The incidences of isolation of *A. baumannii* from soil are low with different rates of isolation (Eveillard et al., 2013). In Lebanon, *A. baumannii* was not isolated from 51 soil samples (Rafei et al., 2015). In Croatia, MDR *A. baumannii* that shared 87% similarity to a local clinical

isolate was isolated from a paleosol environmental samples. It is believed that improper waste disposal through an abandoned quarry near the sampling site might have caused the bacteria to be carried over into the environment and eventually into the paleosol (Hrenovic et al., 2014).

Aquatic environment

A. baumannii has been previously known to be able to utilise a variety of organic compounds as their sole carbon source and nitrate as the sole nitrogen source (Baumann, 1968). Another metabolic advantage of this aerobic microorganism is its ability to grow in low pH and acidic environment. Hence, the aquatic environment may be another possible environmental source. While it was challenging for early studies to resolve the species identity of the isolates among the closely related Acb complex and determine if *A. baumannii* was detected, they provided insights on the prevalence of *Acinetobacter* species in the water environment. Early environmental studies have isolated *Acinetobacter* spp. from deep groundwater wells (Bifulco et al., 1989; Stetzenbach et al., 1986). About 7% of 73 water samples were positive in Lebanon (Rafei et al., 2015). Adopting a One Health perspective, these water samples could have found its way to humans through anthropogenic activities and eventually leading to the transmission of *Acinetobacter.* In the urban environment, water from drainage systems such as canals and rivers may also act as sentinel locations for sampling of *Acinetobacter* species. The use of molecular techniques would allow the identification of *A. baumannii* from these environmental studies. While there was rare isolation of carbapenem resistant or multi-drug resistant *A. baumannii* from river sources, chloramphenicol resistant *Acinetobacter* spp. were isolated in 76.5% of aquaculture environment in Southeast Asia (Huys et al., 2007). These *Acinetobacter* spp. could include members of the Acb complex species which are intrinsically resistant to chloramphenicol. In aquaculture, a study suggested that the colonisation of *A. baumannii* in freshwater fish is dependent on the concentration of *A. baumannii* in the surrounding freshwater environment. It was described that the natural freshwaters have low colonisation potential for freshwater fish (Dekic et al., 2018). Antibiotics use in aquaculture may have selected for antibiotics-resistant bacteria in the environment.

Animals

Wildlife has been described to be an important domain to understand the transmission of ARG between animals, humans and the environments (Dolejska and Literak, 2019). A hypothesis based on the ability of growth at a higher temperature is that *A. baumannii* might be able to grow in animals such as birds where the body core temperature is much higher. The body core temperature of birds can range from 34°C to 44°C (Clarke and Rothery, 2008; Prinzinger et al., 1991), which would be higher than the human body temperature of 37°C, or the environmental ambient temperature of 25°C to 30°C. A sampling study of white stork nestlings in Poland revealed that *A. baumannii* was recovered from 25% of the 661 samples collected (Wilharm et al., 2017). $bla_{\text{OXA-51-like}}$ variants that were related to international clonal lineages, including IC1, 3-6 and 8, were found. However, it was difficult to discern if colonisation in storks was permanent as it was observed that the recovery of *A. baumannii* from the choana region of storks was transient (Wilharm et al., 2017). A follow-up study on free-living birds only yielded 0.2% (2 positive) of the 1051 samples collected, challenging avian hosts as a reservoir for *A. baumannii* (Łopińska et al., 2020).

In another study, up to 8% of various animal samples, including those from cow, dog, cat, horse and goat, were positive in Lebanon (Rafei et al., 2015). Apart from being capable to cause infections in humans, *A. baumannii* has been affecting animals in veterinary settings causing a range of infections such as sepsis, pneumonia, urinary tract infection and skin infection (van der Kolk et al., 2019). It can be challenging to determine if the animals are natural colonisers and to determine the natural environmental sources of *A. baumannii*, such as whether they are found in animal feeds or smaller preys.

Human Colonisation

Previous studies on community skin carriage of *A. baumannii* in healthy individuals ranged from 2.5 to 10% (Dexter et al., 2015). Colonisation of *A. baumannii* in oral biofilms could potentially act as a reservoir for pneumonia and chronic obstructive pulmonary disease (Richards et al., 2015). They are also known to colonise environments which have frequent contact with humans. They can also resist some of the decontamination methods used, making them a clinical significance for hospitals (Peleg et al., 2008). While *Acinetobacter* spp. has been reported to be found widely in nature, high level drug resistance is thought to arise only under antibiotic selection.

Given our hypothesis that the natural environment is a reservoir for antimicrobial resistance, the isolation of *Acinetobacter* spp. in the natural environment will provide insights on the evolution of antimicrobial resistance.

Knowledge gap in environmental reservoir of A. baumannii

Most studies are limited to understanding the relationship of clinical *A. baumannii.* and the hospital environment. In Singapore, a recent local point prevalence study revealed that the hospital environment of medical intensive care unit can be contaminated by carbapenem-resistant *A. baumannii* from patients and transmitted to another patient admitted on a later date (Ng et al., 2018). Limited studies have elucidated the natural environmental reservoir of *Acinetobacter* spp. and *A. baumannii* (Al Atrouni et al., 2016). This presents a research gap to examine the non-hospital natural environmental reservoirs of *A. baumannii*. The natural environment will be important to understand the potential sources for host exposure and community acquired infections, and more importantly, to understand the origins of antimicrobial resistance. It is also important to determine the relationship between clinical isolates and environmental isolates to understand whether there is any transmission or epidemiological link between them. To date, the prevalence of clinically important Acb complex in the non-hospital environment and their potential to enter in or out of the clinical setting, particularly through community-acquired infections, remains to be elucidated. Hence, it is important to determine the natural environmental reservoirs of *A. baumannii* and characterise their resistome.

1.1.8. Emergence of Sequencing Technologies

Next-generation sequencing (NGS) has made tremendous progress over the recent years. It has enabled whole genome sequencing (WGS) of bacterial pathogens in genomic epidemiology and public health studies led by clinical and research microbiology laboratories (Goldberg et al., 2015). This has revolutionalised the diagnostics and analysis of infectious diseases (Westblade et al., 2016). It has provided value information in the surveillance of nosocomial outbreak, as well as food-borne or vector-based diseases (Quainoo et al., 2017). Having the bacterial genome allows phylogenetic studies to determine its clonality and strain types, as well as its antimicrobial resistance determinants (Crofts et al., 2017; Lynch et al., 2016). These are useful to provide an understanding on the transmission and evolution of the bacterial host (Besser et al., 2018).

NGS has also contributed significantly to the study of antimicrobial resistance (Boolchandani et al., 2019). ARG can be characterised directly from raw sequencing reads or from genome assemblies, and subsequently to be compared against reference databases. However, plasmid identification remains as a challenge using short read sequencing technologies (Orlek et al., 2017b). Long read sequencing technologies by Oxford Nanopore Technologies and Pacific Biosciences have overcome this challenge by sequencing across complex genomic regions such as repeats (Besser et al., 2018). These long reads allow assembly of complex regions and circular contigs such as plasmids. The genetic context of ARG can now be closely monitored with the aid of these third-generation sequencing technologies. There are many data analysis web tools and pipelines developed since to allow the analysis of the bacterial WGS dataset (Ekblom and Wolf, 2014). Public database resources have been developed and implemented for the open sharing of microbial genomic data (Sayers et al., 2021).

1.2. Hypothesis

We hypothesise that ARGs originate from the environment. This is supported by the evidence of intrinsic resistance genes, *bla*$_{ADC}$ and *bla*$_{OXA-51-like}$, in *A. baumannii* that has been passed down vertically. The large diversity of *bla*$_{OXA-51-like}$ variants also suggest that there is ongoing evolution of intrinsic ARGs that have yet to be fully understood. Given the genome plasticity and natural competence of *A. baumannii*, it is highly likely that the acquisition of IS elements and plasmids promote mobilisation and horizontal gene transfer events. This is supported by evidence of IS elements, such as IS*Aba1*, contributing to the increased expression and mobilisation of the intrinsic genes. The finding of *bla*$_{OXA-23-like}$ variant from the chromosome of *A. radioresistens* by Poirel et al (2008) might suggest that there may be other environmental sources of ARGs that are yet to be discovered. The *bla*$_{OXA-23-like}$ gene found in *A. radioresistens* could have played a role in conferring resistance to naturally occurring carbapenem, such as thienamycin, in the soil environment (Papp-Wallace et al., 2011). However, *bla*$_{OXA-23-like}$ has now become one of the mainstream beta-lactamase responsible for carbapenem resistance in *A. baumannii*, demonstrating how a series of evolutionary steps could have mobilised the ARG out of *A. radioresistens* chromosome into the genome of Acb complex species. It provides a first case of an intrinsic resistance gene being present as an acquired resistance gene in its closely related species. The presence of large genomic resistance islands harbouring ARGs, IS elements and transposons is most likely an outcome of the accumulation of ARGs through positive selection. For ARGs to persist in the natural environment, we hypothesise that there might be factors, ranging from selection, co-resistance, hitchhiking events, to having other metabolic functions, that have not been fully characterised.

The metabolic ability of *A. baumannii* to grow in higher temperature and survive in different complex mediums lead to the hypothesis that there could be an environmental reservoir and ecological niche that they serve, especially in the urban setting where they could have been involved in community-acquired infections. By studying *A. baumannii* and other *Acinetobacter* spp. in the environment, we will be able to understand the origins and evolution of antimicrobial resistance. We suggest that ARGs could have originated from the natural environment and subsequently been

gradually selected for in the clinical setting. Given the intrinsic resistome, presence of MGE, ability to survive in different conditions and poorly defined environmental sources and ecotypes, we believe that *Acinetobacter* genus will be a good gram-negative microorganism to study the origins and evolution of antimicrobial resistance genes.

1.3. Aims

This study aims to (1) determine the prevalence and antimicrobial resistance genetic determinants of *A. baumannii* in the natural non-hospital environment. The study aims to (2) perform molecular epidemiology and comparative genomic analysis between environmental and clinical isolates to understand the transmission and evolution of antimicrobial genes. Lastly, the study aims to (3) investigate the potential mechanisms behind the transmission and evolution of antimicrobial resistance genes in the environment (Figure 4).

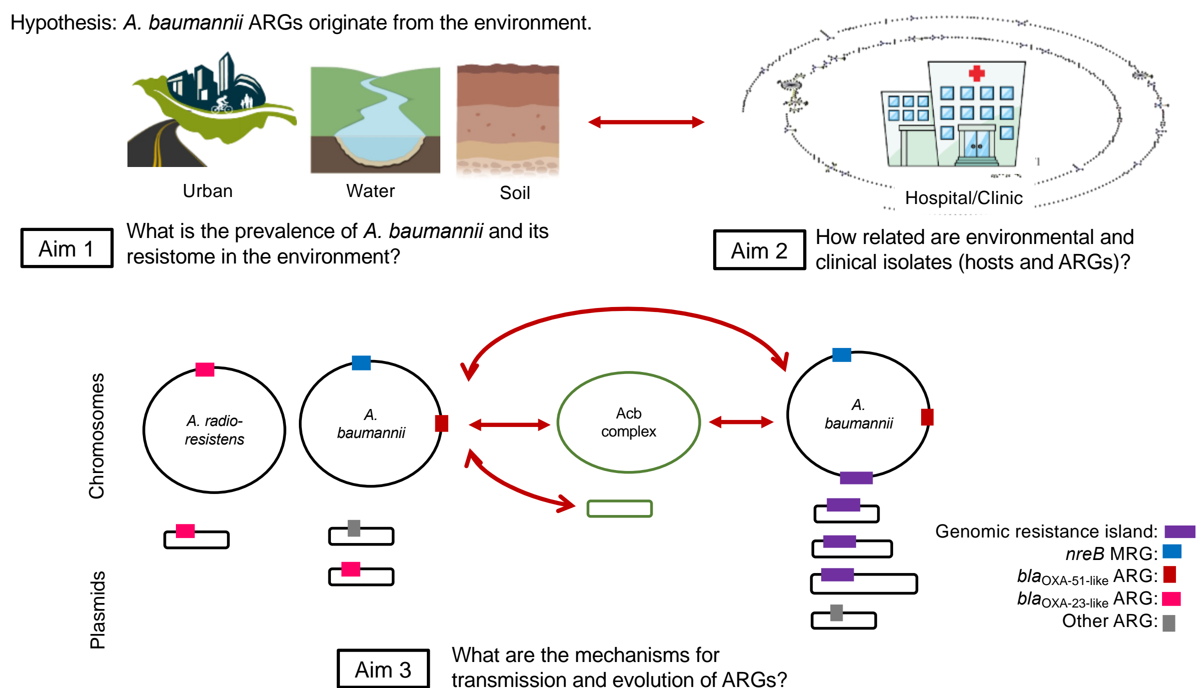

Figure 4. Overview of study aims to understanding the genomic epidemiology and resistome of *A. baumannii* in environmental and clinical settings.

Chapter 2. Materials and Methods

This study involved three approaches to study environmental, clinical and publicly available *A. baumannii* genomes (Figure 5). Firstly, field sampling was conducted to isolate and characterise *A. baumannii* from soil and water environment. Secondly, local clinical Acb complex isolates were retrieved for characterisation. Whole genome sequencing was performed for both environmental and clinical isolates. Lastly, publicly available genome datasets from National Center for Biotechnology Information (NCBI) databases were accessed and curated. Together, these approaches allowed the characterisation and comparison of local environmental and clinical isolates against global *A. baumannii to* perform a comprehensive global epidemiology and resistome study.

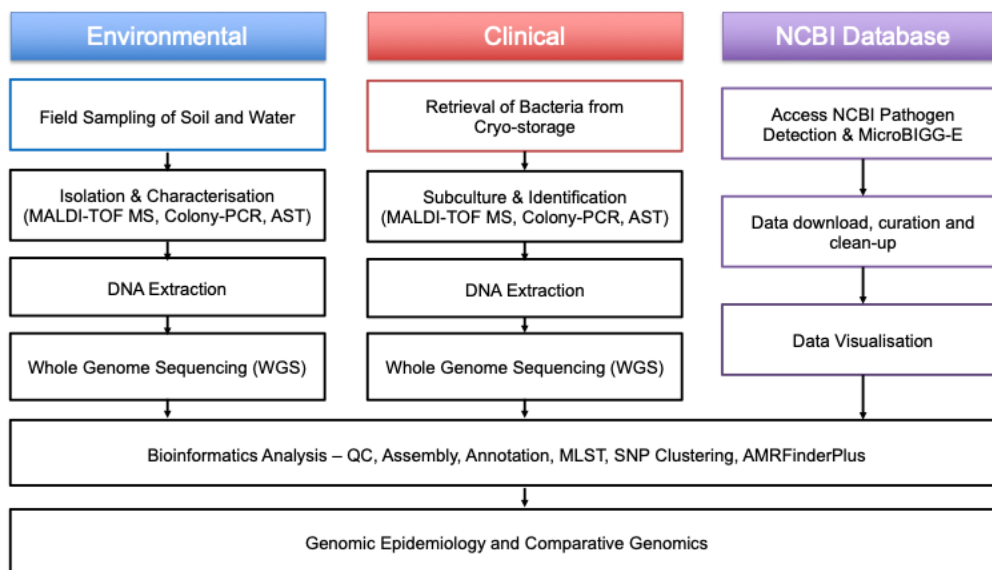

Figure 5. Overview of study approach to obtain environmental and clinical *A. baumannii* for comparison with global datasets in NCBI databases.

2.1. Isolation and characterisation of *A. baumannii*. from the environment

Field Sampling

Environmental sampling consisting of soil and water were conducted in non-hospital environment to determine the prevalence of *A. baumannii* species. The soil samples were collected using an auger at the depth of 10 cm to 30 cm. Sampling locations include pristine soil, urban soil, urban canals, freshwater reservoirs in Singapore, Malaysia and Philippines. The soil and water sampling were conducted with permission from the National Parks (NParks) and Public Utility Board (PUB) under the permits NP/RP19-075 and NP/RP19-047. The sampling date, time, global position system location and photographs of the site were recorded.

Microbiological Culture

The wet weight of the samples were determined, and 10 g of soil were vortexed in PBS-Tween (PBST) for 30 seconds to resuspend the bacteria. Subsequently, the samples were enriched overnight in Baumann's Enrichment Medium at 37°C with 200 rpm (Baumann, 1968). The enriched samples were serially diluted in phosphate-buffered saline and plated on modified selective Leeds Acinetobacter Medium containing 10 mg/L vancomycin and 15 mg/L cefsulodin (Jawad et al., 1994) for incubation at 37°C and 44°C. The higher temperature was selected to isolate *A. baumannii* which can survive when cultured in 44°C. Pink mucoid colonies with pink to mauve colour diffused into the medium were subcultured on tryptic soy agar (TSA) at least twice to obtain a pure culture. The isolates were stored in lysogeny broth (LB) supplemented with a final concentration of 20% glycerol for long term cyrostorage at -80°C.

Species identification

Species identification was carried out using Matrix-Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrophotometry (MALDI-TOF MS) BioTyper following the extended direct transfer manufacturer's protocol. Briefly, a fresh colony was spotted onto the MALDI Target plate using a toothpick before adding 1 µL of 70% formic acid. After drying, 1 µL of HCCA matrix was added and left to dry. The prepared

MALDI plate was loaded for analysis in the MALDI-TOF MS BioTyper (Bruker Daltonics).

The identification was confirmed using direct colony qPCR targeting *A. baumannii* specific ITS region (Table 1) (Chen et al., 2007). *Acinetobacter* genus-specific primers, Ac696F and Ac1093R targeting a 350 bp *Acinetobacter rpoB* hyper-variable region was used to confirm *Acinetobacter* spp. (Gundi et al., 2009). The bacterial colonies were picked using a sterile pipette tip for transfer into 50 µL of water and subsequently heat lysed at 95°C for 10 minutes. The real-time PCR amplification was performed in 10 µL volume with the KAPA2G Fast HotStart ReadyMix PCR Kit (KAPA Biosystems, USA) containing 150 nM of forward and reverse primer set each. EvaGreen saturating dye was used for the qPCR performed in Applied Biosystems StepOnePlus thermocyclers with the following parameters: initial denaturation at 95°C for 20 s, 40 cycles of 95°C for 10 s and 53°C for 20 s, followed by a melt curve stage.

Table 1. List of primers used in this study.

Primer	**Sequence (5' to 3')**	**Reference**
P-Ab-ITSF	CATTATCACGGTAATTAGTG	(Chen et al., 2007)
P-Ab-ITSB	AGAGCACTGTGCACTTAAG	
Ac696F	TAYCGYAAAGAYTTGAAAGAAG	(Gundi et al., 2009)
Ac1093R	CMACACCYTTGTTMCCRTGA	
27F	AGAGTTTGATCMTGGCTCAG	(Frank et al., 2008)
1492R	TACGGYTACCTTGTTACGACTT	

Amplification of 16S rRNA region was carried using 27F and 1492R universal primers (Frank et al., 2008). PCR was conducted using KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems, USA) in 50 µL total reaction volume containing 150 nM of forward and reverse primer set each. PCR was performed in Applied Biosystems Veriti thermocyclers with the following parameters: initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 15 s, 60°C for 15 s, 72°C for 15 s, and a final extension of 72°C for 2 min. The PCR amplicons were purified using Agencourt AMPure XP PCR Purification kit (Beckman Coulter) that was based on magnetic bead-based chemistry.

The purified amplicons were subsequently sent out to 1st BASE (Singapore) for single pass DNA sanger sequencing. Both forward and reverse reads were analysed and curated manually based on their Phred quality score using SeqTrace 0.9.0 software (Stucky, 2012). The 16S rRNA sequence was compared against 16S reference database using BLAST and EzBioCloud platform (Yoon et al., 2017). Novel bacterial species were characterised phenotypically and genotypically in accordance to published taxonomic notes (Chun et al., 2018; Tindall et al., 2010).

Antibiotics susceptibility testing

Antimicrobial susceptibility testing (AST) was carried out using Kirby-Bauer disk diffusion method on Mueller Hinton agar against a panel of antibiotics, namely ampicillin/sulbactam (10/10 µg), piperacillin/tazobactam (100/10 µg), ceftazidime (30 µg), cefotaxime (30 µg), doripenem (10 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), amikacin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg) and colistin (10 µg) following CLSI breakpoints (CLSI, 2018). The minimum inhibitory concentration was determined using microbroth dilution and interpreted using CLSI breakpoints (CLSI, 2018).

2.2 Clinical Acb complex isolates

Acb complex (n = 117) were previously isolated from blood samples in a public tertiary hospital, Tan Tock Seng Hospital, Singapore from February 2006 to December 2010 under ethics approval reference DSRB 2016/00007. The isolates were retrieved from -80°C cyrostorage.

2.3. Bacterial whole genome sequencing

Bacterial whole genome sequencing was carried out to determine the antimicrobial resistance determinants and determine its relationship with public genomes of clinical isolates. The genetic context of resistance genes, such as *bla*$_{OXA}$ beta-lactamase genes, was studied in greater detail to understand the evolution and transmission of the carbapenem resistance genes. Comparative genomic analysis was conducted

between the environmental isolates and clinical host isolates to understand the population structure and antimicrobial resistance determinants with local and global strains.

DNA extraction and library preparation

Genomic DNA was extracted from bacterial colonies using QIAamp DNA Mini Kit (QIAGEN, Germany) according to manufacturer's instruction with a final elution volume of 50 µL. The quality of extracted gDNA was assessed using NanoDrop spectrophotometer to estimate the quantity of DNA and evaluate the purity using the absorbance ratios. A fluorometric-based quantification assay using Qubit HS dsDNA assay (ThermoFisher) was conducted to determine the concentration of gDNA prior to whole genome sequencing. Extracted gDNA was diluted 5X in molecular grade water for gel electrophoresis in 0.6% agarose using TBE buffer for 100V in 20 minutes against 1 kb extend DNA Ladder (NEB). The agarose gel was pre-stained with 5% (v/v) FloroSafe DNA Stain (1st BASE, Singapore) for trans-UV visualisation on Bio-Rad ChemiDoc MP Imaging System. The extracted DNA was kept in -30°C for long term storage.

Next-generation sequencing

WGS was performed using Illumina MiSeq and HiSeq next-generation sequencing platform. Bacterial genomic DNA were fragmented and barcoded using Nextera XT DNA Library Preparation Kit (Illumina) following manufacturer's instructions. The barcoded libraries were quantified using Qubit HS dsDNA fluorometric assay and assessed for the optimal fragment size using High Sensitivity D5000 ScreenTape on the Agilent TapeStation platform. The libraries were normalized and pooled before loading into the sequencer for paired-end sequencing using 2x250bp and 2x150bp Reagent Kit. The sequencing run and output were monitored and downloaded through the BaseSpace Sequence Hub (Illumina).

Long read sequencing

Given the genome plasticity and presence of repeats, IS elements and transposons in *A. baumannii*, one of the limitations of short reads sequencing is that it will result in contigs that are difficult to piece together. Long read sequencing was employed to

assemble the genome to study the genomic organisation, gene context, and complete plasmids. Oxford Nanopore Technology (ONT) long read sequencing library preparation was done using Rapid Barcoding Kit (SQK-RBK004) and Ligation Sequencing Kit (SQK-LSK109) following manufacturer's instruction. Sequencing was performed on a MinION Mk1C device with R9.4 flow cell using MinKNOW software 2.2 (v18.07.10). Base-calling and barcode demultiplexing was performed with ONT Guppy (v4.0.11) on the fast5 read files using the high accuracy mode.

2.4. Bioinformatics analysis

FASTQ sequencing reads were uploaded onto GALAXY (https://usegalaxy.org) platform. Quality check of the reads was conducted using FastQC (Andrews, 2010). The raw data of FastQC output were aggregated using the MultiQC tool (Galaxy Version 1.11+galaxy0) for visualisation (Ewels et al., 2016). De novo assembly and hybrid assembly were generated using Unicycler assembler with default parameters (Galaxy Version 0.4.6.0) (Wick et al., 2017). Assembly statistics were generated using QUAST (Galaxy Version 4.6.3) (Gurevich et al., 2013). Bandage was used to visualise the assembly graph (Wick et al., 2015). Genome annotation was done using Prokka (Seemann, 2014). The GenBank and FASTA sequences of the annotated genomes were used for further analyses using other genomic tools. Putative plasmids were manually assessed based on complete circular contigs and their sequencing depth in hybrid assemblies. The presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) was determined using CRISPRCasFinder (Couvin et al., 2018). Prophages were predicted using PHASTER from the nucleotide sequences (Arndt et al., 2016). Antimicrobial resistance genes and metal resistance genes were identified from the nucleotide sequences using NCBI AMRFinderPlus 3.9.8 with --plus flag (Feldgarden et al., 2019). Toxin-antitoxin system prediction was done using TADB (Xie et al., 2018). Virulence factors genes were predicted using the virulence factor database (VFDB) (Liu et al., 2019b).

Multi-locus sequencing type (MLST) profiles, using Pasteur and Oxford schemes, was be conducted using pubMLST and DTU-MLST (Jolley et al., 2018; Larsen et al., 2012). Novel MLST alleles and sequence types were submitted into the pubMLST database to assign novel MLST profiles. Core SNP phylogeny was carried out using SNP-based analysis against a reference genome, such as *A. baumannii* ATCC 17978

(NZ_CP000521.1), with CSI Phylogeny 1.4 hosted by the Center for Genomic Epidemiology server (Kaas et al., 2014). The following default parameters were used: minimum depth of 10X and minimum relative depth of 10% at SNP positions, minimum distance of 10 bp between SNPs (prune), minimum SNP quality of 30 and minimum Z-score of 1.96. Phylogenetic trees were visualized using the Interactive Tree of Life (iTOL) v6 software (Letunic and Bork, 2019). Newick phylogeny files were uploaded onto the iTOL web server while the associated metadata were updated using iTOL annotation editor v1.4. Gene synteny analyses were performed and plotted using Clinker v0.0.23 with GenBank files as input (Gilchrist and Chooi, 2021). The global alignment results were visualised from the interactive HTML output generated from Clinker and exported as SVG file format. Sequence alignment using BLAST was carried out to compare between nucleotide sequences of NCBI databases (Camacho et al., 2009). Microbial Nucleotide BLAST was used to query against bacterial complete genomes and complete plasmids using megablast algorithm. uGENE software v1.29.0 was used to visualize the sequences and their features.

Pangenome analysis was performed on using roary pipeline (Galaxy Version 3.13.0+galaxy0) with Prokka annotated genomes (gff file format) as input (Page et al., 2015). The proteins would need to share at least 95% blastp identity to be grouped as the same family. Core gene was defined by the gene being present in 99% of the isolates. The newick tree generated by roary using the binary presence and absence of accessory genes was visualised in iTOL together with other metadata of the genomes.

Digital DNA-DNA hybridisation (dDDH) was performed using Type Strain Genome Server (TYGS) with d_4 distance formula (Meier-Kolthoff and Göker, 2019) and interpreted with the cut-off value of 70% for genomes of the same species (Chun et al., 2018). Average nucleotide identity (ANI) analyses were done using JSpeciesWS server (Richter et al., 2015), with the similarity cut-off of 95% for genomes of the same species (Kim et al., 2014). Novel bacterial species were characterised phenotypically and genotypically in accordance to published taxonomic notes (Chun et al., 2018; Tindall et al., 2010). Default parameters were used unless otherwise stated.

2.5. Development of GALAXY Workflow for Genome Assembly and Annotation

Advances in next-generation sequencing (NGS) technologies has enabled routine whole-genome sequencing of bacterial isolates for comparative genomics and molecular epidemiology studies. A code-free bioinformatics protocol was developed for researchers without computational background to assemble bacterial genomes and perform basic tertiary analysis from their sequencing data (Wee and Yap, 2021). This eliminated the need for commercial bioinformatics software that may be costly. The workflow runs on GALAXY, a user-friendly web-based platform that contains many pre-installed bioinformatics tools (Afgan et al., 2018). Using NGS data as input, the workflow performed quality check, de novo assembly, genome annotation, prediction of antimicrobial resistance genes, and multi-locus sequence typing. These genome assembly and annotation bioinformatics steps were described in greater detail in Chapter 2.4 above. This open-source pipeline allowed the documentation, parameterization, sharing and facilitating replication, reuse, and reproducibility of both data and methods that were in line with the FAIR Data Principles – Findability, Accessibility, Interoperability, and Reusability (Goble et al., 2020; Wilkinson et al., 2016).

2.6. Public Genome Database

Genomic survey of resistome was carried out by analysing publicly available genome datasets mined from NCBI databases. As of 31 December 2020, there were 15,262 *A. baumannii* isolates deposited in the NCBI Pathogen Detection (PD) database (https://www.ncbi.nlm.nih.gov/pathogens). Data from NCBI Genome, PD and Microbial Browser for Identification of Genetic and Genomic Elements (MicroBIGG-E) databases were downloaded. The source of isolates deposited were assessed and manually curated the provenance metadata to check the isolation type and source against NCBI BioSample entries and published literature. The public data was cleaned, transformed and presented for visualisation and interpretation using Microsoft Excel. Antimicrobial resistance phenotypic and genotypic data, genomic assemblies, raw short read data, phylogenetic trees (core SNP) together with other metadata (including location, source type, collection date of the samples) were available by FTP and were used for genomic epidemiology analysis.

2.7. Data availability

The whole genome sequencing data from this study have been made publicly available in NCBI BioProject under accession number PRJNA565663.

Chapter 3. Environmental *Acinetobacter baumannii*

Chapter 3 describes the prevalence of environmental *A. baumannii* and their resistome in the non-hospital environment. Field sampling of soil and water were conducted to isolate and culture *A. baumannii* as well as members of the Acb complex and other *Acinetobacter* spp. These isolates were microbiologically and molecularly characterised to determine their ARG and the genetic context of the ARG. Phylogenetic analysis was conducted to assess their genetic relatedness to clinical isolates.

3.1. Prevalence of environmental *A. baumannii*

We have isolated *A. baumannii* and other *Acinetobacter* spp. in our tropical urban and natural environment, not associated with hospitals or healthcare facilities. They have been cultured from various sites such as forests and wells, and are unlikely to be from direct human sources. A total of 266 environmental samples comprising 148 soil, 16 composts and 102 water samples were collected from Singapore, Malaysia and the Philippines and were screened for *Acinetobacter* species (Figure 6; Table 2). Out of the 29 soil samples that cultured positive for *A. baumannii*, 19 (65.5%) samples were associated with urban road soils. The urban road could be a novel under-studied reservoir for *A. baumannii*.

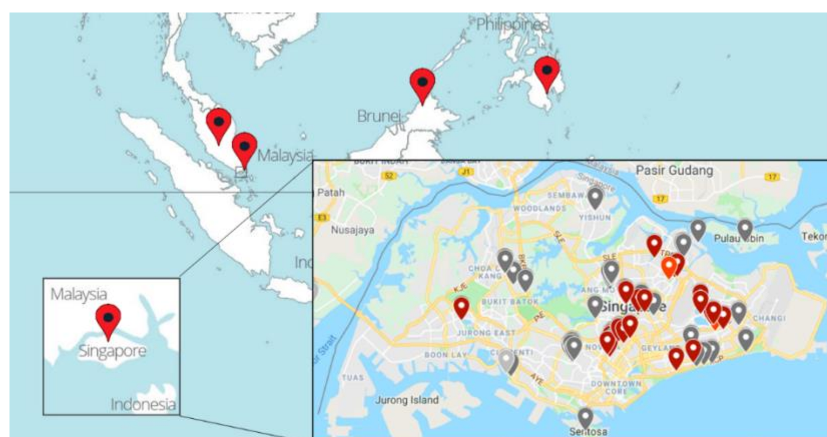

Figure 6. Regional sampling map of locations from which *A. baumannii* was isolated (red: cultured, grey: culture negative).

Table 2. Summary of samples cultured positive for *Acinetobacter* species.

	Number of samples (%)			
	Compost	**Soil**	**Water**	**Total**
Acinetobacter baumannii positive	4 (25.0%)	29 (19.5%)	30 (29.4%)	63 (23.7%)
Other *Acinetobacter* species positive	2 (12.5%)	23 (15.5%)	36 (35.3%)	61 (22.9%)
Other *Acinetobacter* spp. and *A. baumannii* positive	2 (12.5%)	9 (6.1%)	16 (15.6%)	27 (10.2%)
Total	16	148	102	266

Table 3. List of *Acinetobacter* species isolates (n = 108) isolated from environmental samples. Members of the Acb complex are marked with *.

S/N	Species Name	Soil	Water	Grand Total
1*	*Acinetobacter baumannii*	29	30	59
2	*Acinetobacter bereziniae*	2	2	4
3*	*Acinetobacter calcoaceticus*	3	2	5
4*	*Acinetobacter lactucae*	1	5	6
5	*Acinetobacter junii*	2	1	3
6*	*Acinetobacter nosocomialis*	5	1	6
7*	*Acinetobacter pittii*	4	7	11
8	*Acinetobacter radioresistens*	2	1	3
9	*Acinetobacter schindleri*	1	0	1
10*	*Acinetobacter seifertii*	2	2	4
11	*Acinetobacter tandoii*	1	4	5
12	*Acinetobacter towneri*	1	0	1

A total of 12 different *Acinetobacter* species out of 63 known *Acinetobacter* species have been isolated in this study (Table 3). All six pathogenic members of the Acb complex were isolated from the environment in our study.

3.1.1. Linkage of environmental isolates to clinical isolates

Environmental *A. baumannii* isolates had diverse MLST profiles, including novel MLST profiles that did not belong to the global clonal complexes responsible for the nosocomial hospital infections. There were 8 novel MLST Pasteur scheme ST found in the *A. baumannii* environment isolates, while some of the STs have been associated with clinical isolates from other parts of the world (Supplementary Table S1). These STs have been reported in diverse sources ranging from food and animals to medical environments, including sputum, blood and wound cultures. Environmental ST10 *A. baumannii* isolates that clustered with local clinical ST10 *A. baumannii* were isolated. Apart from shared MLST, a higher resolution sequence typing method, such as core genome SNPs, or phylogenetic BLAST against the genome tree would be required for a clearer understanding of the environmental linkages to the clinical isolates. Further analysis could map isolates onto the genome tree to have a better understanding of their relationship with other international clones. Currently, there were no shared STs between environmental isolates collected from different locations. This could be a result of under-sampling and neutral diversity being observed in the sampled isolates. However, the potential unique ecological niches for the isolates cannot be excluded too.

3.1.2. Characterisation of community-associated ST10 environmental isolates

We successfully isolated two *A. baumannii* ST10 isolates, strain N3-45 and strain W3-157, from the urban water canal environment in Singapore. To the best of our knowledge, this is the first report of ST10 isolated from a non-hospital setting globally. In a recent study by Meumann et al. (2019), ST10 was reported as the most common ST for community-acquired infections in Australia. Whole genome sequencing of the two environmental ST10 isolates, N3-45 and W3-157, were conducted to characterise their genomes and understand their genetic relatedness to clinical strains. These urban environmental isolates shared genetic similarity to *A. baumannii* Pasteur scheme ST10, a sequence type associated with CAI. This presents a potential urban environmental reservoir for *A. baumannii* causing CAI.

3.1.3. Complete genome of ST10 *A. baumannii* N3-45

A. baumannii N3-45 was isolated in 2019 from an urban water canal in central Singapore in this study. The hybrid assembly of *A. baumannii* N3-45 yielded a complete chromosome which is 3,664,990 bp in size with a GC content of 39.0%. It also harboured two plasmids, pN3-45_1 and pN3-45_2 that are 7,146 bp (GC content 32.0%) and 5,398 bp (GC content 31.1%) in size. The genome has 3,420 coding sequences (CDS), 6 copies of 16S-23S-5S rRNA operons, and 68 tRNA genes. The genome had slightly less CDS than the median of 3,680 CDS reported in NCBI Genome for *A. baumannii*. This isolate did not harbour any CRISPR array or complete prophage but had 3 incomplete and 1 questionable prophage regions predicted. The isolate belonged to Pasteur ST10 and Oxford ST2373. Antimicrobial resistance genes *bla*$_{ADC-76}$, *ant(3")-IIa*, *bla*$_{OXA-68}$ (*bla*$_{OXA-51-like}$) and *nreB* were found to be chromosomally encoded in this isolate. This is consistent with the intrinsic resistance genes reported in *A. baumannii* species.

To understand more about the origins and roles of the small plasmids found in this environmental ST10 isolate, pairwise comparison using BLASTN against the NCBI *Acinetobacter* plasmid database showed that the plasmid pN3-45_1 shared a high identity of 95.6% to plasmid pAba10324b (NZ_CP023024.1) that is 7,143 bp in size. Plasmid pAba10324b was found in *A. baumannii* strain 10324, which belonged to Pasteur Scheme ST10 with a novel Oxford Scheme ST. This strain was isolated in 2012 from a clinical bronchial fluid sample of a 31-year-old male patient in Mexico. This small plasmid containing 9 coding sequences, which were inclusive of a *rep* replicase gene, a type II toxin-antitoxin system and hypothetical proteins. The toxin-antitoxin gene may be responsible for the maintenance of this plasmid in environmental bacterial host and could have possibly been disseminated across *A. baumannii* ST10, though further investigations are needed to validate this.

The sequence of the other plasmid, pN3-45_2, had 80% query cover hit against *A. calcoaceticus* CA16 plasmid pCA16 with 88.2% identity. The plasmid pN3-45_2 contained mostly hypothetical proteins. Interestingly, both isolates had been cultured from environmental origins. *A. calcoaceticus* CA16 was isolated from canola root in Canada in 2015 while *A. baumannii* N3-45 was isolated from environmental water

canal in Singapore. This suggests the sharing of plasmid sequences between Acb complex members from different geographically distinct locations.

3.1.4. Complete genome of ST10 *A. baumannii* W3-157

The other environmental isolate, *A. baumannii* W3-157, was also isolated in 2019 from an urban water canal in Singapore in our study. The hybrid assembly yielded a complete chromosome which is 3,904,433 bp in size with a GC content of 39.03% with no plasmid. The isolate belonged to Pasteur ST10 with a novel Oxford ST being closest to ST1426, 585, and 1317. The genome has 3,575 predicted CDS, 6 copies of 16S-23S-5S rRNA operons, and 66 tRNA genes. This isolate did not harbour any CRISPR array and had 2 intact and 1 questionable prophage regions predicted. The first intact prophage region was 49.3 kb in size with GC content of 37.9%, encoding 30 phage proteins and 17 hypothetical proteins. This intact region shared 31.9% of proteins with PHAGE_Pseudo_phiCTX (GenBank accession: NC_003278). The questionable region was 51.3 kb in size with GC content of 38.4%. Out of its 68 proteins, 37 proteins (54.4%) were similar with PHAGE_Acinet_Bphi_B1251 (GenBank accession: NC_019541). The last intact prophage region was 42.1 kb in size with GC content of 39.5%, encoding 39 phage proteins and 20 hypothetical proteins and similarly to the questionable region. It also shared some proteins with PHAGE_Acinet_Bphi_B1251 (GenBank accession: NC_019541), albeit slightly lower (16.9%). Compared to *A. baumannii* N3-45, *A. baumannii* W3-157 had an additional ARG, *adeC*, and had *bla*$_{ADC}$ instead of *bla*$_{ADC-76}$.

3.1.5. Genomic epidemiology of ST10 environment and clinical isolates

To understand the genetic relationship of the environmental ST10 isolates against other ST10 isolates, a whole genome SNP-based phylogenetic tree was plotted with 58 *A. baumannii* ST10 isolates downloaded from NCBI database, as well as local ST10 clinical isolates that were studied in greater detail in Chapter 4. The closest local clinical isolate in the phylogenetic tree to environmental ST10 *A. baumannii* N3-45 was *A. baumannii* AB141 that we had sequenced in this work (Figure 7).

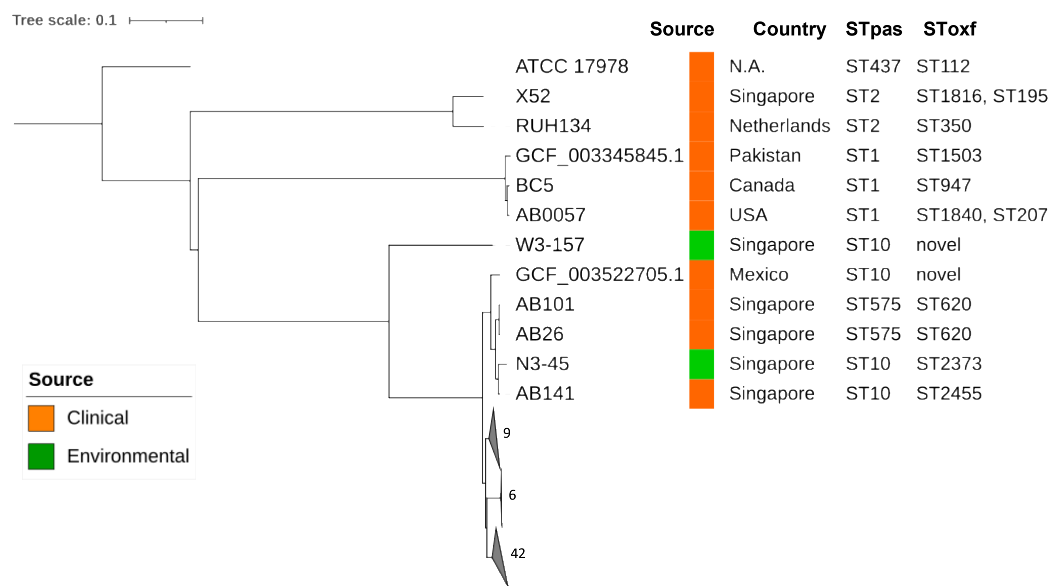

Figure 7. SNP phylogenetic tree of *Acinetobacter baumannii* environmental ST10 isolates (annotated in green) revealed clustering with clinical ST10 isolates. SNP Clustering was done with *A. baumannii* ATCC17978 as the reference strain together with other isolates from International Clone 1 (ST1) and International Clone 2 (ST2) using CSI Phylogeny. Environmental *A. baumannii* N3-45 isolated in this study was phylogenetically closest to clinical *A. baumannii* AB141. Phylogenetic tree was visualized using iTOL v6 software.

A. baumannii AB141 was a blood culture isolate in Singapore isolated in 2010 from a 76-year-old male patient, with multiple co-morbidities, such as cardiovascular disease, renal disease and diabetes mellitus. The patient was admitted to the general medical ward when the diagnosis of *A. baumannii* was made on the third day of admission.

The isolate was resistant to amoxicillin, amoxicillin/clavulanic acid, ciprofloxacin, ceftriaxone, cefuroxime, and susceptible to amikacin, ceftazidime, cefepime, gentamicin, imipenem, meropenem, polymyxin B, ampicillin/sulbactam, cotrimoxazole and piperacillin/tazobactam. The phenotypic resistance found, except for ciprofloxacin, were known to be intrinsic to Acb complex. Given the short duration of admission and positive culture on Day 3, we believe that there could be an open possibility that the patient may be infected prior to hospitalization. He was described to have low APACHE II Score of 9 indicating that he was not severely ill. There was no lung disease noted in the metadata records and the patient was later discharged after 11 days. The clinical context suggested that *A. baumannii* AB141 could be of a less virulent strain since the patient recovered well under definitive antibiotics treatment with ampicillin/sulbactam and combination therapy.

While both environmental *A. baumannii* N3-45 and clinical *A. baumannii* AB141 isolates were phylogenetically related and shared similar ARG, there were some genomic differences between them. Both isolates were ST10 using Pasteur Scheme but have different sequence types using Oxford Scheme. N3-45 belonged to Oxford ST2373 (*gpi* allele 331) while AB141 belonged to Oxford ST2455 (*gpi* allele 16). Both ST were a single locus variant where they differed in *gpi* locus allele with 93.8% nucleotide difference due to 19 nucleotide substitution mutations. The *gpi* gene locus allele encoding for glucose-6-phosphate isomerase in the Oxford scheme has been described to undergo high frequency of recombination, resulting in the large number of sequence types in Oxford scheme (Gaiarsa et al., 2019).

Based on the SNP-based phylogenetic tree, environmental *A. baumannii* W3-157 may belong to an outlier of ST10 isolates given the difference in the tree structure and types of ARG found (Figure 8). This suggests that the isolate might be adapted to a novel ecological niche and have different accessory genes compared to rest of the ST10 isolates. In terms of intrinsic ARG, it harboured bla_{ADC} instead of bla_{ADC-76} that was found in most of the ST10 isolates. However, bla_{OXA-68} ($bla_{OXA-51-like}$) intrinsic resistance gene was found in *A. baumannii* W3-157 too.

SNP phylogeny of 63 *A. baumannii* ST10 isolates revealed that while majority of the isolates share similar ARG profile. There were groups of isolates (highlighted as A to E; Figure 8) sharing additional ARG that were likely to be plasmid mediated in each of

these groups. The isolates were assigned to various groups based on the co-occurrence of ARGs present. These isolates shared similar ARG among their groups that were clustered together based on their SNP phylogeny. These suggest that ARG may be introduced to this sequence type and may persist in infections. We observed geographical clustering of these isolates with their ARGs, indicating potential endemic infections (highlighted as B and E; Figure 8). We found that local clinical *A. baumannii* AB26 from ST575 (a ST that was closely related to ST10 isolates by phylogenetic analysis) contained a megaplasmid that harboured multiple ARG to confer multi-drug resistance (highlighted as A; Figure 8). This resulted in the isolate having significantly more ARG compared to its close phylogenetic neighbours. More details of this clinical isolate were discussed in Chapter 4.5.3.

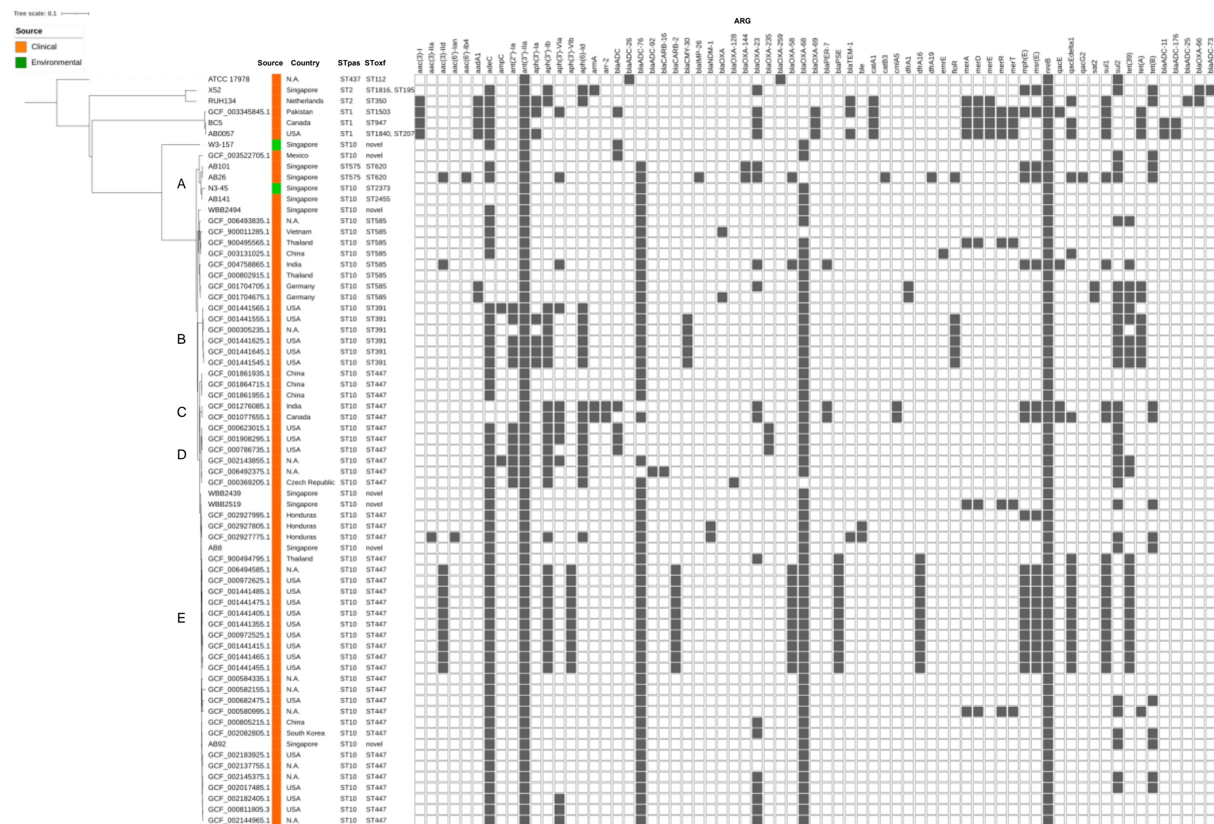

Figure 8. SNP phylogenetic tree of *A. baumannii* ST10 isolates. SNP clustering of *A. baumannii* ST10 environmental (local isolates highlighted in green; part A) and clinical isolates from ST10 together with International Clone 1 (ST1) and International Clone 2 (ST2) using CSI Phylogeny. Groups were highlighted based on the co-occurrence of ARGs. ARGs were determined using AMRFinderPlus. Phylogenetic tree was visualized using the iTOL v6 software.

3.2. Resistome of environmental *Acinetobacter* isolates

3.2.1. Antimicrobial Resistance Genes

The antimicrobial resistance profile of the *Acinetobacter* isolates demonstrated that most of them were phenotypically sensitive to the panel of tested antibiotics. This finding largely supports the sensitivity profile of community-acquired infection by *Acinetobacter* species (Dexter et al., 2015). A minority of these isolates (4/108=3.7%) were phenotypically resistant to various antibiotics, including sulfonamide, tetracycline and carbapenem (Figure 9). Whole genome sequencing revealed ARG ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-72}}$, *tet(B)*, *sul2*) that are known to confer resistance to these antibiotics. These ARG were identical in sequence to ARG found in clinical *A. baumannii*, suggesting that they might share common origins or reservoirs. One of the environmental soil isolates, *A. baumannii* K09-14, was carbapenem resistant and possessed $bla_{\text{OXA-72}}$ carbapenemase gene. Another soil isolate, *A. baumannii* E88-42, was resistant to trimethoprim-sulfonamide combination drugs with *sul2* and *tet(B)* resistance genes. Tetracycline and co-trimoxazole resistant *A. baumannii* S1-50, a water isolate, harboured *tet(B)* and *sul2* ARG. Another *Acinetobacter* spp. isolate S1-76 from the same water source as *A. baumannii* S1-50 also harboured *sul2*, a sulfonamide resistance gene. *A. towneri* isolates from sludge samples harboured *mph* and *msr(E)* macrolide resistance genes. All *A. baumannii* isolates had an intrinsic $bla_{\text{OXA-51-like}}$ intrinsic resistance gene. In addition, we discovered the presence of *A. baumannii* identical $bla_{\text{OXA-23-like}}$ genes in 3 environmental isolates of *A. radioresistens* that were phenotypically sensitive to the panel of tested antibiotics, suggesting that $bla_{\text{OXA-23-like}}$ may confer intrinsic resistance (e.g. to ampicillin) or play a metabolic, signalling or other novel biochemical role. The presence of the $bla_{\text{OXA-23-like}}$ gene in our *A. radioresistens* isolates supports the current understanding that $bla_{\text{OXA-23}}$ have evolved and mobilized from *A. radioresistens* (Poirel et al., 2008).

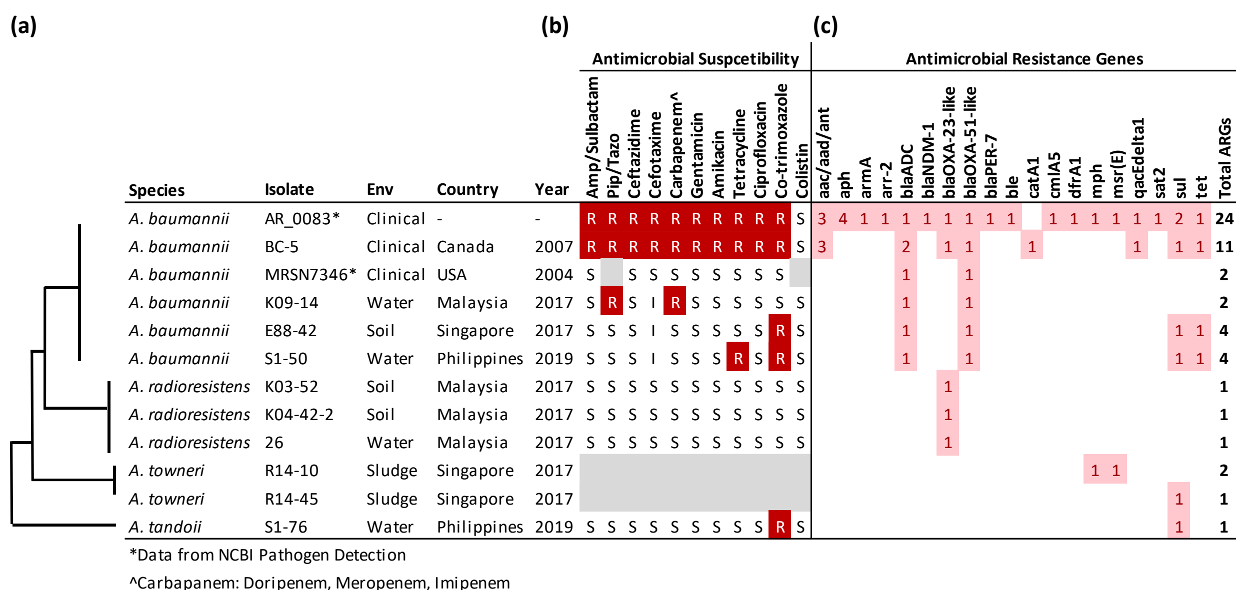

Figure 9. ARG of selected environmental *Acinetobacter* spp. in this study. Both environmental and clinical strains of *Acinetobacter* spp. (a) were arranged using molecular phylogenetic analysis of 16S rRNA gene by Maximum Likelihood method in MEGA7. (b) Antimicrobial resistance phenotypes and (c) ARGs were detected in environmental *Acinetobacter* isolates. AST phenotypes (susceptible: S, intermediate: I, resistant: R) and the count of ARG were plotted. Data that was not available was shaded in grey.

3.2.2. Nickel Metal Resistance Gene

All *A. baumannii* environmental isolates had *nreB* nickel resistance genes (Supplementary Table S2 and Supplementary Figure S1). However, interestingly, the isolates had different alleles of the *nreB* gene with different identity percentages, suggesting evolution and diversity in *nreB* that is currently not well documented for *A. baumannii*. Further analysis on 4062 *A. baumannii* genomes (which included environmental, animal and clinical isolates) in the NCBI Pathogen Detection database revealed that the *nreB* genes were present in these isolates. While *nreB* has been shown to mediate nickel efflux in *Achromobacter xylosoxidans* (Grass et al., 2001), there are limited studies describing nickel metal resistance in *A. baumannii*. However, it was unclear whether the *nreB* metal resistance gene (MRG) were located on the same genomic locus in *A. baumannii*. The presence of *nreB* at the same chromosomal loci throughout the genomes would suggest vertical transmission of this highly

conserved gene in *A. baumannii*. Given the presence of this ubiquitous gene in *A. baumannii* genomes, we hypothesise that *nreB* nickel metal resistance allele may play an important role in the environment or metabolism of *A. baumannii* and warrants further investigation, though it was not done in this study.

3.2.3. Diversity of the *bla*$_{OXA}$ gene

There was high diversity of the *bla*$_{OXA-51}$ family intrinsic beta-lactamase in the environmental *A. baumannii* strains sequenced. Out of 26 isolates, 17 variants of *bla*$_{OXA-51-like}$ were identified on the translational level, suggesting the ongoing evolution of the ARG. There were 3 isolates with novel variants that were not represented in the AMRFinderPlus database used. These genes were predicted to be functional, without any introduction of internal stop codons. In other *Acinetobacter* spp. sequenced, there were 8 novel variants of *bla*$_{OXA}$ that shared at least 94.8% similarity to known *bla*$_{OXA}$ in the database. This suggests the presence of novel diverse *bla*$_{OXA}$ alleles that could have a functional role in these environmental isolates.

3.2.4. Environmental isolates exhibit *bla*$_{OXA}$ variants known from international clonal lineage

One of the environmental *A. baumannii* isolates, strain K07-22, harboured *bla*$_{OXA-71}$, which is a variant of *bla*$_{OXA-51-like}$ intrinsic resistance gene. This gene variant has been described to be found in the International Clone 3 (IC3) and was also reported in a published study on avian isolates (Wilharm et al., 2017). The K07-22 isolate had novel MLST Pasteur and Oxford scheme STs that have not been reported before. The finding of *bla*$_{OXA-71}$ in a non-IC3 clone suggests that there may be convergent evolution of *bla*$_{OXA-51-like}$ genes, or could be an artefact due to recombination events. However, this is further complicated by having different DNA sequences encoding for the same OXA, and would need to be further examined by studying if they shared similar *bla*$_{OXA-51-like}$ DNA sequences between environmental and clinical isolates. That said, the role of *bla*$_{OXA-51-like}$ genes in the natural environment remains unclear. The diversity of

bla$_{OXA-51-like}$ variants in the environment could be studied further to determine if there would be more evidence of convergent evolution.

3.2.5. Characterisation of *bla*$_{OXA-72}$ encoding plasmid from environmental carbapenem resistant *A. baumannii*

As carbapenems are parenterally administered and not widely used in agriculture, we investigated the mechanism for carbapenem resistance in the environmental *A. baumannii* isolate K09-14 which was isolated in this study. The environmental CRAB harbouring a *bla*$_{OXA-72}$ encoding plasmid was isolated from a soil sample near Singapore in 2017. The isolate was resistant to carbapenems (imipenem, meropenem, doripenem) and a penicillin/beta-lactamase inhibitor combination (piperacillin/tazobactam) but sensitive to other tested antibiotics. The minimum inhibitory concentrations of imipenem and meropenem were ≥ 64 mg/mL. Whole genome sequencing using both short and long read sequencing revealed that A. baumannii K09-14 belonged to ST46 using the MLST Pasteur scheme and a novel ST2098 using the MLST Oxford scheme. Other ST46 isolates found in pubMLST database were isolated from sputum in Czech Republic and from blood in China (

Table 4). Using the Oxford MLST scheme, this isolate has a novel glucose dehydrogenase *gdhB* allele due to a single base substitution from ghb-59 allele 201A>T, resulting in R67S amino acid mutation. The novel allele has been assigned as gdhB-211 while the sequence type has been assigned as ST2098 by pubMLST.

Table 4. Antibiotics susceptibility profile of *A. baumannii* K09-14 with other known MLST-Pasteur ST46 isolates.

Isolate	Source, Country	Isolation Year	Antibiotics Susceptibility Profile													Reference
			Ampicillin/ Sulbactam	Piperacillin/ Tazobactam	Piperacillin	Ceftazidime	Cefotaxime	Doripenem	Imipenem	Meropenem	Gentamicin	Amikacin	Tetracycline	Ciprofloxacin	Trimethoprim/ Sulfamethoxazo	
K09-14	Soil, Malaysia	2017	S	R		S	I	R	R	R	S	S	S	S	S	This study
LUH 7852	Sputum, Czech Republic	1994	S		R	R			S		R	S	R		R	Diancourt et al., 2010
AB204	Blood, China	2015	Data not available													

Complete genome of A. baumannii K09-14

A. baumannii K09-14 hybrid assembly obtained from whole genome sequencing yielded one complete chromosome which is 3,972,439 bp in size with a GC content of 39.0%, and one plasmid, pK09-14, consisting of 7,791 bp with a GC content of 33.7%. The genome has 3,608 CDS, 58 pseudo genes, 6 copies of 16S-23S-5S rRNA operons, 72 tRNA genes and 4 ncRNA genes. Antimicrobial resistance genes, *ant(3")-IIa*, bla_{ADC-26}, $bla_{OXA-104}$ ($bla_{OXA-51-like}$) were found on its chromosome while bla_{OXA-72} ($bla_{OXA-40-like}$) was identified in the plasmid. The complete sequences of the chromosome and plasmid have been deposited in GenBank under accession numbers CP043953.1 and CP043954.1 respectively. As of time of writing on 14 January 2022, it has been currently selected as the representative genome of *A. baumannii* in the NCBI Genome database.

Carbapenem resistance is mediated by plasmid-encoded bla_{OXA-72}

Plasmid pK09-14 has 13 protein-coding genes, with 8 of the genes as encoded hypothetical proteins (Figure 10). The plasmid size of 7,791 bp was also confirmed using PCR. Through next-generation sequencing data, the plasmid was estimated to have 31 genome copies. The carbapenemase bla_{OXA-72} ($bla_{OXA-40-like}$) gene found on this plasmid was flanked by XerC-XerD recombination site (Figure 10). A Xre-RelE

toxin-antitoxin (TA) system and a ParA-like encoding partitioning system was found on the plasmid. The lack of IS*Aba1* upstream of *bla*$_{OXA-104}$ (*bla*$_{OXA-51-like}$) chromosomally encoded intrinsic resistance gene suggest that it is unlikely to be responsible for the carbapenem phenotype. Hence, *bla*$_{OXA-72}$ encoding plasmid is hypothesised to be responsible for the carbapenem resistance.

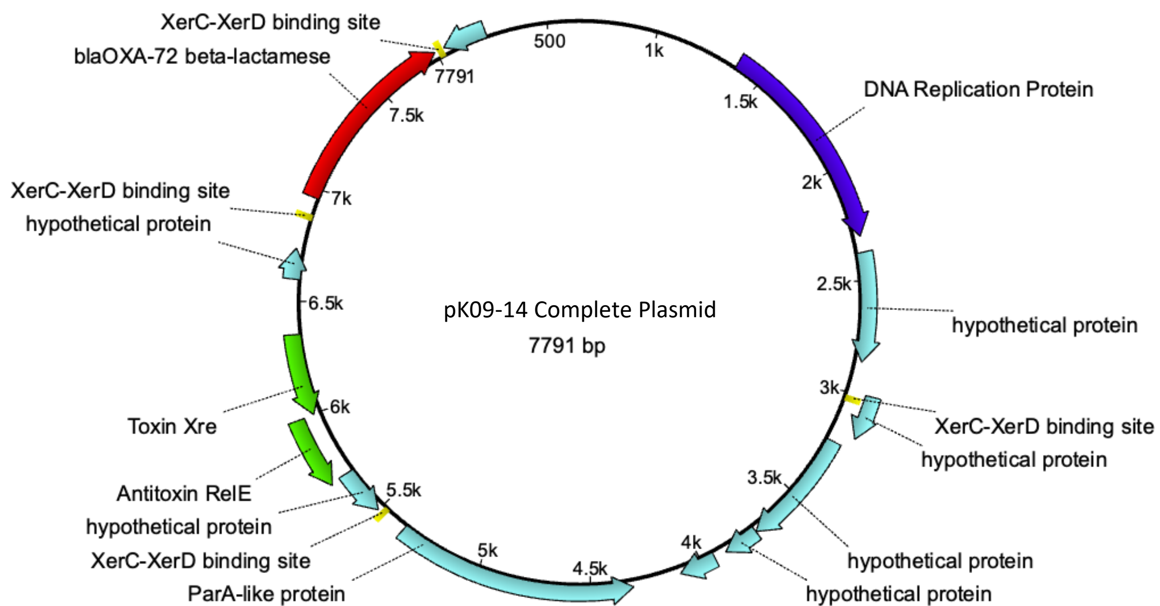

Figure 10. *bla*$_{OXA-72}$ carbapenemase gene found in environmental *A. baumannii* plasmid. Schematic annotation of *A. baumannii p*K09-14 complete plasmid (GenBank accession: CP043954.1) harbouring *bla*$_{OXA-72}$ beta-lactamase gene (annotated in red). The coloured arrows represent the gene CDS drawn to scale. Figure was generated using Unipro UGENE v39.0 software.

Plasmid pK09-14 may belong to a novel group as the *rep* gene was less than 74% identity against pAB1, which has been classified as GR17 (Bertini et al., 2010). Two plasmids from NCBI Database with the same *rep* gene were found to share 84% plasmid similarity to pK09-14 with 100% identity (Table 4). The plasmids were isolated from carbapenem-resistant *A. baumannii* of different sources. Plasmid pAbIHIT32296 was found on Pasteur scheme ST294 *A. baumannii* isolated from a choanal swab of a grey parrot presented to the veterinarian in Luxembourg (Prenger-Berninghoff et al., 2017), while plasmid pA52-OXA-72 was isolated from a community-acquired

pneumonia sputum sample in China (Jia et al., 2019). These plasmids shared the same replication *rep* gene, *blaOXA-72* flanked with XerC-XerD recombination sites, hypothetical proteins and parA-like partitioning protein as pK09-14 (Figure 11). Carbapenemase gene flanked by XerC-XerD binding site for recombinase may suggest potential for genome integration and mobility (Merino et al., 2010). The encoded Xre-RelE toxin-antitoxin (TA) system may be involved in plasmid maintenance and stability, allowing *bla*$_{OXA-72}$ to be maintained in the plasmid system without the need for antibiotics selection in the environment.

Table 5. List of closest matched plasmids to pK09-14 determined by replicase gene (*rep*) nucleotide identity.

Species	**Isolation Source, Country**	**Isolation Year**	***rep* match: plasmid (identity)**	**Plasmid Size (kb)**	**Plasmid similarity: Query cover (identity)**	**Resistance genes carried**
A. baumannii IHIT32296 (ST294)	Grey Parrot choanal swab, Luxembourg	2016	pAbIHIT32296 (100%)	8.4	84% (100%)	*bla*$_{OXA-72}$
A. baumannii A52 (ST77)	Sputum, China	2015	pA52-OXA-72 (100%)	8.4	84% (99%)	*bla*$_{OXA-72}$
A. baumannii MRSN15313 (ST79)	Cerebrospinal fluid, Brazil	2008	p597A-14.8 (100%)	14.8	70% (99%)	None
A. johnsonii XBB1	Sewage, China	2010	pXBB1-5 (97%)	13.9	23% (95%)	None
Acinetobacter spp. WCHA45	Sewage, China	2015	P2_010045 (96%)	7.2	25% (95%)	None
A. baumannii MDR-UNC	Wound, USA	2012	pAB120 (66%)	10.8	46% (95%)	*bla*$_{OXA-72}$
A. pittii XJ88	Sputum, China	2013	unnamed1 (-)	9.5	32% (93%)	*bla*$_{OXA-72}$

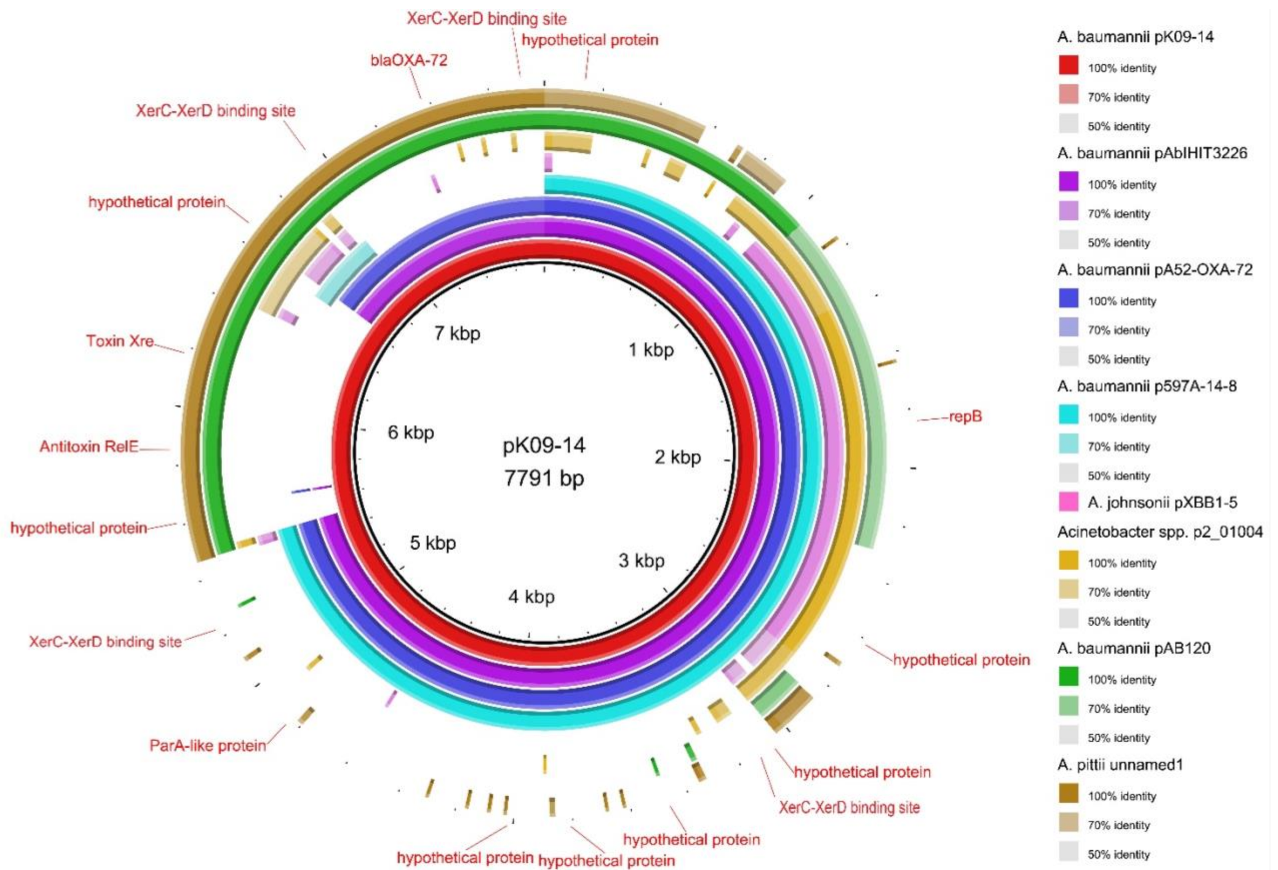

Figure 11. Plasmid pK09-14 shares similarity to other *Acinetobacter* plasmids. Comparison of plasmid sequences sharing similar *rep* gene or *bla*$_{OXA-72}$ gene to plasmid pK09-14 generated using BLAST Ring Image Generator (BRIG) from performing a BLASTN analysis. Rings correspond to each plasmid sequence input in the same order as Table 5 with pK09-14 in the innermost ring (in red).

According to data in NCBI Pathogen Detection database accessed 30 September 2019, *blaOXA-72* has been identified in *A. baumannii* since 2007 and has been now described in other parts of the world. *bla*$_{OXA-72}$ has also been found in other close relatives of *A. baumannii*, namely *A. calcoaceticus*, and *A. pittii* in Lebanon and Brazil. *bla*$_{OXA-72}$ was found on a small 10,498 bp plasmid, pIEC338SCox, from *A. pittii* isolated in the Amazon region of North Brazil (Brasiliense et al., 2019). Plasmid harbouring *bla*$_{OXA-72}$ was also isolated from a carbapenem-resistant *A. nosocomialis* strain ST410 in Taiwan (Chen et al., 2018a). In companion animals, *bla*$_{OXA-72}$ has been found *in A.* baumannii isolated from a dog in Serbia (Misic et al., 2018). The ARGs found in these plasmids were flanked with XerC-XerD recombinase sites, suggesting its potential transfer between plasmids and integration into chromosomes. The presence of *bla*$_{OXA-}$

$_{72}$ gene in different *Acinetobacter* species highlights that the gene could have been transmitted through horizontal gene transfer mechanisms. The genetic context of the *bla*$_{OXA-72}$ and TA system on the plasmid suggests that the plasmid has disseminated into different lineages of *A. baumannii*, as well as onto other members of Acb complex.

3.2.6. Characterisation of *sul2* and *tet(B)* gene in environmental *A. baumannii*

A. baumannii E88-42 was isolated from an urban road in Singapore in 2017 in our study. It was phenotypically resistant to sulfonamide, and sensitive to the panel of tested antibiotics. Whole genome sequencing using both short and long read sequencing revealed that the isolate belonged to ST150 and ST744 according to MLST Pasteur scheme and Oxford scheme respectively.

Complete genome of A. baumannii E88-42

A. baumannii E88-42 hybrid assembly obtained from whole genome sequencing yielded one complete chromosome which is 3,879,180 bp in size with a GC content of 39.0%, and three plasmids, pE88-42_1 (201,020 bp in size with a GC content of 39.1%), pE88-42_2 (39,011 bp in size with a GC content of 35.1%) and pE88-42_3 (8,965 bp with a GC content of 35.6%). The genome has 3,849 CDS, 6 copies of 16S-23S-5S rRNA operons, 74 tRNA genes and 1 tmRNA genes. Antimicrobial resistance genes, *ant(3")-IIa*, *bla*$_{ADC-163}$ *and bla*$_{OXA-121}$ (*bla*$_{OXA-51-like}$) were identified in the chromosome. Other ARGs, *sul2*, *tet(B)*, *aph(6)-Id*, *aph(3"-Ib)*, and MRG, *merD*, *merA*, *merT*, *merR,* were found in the large plasmid pE88-42_1.

The pE88-42_1 megaplasmid has 209 coding sequences, with 141 of the genes encoded as hypothetical proteins and 21 of the genes encoded as IS transposases. The plasmid had a top BLAST hit against *A. baumannii* strain MC1 plasmid pMC1.1 (NZ_MK531536.1) with 81% query cover and 99.9% identity, as well as against other *A. baumannii* and *A. seifertii* plasmids with 53-69% query cover. The metadata associated with plasmid pMC1.1 indicated that the 184,770 bp plasmid was isolated from Bolivia. Here, we have found an environmental megaplasmid pE88-42_1 harbouring ARG and mercury resistance operon in the non-hospital environment.

3.3. Isolation of *A. seifertii* and its pangenome

We have isolated *A. seifertii*, which is an emerging clinically significant species of the Acb complex, from an environmental soil sample collected near Singapore in 2017. At the point of analysis on 9 Dec 2020, there were 31 published *A. seifertii* genomes that were in scaffold and contig level deposited in NCBI Genome database. Hence short read and long read WGS were performed to obtain a hybrid assembly to complete the genome. Having a complete genome allowed us to understand the genomic backbone of *A. seifertii* and its intrinsic resistance genes.

The hybrid assembly of *A. seifertii* S21 revealed a completed chromosome (NZ_CP065820.1) of 4,070,688 bp with GC content of 38.4%. No plasmids were found in the genome. The genome has 3,650 CDS, 49 pseudo genes, 6 copies of 16S-23S-5S rRNA operons, 73 tRNA genes and 4 ncRNA genes. Antimicrobial resistance genes, *bla*$_{ADC}$, *adeD, adeE and nreB* were identified in the genome. No *bla*$_{OXA}$ family carbapenemase gene was found in the genome. CRISPR was not detected in the genome, and 1 questionable prophage was found. The isolate was phenotypically susceptible to the panel of tested antibiotics. The complete sequence of the chromosome has been deposited in GenBank under accession number CP065820.1.

3.3.1. Pangenome of *A. seifertii*

Given the limited studies on the genomics of *A. seifertii*, a preliminary analysis of *A. seifertii* pangenome was conducted to understand the number of core and accessory genes of *A. seifertii* genomes. Compared to our first analysis on *A. seifertii* genomes, there were 37 additional complete genomes published by a Taiwan study that was released on NCBI that we have included when we performed the pangenome analysis. All *A. seifertii* genomes from NCBI (n = 66, downloaded on 17 Dec 2020) were downloaded and included for pangenome analysis using Roary in GALAXY server.

Out of the 18,060 total genes in these genomes, we found 1,182 core genes present in at least 99% of the genomes, 723 soft core genes (found in 95-99% of genomes), 3,372 shell genes (found in 15-95% of genomes) and 12,783 cloud genes (found in 15% of the genomes) (Table 6). The increase in accessory genes with the higher number of genomes analysed in the rarefaction curve suggested that there is high diversity of accessory genes and possibly many more isolates that have yet to be

sequenced out there (Figure 12). There is an open pangenome that remains to be fully understood especially given that majority of the isolates analysed were obtained from Asia.

Table 6. Number of core and accessory genes in *A. seifertii* pangenome.

Category	Prevalence in strains	Number of genes
Core genes	(99% <= strains <= 100%)	1,182
Soft core genes	(95% <= strains < 99%)	723
Shell genes	(15% <= strains < 95%)	3,372
Cloud genes	(0% <= strains < 15%)	12,783
Total genes	**(0% <= strains <= 100%)**	**18,060**

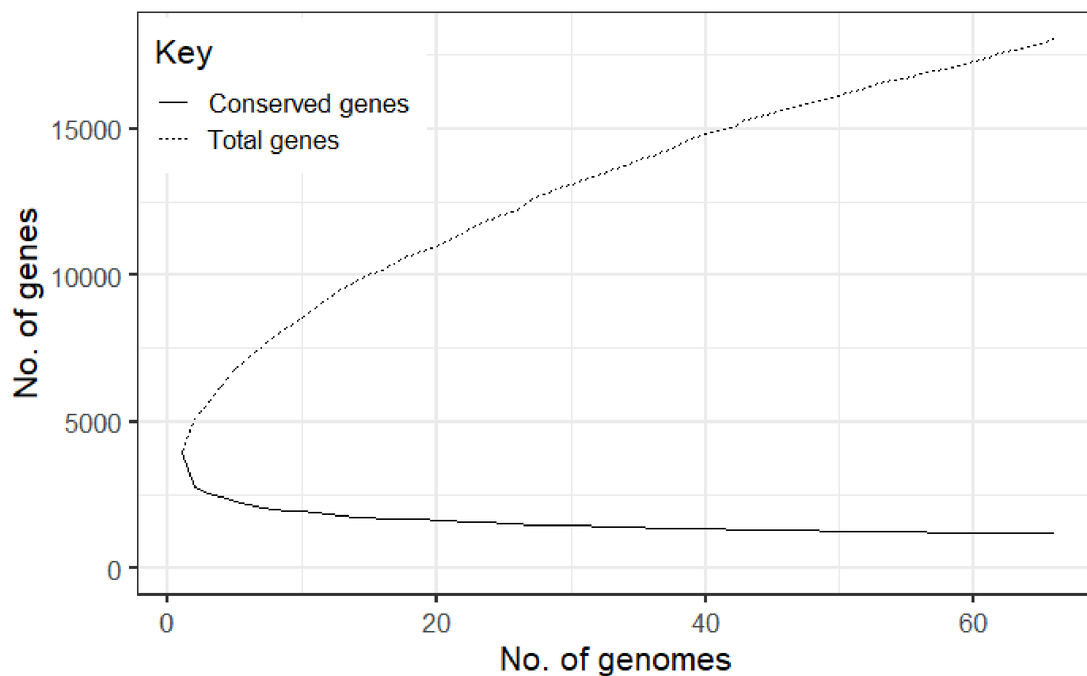

Figure 12. Accumulation curve for *A. seifertii* pangenome. The number of total genes (dotted line) and conserved genes (solid line) were plotted for *A. seifertii* pangenome. Increasing number of accessory genes observed with more genomes analysed.

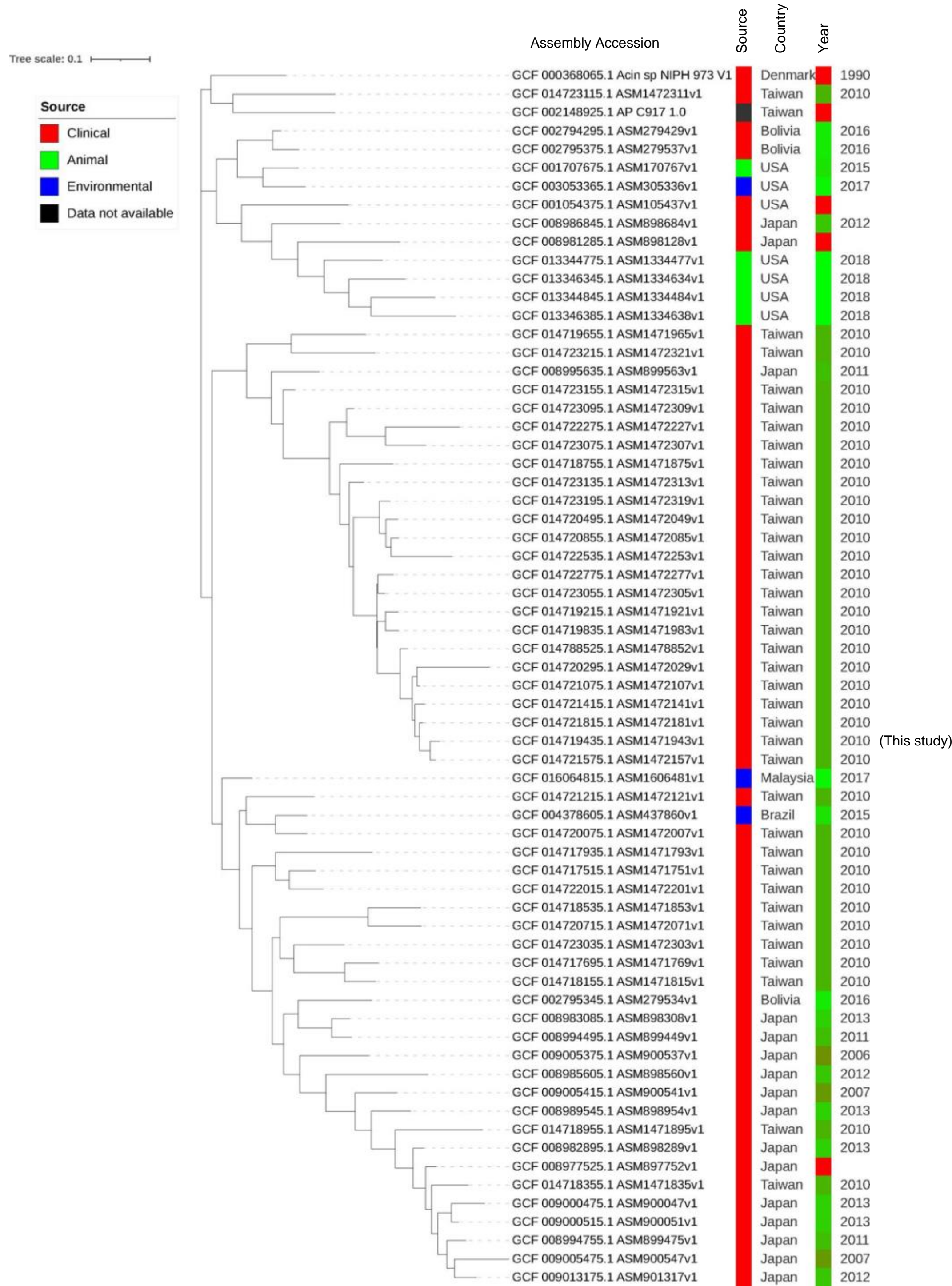

Figure 13. Pangenome phylogenetic tree of *A. seifertii*. Genomes assemblies were downloaded from NCBI (n = 66, downloaded on 17 Dec 2020) with *A. seifertii* S21 isolated from this study highlighted in yellow. The phylogenetic tree was created using the binary presence and absence of accessory genes determined using Roary pangenome analysis. Each assembly is plotted together with its metadata of source,

country and year of isolation obtained from NCBI PD database when available. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).

The pangenome phylogenetic tree revealed that the isolates largely clustered accordingly to their geography but there is also evidence of isolates from different parts of the world clustering together with Asian isolates (Figure 13). We observed that out of the 37 complete genomes of clinical *A. seifertii* from the Taiwan study, 36 genomes contained plasmids with an average number of 3.39 ± 1.54 per genome. The plasmids were of different sizes, ranging from 2,450 bp to 336,046 kb with a median of 66,818 bp in length. The GC% content of the plasmids ranged from 32.5% to 42.2% with a median of 38.6%. The different types of plasmids *in A. seifertii* and their genes may have contributed to the large accessory genome of this species.

3.3.2. Resistome of *A. seifertii*

Using NCBI Pathogen Detection and MicroBIGG-E database, there were a total of 58 *A. seifertii* genomes present in the Acb complex category (accessed on 30 December 2021). To understand the genetic context of the ARGs found, we further examined the 25 complete genomes (22 clinical, 1 environmental, 2 unknown sources) that were available in this dataset. In the earlier analysis, majority of the clinical *A. seifertii* isolates harboured plasmids that might have contributed to the accessory genome of the species. Here, we found that the presence and number of plasmids correlated with the number of ARGs in *A. seifertii* (Figure 14). Mercury resistance operon and other acquired ARG were found to be plasmid mediated on separate plasmids that were likely to be able to disseminate between species of Acb complex.

	ARG & MRG																														
Isolate	adeD	adeE	amvA	ant(2'')-Ia	ant(3'')-IIa	aph(3'')-Ib	aph(3')-VIa	aph(6)-Id	bla	blaADC	blaADC-133	blaCTX-M	blaOXA-82	gyrA_S81L	hdeD-GI	merA	merC	merD	merE	merP	merR	merT	nreB	sul2	tet(39)	tet(B)	trxLHR	yfdX2	Total ARG & MRG	Number of Plasmids	Country
PDT000847998.2	1	1	1						1	1													1						6	0	Taiwan
PDT000914431.1	1	1	1						1	1													1						6	0	Malaysia
PDT001014276.1	1	1	1						1	1													1						6	0	Chinay
PDT001078309.1	1	1	1						1	1													1						6	0	Germany
PDT000848010.2	1	1	1						1	1													1						6	1	Taiwan
PDT000848024.2	1	1	1						1	1													1						6	1	Taiwan
PDT000848030.2	1	1	1						1	1						1	1	1	1	1	1	1	1						13	1	Taiwan
PDT000848016.2	1	1	1						1	1													1						6	2	Taiwan
PDT000848025.2	1	1	1	1					1	1						1	1	1	1	1	1	1	1						14	2	Taiwan
PDT000848027.2	1	1	1						1	1			1			1	1	1	1	1	1	1	1						14	2	Taiwan
PDT000848029.2	1	1	1	1					1	1						1	1	1	1	1	1	1	1						14	2	Taiwan
PDT000848002.2	1	1	1							1						1	1	1	1	1	1	1	1						12	3	Taiwan
PDT000848006.2	1	1	1	1					1	1						1	1	1	1	1	1	1	1						14	3	Taiwan
PDT000848009.2	1	1	1	2					1	1						1	1	1	1	1	1	1	1						15	3	Taiwan
PDT000848020.2	1	1	1						1	1													1		1				7	3	Taiwan
PDT000848023.2	1	1	1	1					1	1			1			1	1	1	1	1	1	1	1						15	3	Taiwan
PDT000848032.2	1	1	1	1		1	1	1	1	1						1	1	1	1	1	1	1	1	1		1			19	3	Taiwan
PDT000850851.1	1	1	1			1	1	1	1	1						1	1	1	1	1	1	1	1	1		1			18	3	Taiwan
PDT000848017.2	1	1	1	1		1	1	1	1	1			1			1	1	1	1	1	1	1	1	1		1			20	4	Taiwan
PDT000848012.2	1	1	1	1		1	1	1	1	1						1	1	1	1	1	1	1	1	1		1			19	5	Taiwan
PDT000848015.2	1	1	1	1		1	1	1	1	1			1			1	1	1	1	1	1	1	1	1		1			20	5	Taiwan
PDT000848018.2	1	1	1	1		1	1	1	1	1			1			1	1	1	1	1	1	1	1	1		1			20	5	Taiwan
PDT000848019.2	1	1	1	1		1	1	1	1	1			1			1	1	1	1	1	1	1	1	1		1			20	5	Taiwan
PDT000848007.2	1	1	1	1		1	1	1	1	1			1			1	1	1	1	1	1	1	1	1		1			20	6	Taiwan
PDT000848014.2	1	1	1	1		1	1	1	1	1						1	1	1	1	1	1	1	1	1		1			19	6	Taiwan

Figure 14. Resistome of *A. seifertii* complete genomes. Number of ARG, MRG and plasmids of the complete genomes from NCBI PD, MicroBIGG-E and Plasmids databases were analysed. Each row represents the complete genome of an *A. seifertii* isolate with its ARG and MRG count.

Using clinical *A. seifertii* AS40 complete genome (GCA_014721815.1), we sought to understand the genetic context of its ARG. While the authors stated that the carbapenem resistance gene, *bla*$_{\text{OXA-82}}$ (*bla*$_{\text{OXA-51-like}}$) was found to be plasmid-mediated with IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ genetic structure (Li et al., 2020), the types of plasmids were not described. BLAST analysis of plasmid pAS40-3 (NZ_CP061643.1) that was 74,127 bp in size containing *bla*$_{\text{OXA-82}}$ against other *Acinetobacter* complete plasmid revealed that other similar plasmids were found in *A. baumannii* species with varying query cover of 79% to 96% and percentage identity of 98.8% to 99.9%. One such plasmid of interest was *A. baumannii* strain SGH0905 plasmid pS32-2 that was 75,987 bp in size and had 91% query cover and 99.3% similarity with plasmid pAS40-3. This large plasmid was isolated from a clinical *A. baumannii* isolate in Singapore. Though the plasmid itself did not harbour any ARG, we described other plasmids that share high similarity to it harbouring carbapenemase genes in Chapter 4.5.2 and Chapter 5.3.3.

Other acquired ARGs, *sul2*, *tet(B)*, *aph(3')-Via*, *aph(3'')-Ib* and *aph(6)-Id*, were found on plasmid pAS40-1 (NZ_CP061641.1) which was 134,105 in size. Other *A. seifertii* isolates also had identical plasmid encoding these resistance genes. Other similar plasmids were found in *A. baumannii* isolates with 77% to 92% query cover and 98.7% to 99.9% percentage identity. *A. seifertii* plasmid pAS40-1 had 82% query cover and 99.9% percentage identity against *A. baumannii* strain MC1 plasmid pMC1.1 (NZ_MK531536.1), which was a plasmid described earlier to be similar to an environmental megaplasmid pE88-42_1 that we have isolated in Chapter 3.2.6.

Mercury resistance genes, namely *merR*, *merT*, *merP*, *merC*, *merA*, *merD*, *merE*, were found to be in an operon context in plasmid pAS40-2 (NZ_CP061642.1) that is 106,923 bp in size. These were consistent with the other complete plasmids available in the clinical *A. seifertii* genomes where each large plasmid conferred different ARG and MRG to the isolates.

3.4. Sequencing of environmental *A. tandoii* host and phage

In the course of environmental sampling, we isolated *A. tandoii* strain W4-4-4 from coastal water off the shores of Singapore in 2017. Subsequently, *A. tandoii* W4-4-4 was used as the host bacteria for phage isolation. Antibiotics susceptibility testing by disk diffusion according to CLSI Standards revealed that the isolate was susceptible to the tested panel of antibiotics.

The genome was sequenced on Illumina HiSeq platform. A total of 550,624 sequencing reads using 150-bp paired-end sequencing were obtained. Read quality was assessed with FastQC version 0.72 (Galaxy). The genome was assembled *de novo* using Unicycler (Galaxy version 0.4.6.0), and 63 contigs (373-fold coverage depth, N_{50} of 312,393 bp) were obtained. The *A. tandoii* W-4-4-4 has a genome size of 3,575,445 bp with GC content of 40.2%. The draft assembly has 3,444 coding genes, 66 tRNAs and 3 rRNAs using the RASTtk pipeline (Brettin et al., 2015). It contains beta-lactamase that shared 92.6% nucleotide identity to $bla_{\text{OXA-664}}$, an intrinsic carbapenemase described for *A. tandoii* species (Tian et al., 2018). No intact or questionable CRISPR array was found. Three candidate prophages were predicted, with one intact (34.5 kb, 38 CDS), one questionable (22.9 kb, 32 CDS) and one incomplete (18.8 kb, 29 CDS) prophage. The intact prophage region had a GC content of 40.5% with 28 phage related proteins and 10 hypothetical proteins. It shared 39.5% homolog with the closest hit to PHAGE_Pseudo_phiCTX (GenBank accession: NC_003278).

The lytic *Acinetobacter* phage Φ-S1 was isolated from another water sample using *A. tandoii* W4-4-4, from the same environmental coastal site. Phage Φ-S1 was amplified, and the DNA extracted using QIAGEN DNA Mini Kit. The draft genome of phage Φ-S1 consists of circular double-stranded DNA of 75,793 bp with a GC content of 39.5% (3,131X coverage depth). The genome was predicted to have 99 CDS and 2 tRNA. Of all the CDS, 87 (87.8%) are hypothetical proteins.

Phage Φ-S1 encodes two N4-like RNA polymerases, namely a virion-associated RNA polymerase vRNAP and RNA polymerase II, as well as a single-stranded DNA-binding protein (SSB), which suggests a possible genome injection mechanism as phage N4 (Choi et al., 2008). Other replication genes present include DNA polymerase I, DNA helicase, DNA primase, ATPase, ribonucleotide reductase, thymidylate synthetase,

ATPase. It also encodes prohead core protein protease, lytic tail fiber, putative baseplate hub subunit and tail lysozyme, N4 gp16-like terminase, N4 gp44-like, N4 gp68-like, and N4 gp69-like proteins.

The closest relative of phage Φ-S1 is *Acinetobacter* phage Presley (NC_023581.1), sharing 26 homologs. *Acinetobacter* phage Presley has been described to be an N4-like podophage, belonging to the *Caudovirales*, *Podoviridae* family (Farmer et al., 2013). It was isolated from a sewage sample collected in College Station, Texas, United States, against *A. baumannii*, and has been proposed to share the same N4-like genome injection and replication mechanism.

This whole-genome shotgun project has been deposited in NCBI under BioProject number PRJNA474898 and accession number QKRJ00000000. The raw reads are available in the Sequence Read Archive (SRA) under accession number SRR8187417.

3.5. Novel *Acinetobacter* species isolated from water sources

Novel *Acinetobacter* species were isolated from in the course of environmental sampling of water specimens. These isolates yielded low confidence score for species identification using MALDI-TOF MS BioTyper. Full-length 16S sequencing was conducted before whole genome sequencing was done for further investigation. Genome-based approaches confirmed that these isolates were novel species. Notably, one of the isolate *Acinetobacter* spp. S1-76 harboured resistance to co-trimoxazole.

3.5.1. Characterisation of co-trimoxazole resistant *Acinetobacter* spp. S1-76

Co-trimoxazole resistant *Acinetobacter* spp. S1-76 was isolated in 2019 from a water sample in Davao, Philippines, in our study. Preliminary phenotypic characterisation revealed that it was resistant to co-trimoxazole antibiotics. The isolate was oxidase-negative and catalase-negative. Sole carbon sources assimilated include capric acid, malic acid, trisodium citrate and phenylacetic acid. No growth occurred on D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate and adipic acid. Cells were negative for indole production, fermentation of D-glucose, urease and beta-galactosidase activity. Esculin and gelatin were not hydrolysed. The best match profile from MALDI-TOF MS identification was against *A. tandoii* DSM 14970T HAM with a score value of 1.91 followed by *A. johnsonii* DSM 6963T HAM with a score value of 1.84. The match profile scores were between 1.7 and 2.0 which suggest the identification is only able to discriminate at the genus level.

The draft genome assembly of *Acinetobacter* spp. S1-76 contained 329 contigs (N50: 20,269 bp) with a total length of 3,663,906 bp and GC content of 42.2%. It harboured *aph(3'')-Ib*, *aph(6)-Id* and *sul2* antimicrobial resistance genes. *Acinetobacter* spp. S1-76 had 84% average nucleotide identity (ANI) similarity to *A. tandoii* DSM 14970 (CIP 107469) genome when calculated using ANIb in the JSpeciesWS server (Richter et al., 2015). The similarity cut-off for ANI is 95% for genomes of the same species (Kim et al., 2014). Tetra Correlation Search (TCS) revealed that *A. tandoii* was one of the closest matches with z-score of 0.971, which was below the cut-off of 0.989. Digital DNA-DNA hybridisation (dDDH, d_4) showed 29.5% similarity to *A. tandoii* which was less than the recommended cut-off of 70% for DDH analysis of same species (Chun et al., 2018).

The full length 16S rRNA gene of 1,529 bp shared 99.1% similarity to LBNL_s hit taxon name, strain SC36 in the EzBioCloud database. Isolate SC36 was isolated from a Mangrove Wetland Ecosystem in China in 2013 and was identified as *Acinetobacter tandoii*. SC36 isolate was studied for its strong phosphorus accumulating ability and potential for application in the removal phosphorus in the environment. Though it had a lower ANI values of 85.6% and 85.6% when compared with published *A. tandoii* genomes (DSM 14970 and KCTC 12417), the authors noted that it could be a result of a different genomospecies (Zhang et al., 2019). Both S1-76 and SC36 isolates have been described to be isolated from environmental water sources. Comparing between S1-76 and SC36, both isolates had an ANI value of 95.6% to 95.9%, suggesting that they were likely to be the same genomospecies. The GC content of these two isolates were 2% higher than known *A. tandoii* genomes in NCBI database. Genomes belonging to the same species should have less than 1% different in GC content (Meier-Kolthoff et al., 2014). Put together, these data suggest that these isolates were unlikely *A. tandoii* species.

Our analysis suggests that S1-76 is a novel *Acinetobacter* spp. that is resistant to co-trimoxazole. We propose that based on the established species delineation standards, S1-76 and SC36 are likely to be same genomospecies that is closely related to *A. tandoii*.

3.5.2. Characterisation of *Acinetobacter* spp. BD4-125

Acinetobacter spp. BD4-125 was isolated in 2019 from a freshwater quarry in Singapore. Preliminary phenotypic characterisation revealed that the cells were oxidase-negative and catalase-negative. Source carbon sources assimilated include capric acid and malic acid. No growth occurred on D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, adipic acid, trisodium citrate and phenylacetic acid. Cells were negative for indole production, fermentation of D-glucose, urease and beta-galactosidase activity. Esculin and gelatin were not hydrolysed. Species identification using MALDI-TOF MS did not yield reliable identification, prompting further analysis using 16S sequencing. The 16S rRNA gene was amplified using 27F and 1492R primer set and sequenced by Sanger sequencing to obtain a read of 1,385 bp that was searched against EzBioCloud 16S database.

However, there was no identification that have a value greater than the cut-off of 98.65%, suggesting that it could be a novel species of *Acinetobacter*.

The draft genome assembly of *Acinetobacter* spp. BD4-125 contains 155 contigs (N50: 45,022 bp) with a total length of 3,287,988 bp and GC content of 41.4%. BD4-125 had 83.1% ANI similarity to *A. brisouii* CIP 110357 genome and 82.8% similarity to *Acinetobacter sp.* ANC 3789 genome when calculated using ANIb in the JspeciesWS server (Richter et al., 2015). Both similarity cut-off were below the cut-off value of 95% for ANI analysis (Kim et al., 2014). Tetra Correlation Search (TCS) revealed that *Acinetobacter sp.* ANC 3789 was one of the closest matches with z-score of 0.974, which is below the cut-off of 0.989. Digital DNA-DNA hybridisation (dDDH, d_4) showed 27.3% similarity to *A. brisouii* CIP 110357 which was less than the recommended cut-off of 70% for DDH analysis of same species (Chun et al., 2018).

Alignment search of the 16S rRNA gene was conducted using BLAST against NCBI non-redundant nucleotide database to obtain it shared 99.3% similarity to Uncultured *Acinetobacter sp.* Clone 12L_68 16S ribosomal RNA gene, partial sequence (GenBank accession number: KP183059.1). This entry was submitted in March 2015 into NCBI GenBank database under the working title of “Malachite green-degrading uncultured bacterial diversity of surface seawater specimen of South China Sea”. It was probably isolated from an aquatic environment. No further information or publication was available for this isolate. The second hit was against *A. calcoaceticus* strain MnW2201009 16S ribosomal RNA gene, partial sequence (GenBank accession number: MG011596.1) with 99.2% similarity by BLAST. It was deposited in November 2017 under the working title of “Innate immunity in the skins of *Odorrana grahami* at three sites”. The metadata suggested that it could be isolated from frog skin potentially from a highland environment in China at 1,792 m altitude. However, it was possible that this entry of a partial sequence has been mislabelled as *A. calcoaceticus* during submission. The other BLAST hits were well below the cut-off value of 98.7% for 16S species identification.

Our analysis suggested that BD4-125 is a novel *Acinetobacter* spp. based on the established species delineation standards. The 16S analysis suggested that it is probably related to other uncultured *Acinetobacter* isolates which may be studied using 16S metagenomics and could be related to an aquatic environment.

3.6. Discussion

3.6.1. Prevalence and diversity of environmental *A. baumannii*

We have successfully isolated diverse *A. baumannii* ST from the urban road environment. This environment is understudied as majority of environmental studies consider pristine soil to be the standard environment. Conventional soil sampling studies have attempted to isolate *A. baumannii* from the natural soil environment. We had a low isolation rate of 19.5% from soil, while some other groups have reported a higher isolation rate. Diverse *A. baumannii* isolates with novel STs have been isolated from soil (8/8, 100%) associated with various crops in Brazil. Beta-lactamases bla_{SHV} and bla_{GES} were found in these isolates (Furlan et al., 2018). In an Iraq study, *A. baumannii* isolated from soil samples (22/50, 44%) were found to be generally sensitive to antibiotics (Hassan and Khider, 2020). The study further demonstrated that environmental isolates were be able to produce stronger biofilms when compared to local clinical isolates. The environmental isolates in our study were diverse, with different ST detected, some of which have been previously reported in clinical isolates.

3.6.2. Urban environment as a reservoir for *A. baumannii*

Given the close proximity of human interaction and rapid development of the urban environment, we hypothesised that the urban roads could play a role as an environmental reservoir of clinically significant bacterial species. It might be possible for the bacteria in this environment to be aerosolised in wetter seasons by the rain (Joung et al., 2017). These urban road samples were collected from sediments on the road surfaces representing the built environment as opposed to the pristine soil environment from nature parks.

The urban road can be argued as an abiotic surface due to its high surface temperature and exposure to unpredictable elements of weather. However, *A. baumannii* have been described to be able to survive and persist in harsh environments, a survival advantage due to its large metabolic ability. For example, it can grow at a higher temperature of 44°C, which we have also used to selectively isolate *A. baumannii*. While petroleum hydrocarbons and organic compounds have been found on the urban roads (Liu et al., 2019a), it is unclear whether *A. baumannii*

can thrive on the roads by metabolising these hydrocarbons and organic compounds, although *A. baumannii* has been previously isolated from petroleum hydrocarbon contaminated soil obtained from different oil exploration sites and oil refineries (Sarma et al., 2004). There have also been reports of its ability to metabolise complex hydrocarbons, which has been explored as a possible microorganism for bioremediation uses (Mishra et al., 2004; Oanh et al., 2020). Furthermore, various *Acinetobacter* species, including Acb complex, have been cultured and isolated from the soil and inanimate environments which come into human contacts frequently (Choi et al., 2012). Using metagenomics approach, *Acinetobacter* genus, particularly *A. radioresistens*, *A. nosocomialis* and *A. lwoffii*, were found in high prevalence on inanimate surfaces in US subway stations (Afshinnekoo et al., 2015).

3.6.3. ST10 in Urban Environment

To the best of our knowledge, this is the first report of Pasteur ST10 *A. baumannii* isolated from the non-hospital setting. In a recent study by Meumann et al (2019), ST10 has been reported as the main ST for most of the community-acquired infections in Australia, with other diverse sequence types forming the rest. In our study, we have isolated ST10 isolates from the urban aquatic environment. The urban canal network likely contains seepage of urban water sources or runoff from the soil and roads. This environment could be a proxy to understand the urban environments that we live in and interact with.

Another potential reservoir for *A. baumannii* is animals. We have found *A. baumannii* ST10 that was isolated from dogs, suggesting that this ST is capable of causing infections in animals. The hypothesis of ST10 originating as an environmental isolate suggests that it has been circulating in the natural environment prior to infecting humans, subsequently seeping into environments that can be sampled, such as the urban water runoffs. The other possibility would be ST10 is a commensal on humans, though there are limited studies exploring the colonization of *A. baumannii* in healthy humans.

While we have shown that our environmental ST10 isolates were phylogenetically similar to local clinical ST10 isolates, we also found that there were slight disparities

between the isolates. For instance, the *gpi* allele of the MLST Oxford scheme differed amongst these isolates, suggesting that there could be other genomic recombinations and differences between the isolates (Gaiarsa et al., 2019). It also demonstrated that multi-locus sequence typing (MLST) is a coarse typing tool and may not be representative of the subclades or strain-specific associations. In this work, we have used Pasteur scheme ST10 to understand the possible context of community-acquired *A. baumannii*. We observed that the Oxford Scheme ST can further differentiate and discriminate between different isolates amongst the Pasteur Scheme ST10 clade. The combinatorial use of both MLST Pasteur and Oxford schemes, together with capsular typing OCL and KL has been proposed recently as a quick typing index presented as $ST_{Pasteur}$:ST_{Oxford}:KL:OCL (Hamidian et al., 2021). This is akin to an expanded MLST with more gene loci considered by including the typing of the capsule and outer polysaccharide loci. SNP-based phylogenetics tools would provide a more superior and discriminatory whole genome typing compared to these gene-based approaches (Maiden et al., 2013; Uelze et al., 2020). Therefore, it is important to utilise whole genome sequencing data to provide a higher resolution and deeper understanding on the genomic relatedness between isolates. These would be more discriminatory than MLST, which only involves the use of seven housekeeping genes.

3.6.4. Resistome of environmental *A. baumannii*

In our study, we have found that environmental *A. baumannii* were largely sensitive to the panel of tested antibiotics. This is consistent with previous studies isolating *A. baumannii* from the environment, which reported that non-hospital associated and community-acquired isolates were generally susceptible (Eveillard et al., 2013). The absence of abundant ARG in non-clinical isolates would suggest that these ARG seemed to be limited to the circulating clones in the hospital environment. In addition to this, we have found environmental *A. baumannii* isolates that harboured different acquired ARG from plasmids in the soil, such as carbapenem resistance.

To our understanding, this is the first known report of carbapenem resistance in non-hospital environmental *A. baumannii* not from human or animal source. This K09-14 isolate had a novel Oxford MLST profile, supporting that there is a high diversity of *A. baumannii* ST in the environment. The carbapenem resistance was mediated by a

unique *bla*$_{OXA-72}$ carrying small plasmid. Clinically, the carbapenem class of antibiotics are parenterally administered (with one exception that is available orally, Tebipenem) and it was very unlikely that the soil sample contained carbapenems because the site was far from human habitation. Hence we hypothesise that the acquisition of carbapenem resistance could be a form of defence against naturally occurring beta-lactam antibiotics in the soil environment. The present carbapenems have been developed and derived from a model compound of a naturally occurring beta-lactam antibiotics, thienamycin, from *Streptomyces cattleya* (Papp-Wallace et al., 2011). The presence of *bla*$_{ADC}$ and *bla*$_{OXA-51}$ intrinsic resistance genes in *A. baumannii* would confer evolutionary advantages in the form of resistance to beta-lactams as well as first and second-generation cephalosporins. In addition, the *bla*$_{OXA-72}$ encoding plasmid shared similarities to other plasmids isolated from clinical samples around the world (Figure 11). These small plasmids contained genes encoding for partitioning protein, toxin-antitoxin and replicase genes. We propose that plasmid-mediated transfer of antimicrobial resistance genes together with toxin-antitoxin plasmid maintenance genes may result in successful persistence of the plasmid in the natural environment, ultimately aiding in its geographical dissemination. This supports our hypothesis that antimicrobial resistance genes could be actively circulating and horizontally transferred amongst bacterial populations in the natural environment, increasing the risk of drug-resistant community-acquired infections while threatening the efforts to prevent antimicrobial resistance.

We found more evidence that environmental *A. baumannii* can harbour different plasmids which shared similarities with known plasmids from clinical strains of *A. baumannii*. For instance, we found plasmid pN3-45_1 from an environmental ST10 isolate that was similar to a plasmid found in another ST10 clinical isolate. These plasmids might have been passed down vertically by the host bacteria. ARG against sulfonamide and tetracycline were found on a megaplasmid in *A. baumannii* E88-42, an environmental isolate from Singapore. The presence of a megaplasmid in our environmental isolate could have conferred an evolutionary advantage and lower fitness cost to the isolate for its ecological niche (Ghaly et al., 2020). ARG could have hitchhiked or play other functions in these niches where there might be lower antibiotics selection pressure. The megaplasmid also harboured metal resistance against mercury which could be present in the terrestrial environment as a pollutant or

as a result of mercury-based compounds (e.g. pesticide, herbicides, disinfectants) use (Boyd and Barkay, 2012; Gworek et al., 2020). Metals have also been added to animal feeds as growth promoters and prophylaxis which may select for MRG (Zhu et al., 2013). More importantly, these findings suggest that megaplasmids could be evolving and circulating among non-clinical strains. The environment could be a reservoir for plasmid exchange and evolution.

3.6.5. Pangenome and resistome of *A. seifertii*

A. seifertii has a large open pangenome with plasmids contributing to the accessory gene pool. Currently, the genomes of *A. seifertii* were limited to mostly Taiwan, Japan and US. We expect that the pangenome will continue to increase with more *A. seifertii* identified from different geographical regions and environments. We initially encountered challenges when identifying environmental *A. seifertii* isolates, possibly due to its close relatedness with its other Acb complex members. It was formerly known as *Acinetobacter* genomic species ‘close to 13TU’ and was later renamed in 2015 as *A. seifertii* (Nemec et al., 2015). *Acinetobacter* genomic species 13TU has also been named as *Acinetobacter nosocomialis* (Nemec, 2011). The rapid identification of Acb complex species would require faster and more accurate species typing tools such as MALDI-TOF MS or confirmatory qPCR (Buchan and Ledeboer, 2014). Given the clinical relevance of Acb complex and its potential to acquire drug resistance genes, it is important to expand the studies to include members of Acb complex. To the best of our knowledge, there is no published and sequenced clinical *A. seifertii* isolate from Singapore. Earlier literatures could have identified this organism as *Acinetobacter* genomic species ‘close to 13TU’.

In this study, the environmental *A. seifertii* isolated was susceptible to the panel of tested antibiotics. No acquired resistance genes were detected in its genome. It harboured similar *bla*$_{\text{ADC}}$ and *nreB* intrinsic resistance genes as *A. baumannii*. However, unlike *A. baumannii*, there is no intrinsic *bla*$_{\text{OXA}}$ gene in *A. seifertii* genomes. Hence, acquired carbapenem resistance in *A. seifertii* is likely plasmid-mediated. We have not observed any mobilisation or integration of ARG into *A. seifertii* chromosomes yet. In a study examining clinical *A. seifertii* isolates collected over 8-

year period, the authors found plasmid-borne IS*Aba1*-*bla*$_{OXA-51-like}$ genetic structure for carbapenem resistance (Li et al., 2020). We observed that all except one of the 37 complete genomes in *A. seifertii* from this study had plasmids. These plasmids tend to be large and could co-exist with each other to harbour different sets of ARG and MRG. Acquired beta-lactamase resistance by *bla*$_{OXA-58}$ have been found in *A. seifertii* isolated from old clinical samples back in 1993 and 1997, as well as in black swan samples in Brazil (Cayô et al., 2016; Narciso et al., 2017). This presents a risk of ARG transfer between *A. seifertii* and *A. baumannii*.

Being a close relative of *A. baumannii*, *A. seifertii* could play a potential role as a reservoir for the dissemination of carbapenem resistance. Multi-drug resistance has been reported in *A. seifertii* SAb133 isolated from environmental soil in Brazil (Furlan et al., 2019). Notably, this isolate had *bla*$_{TEM}$, efflux pumps and various metal resistance genes including those against copper, arsenic, magnesium, cobalt, zinc, cadmium, and tellurium. While the study did not manage to discern if these ARG and MRG were plasmid-mediated, we postulate that this was likely the case. We have observed plasmids conferring resistance in the complete *A. seifertii* genomes analysed. The prevalence of *A. seifertii* in the natural environment points to its possible source for CAI in humans, and clinical laboratories should be alert in detecting these non-baumannii *Acinetobacters* in their samples. Future studies should seek to isolate Acb complex species to understand its resistome and clinical significance.

A. seifertii could be a good potential model to study non-clonal *Acinetobacter* infection. The ARG gene pool of *A. seifertii* is shared with Acb complex and could be laterally transferred into *A. baumannii*. Given the increasing trend of *A. seifertii* being responsible for clinical infections, the species might recapitulate the introduction of *A. baumannii* into clinical settings. It is an emerging pathogen with a population structure that seemed to be similar to community acquired *A. baumannii*. The pangenome analysis of *A. seifertii* revealed a large diversity of accessory gene pool despite majority being clinical isolates, and a bulk of it from the same geographical location. This highlights that there would likely be more accessory genes identified with more *A. seifertii* being sequenced. With more data on *A. seifertii*, it could potentially recapitulate the emergence of clonal lineages that may eventually be circulating in the clinical settings.

3.6.6. Draft genomes of *A. tandoii* and phage

Here, we have isolated *A. tandoii* from a local aquatic environment and presented the genomic sequence of the *A. tandoii* bacterial host and phage that were both isolated from the same environment at a different time. A preliminary analysis was conducted on the environmental A. *tandoii* though the aims of this thesis did not initially set out to perform an in-depth study of other non-*A. baumannii* species. *A. tandoii* has been known to be an environmental isolate and was first described to be isolated from activated sludge in Australia in 2003 (Carr et al., 2003). It has also been isolated from the gut of termites and later shown to have phenol degradation properties (Van Dexter and Boopathy, 2018).

The sequenced phage was similar to a N4-like podophage isolated from wastewater which targets *Acinetobacter*. Bacteriophages could be a potential alternative therapeutic or biocontrol agent against *A. baumannii* (Chen et al., 2017). Phage therapy has been explored for inactivating pathogenic bacteria and lowering environmental risks in the soil environment (Ye et al., 2019). Apart from the bacteriophages, the endolysins that the phages encode have been of recent interest for their antibacterial properties and applications in lysin-based therapies (Gondil and Chhibber, 2021). The endolysin produced by bacteriophage p54 isolated from a hospital water sample has shown promising antimicrobial properties against MDR *A. baumannii* and a few other clinically relevant Gram-negative bacteria (Khan et al., 2021). This has led to an interest in isolating and characterising bacteriophages against MDR *A. baumannii* for potential use as phage therapy (Gordillo Altamirano et al., 2021; Hua et al., 2018). This host-environment relationship can be considered for future study designs in attempt to screen for host and phages from the same environment. Alternatively, a host bacteria of clinical origins could be used for the screening process.

Another aspect on the role of environment phages in AMR is whether these phages could be a reservoir for ARG or mediate the transmission of ARG (Calero-Caceres et al., 2019). Phages have been suggested to carry ARG in several studies (Jebri et al., 2021; Strange et al., 2021). On the other hand, ARG was rare in phage genomes but tenfold higher in prophages (Enault et al., 2017; Kleinheinz et al., 2014). One possible explanation was that the ARG could have been mobilised by an MGE into the

prophage, causing the prophage to be inactivated and unable to contribute itself into the phage reservoir (Enault et al., 2017). Temperate phages have been understood to be able to mobilise and carry AMR genes through lateral transduction (Hall, 2021). Lateral transduction by phages has been an emerging concept to understand the evolution of bacterial genome (Chen et al., 2018b; Hall, 2021; Humphrey et al., 2021). Further studies on host-phage relationship would be beneficial to determine the extent of lateral transduction in *A. baumannii*. Generally, the role of phages in the natural environment remains to be further studied to better understand how they contribute to AMR as well as bacterial genome adaption and evolution.

3.6.7. Novel *Acinetobacter* species from aquatic environment

Novel *Acinetobacter* spp. had been isolated as part of this environmental study. These isolates were examined further when they did not yield confident score for MALDI-TOF MS BioTyper species identification. Whole genome sequencing allowed us to identify two novel *Acinetobacter species* from the water environment. The natural environment is an untapped reservoir with novel species that have yet to be characterized. Novel species with tentative taxonomic identification are commonly described in metagenomics studies (Chen et al., 2021; Feng et al., 2021). More recently, the concept of culturomics emerged where different culture conditions were explored in an attempt to culture novel species. These often require complex substrates or unique growth conditions (Lagier et al., 2012; Lagier et al., 2018). Here we managed to isolate and characterise these novel species which could grow on commonly available culture mediums. This has greatly eased the characterisation of these novel isolates.

In terms of resistome, these novel *Acinetobacter* spp. can potentially harbour clinically relevant ARG. In *Acinetobacter tandoii*-like S1-76, we found that novel species isolate could be capable of harbouring clinically relevant ARG, such as *aph(3'')-Ib*, *aph(6)-Id* and *sul2*. Further long read sequencing would be required to distinguish if these ARG were plasmid-mediated. This presents a risk of ARG circulating and evolving in novel *Acinetobacter* spp. reservoirs.

Currently the two novel water isolates were singletons in our study though genomic matches in the public databases were found. We suspect that *Acinetobacter tandoii*-

like S1-76 together with another previously reported isolate belonged to another species that is different from *A. tandoii.* The other novel isolate *Acinetobacter* spp. BD4-125 matched an uncultured 16S sequence from a metagenomics sample from an aquatic environment. Public databases could be a useful genomics resource to aid in the investigations of novel isolates if there were accurate and informative metadata accompanying the sequence submission. Bacterial taxonomy may be challenging but genomics tools have greatly empowered species identification. The taxonomy naming may continue to shift and evolve as more genomes are available for the interpretation of their population structure.

3.6.8. Non-hospital environment as an untapped reservoir

There is a lack of systematic studies assessing the non-hospital environmental reservoir of the urban and pristine natural environment. The study of these extra-hospital settings could provide insights on the prevalence and resistome of *A. baumannii* to shed light on the origins of community-acquired infections and the role of the urban environment (Eveillard et al., 2013). There could be unique ecological niches between the different environments as shared STs were not observed between our collection of environmental isolates. The diverse ST and presence of ARG harbouring novel *Acinetobacter* spp. indicate the non-hospital environment as an untapped reservoir for the study of *Acinetobacter* species. These studies can be further broken down to identify the various aspects of bacterial hosts, plasmids and genes (e.g. resistome). There is a need for diverse and non-clonal strains to be sequenced for comparative genomics studies. These isolates can be used as outgroups to better understand the genomics backbone of these susceptible isolates. The study of environmental *A. baumannii* isolates obtained from pre-antibiotic era have provided clues on the genomic diversity and virulence potential of this species (Repizo et al., 2020; Repizo et al., 2017). Similarly, having additional environmental isolates could provide the genetic context to understand genomic evolution, adaption and assess how acquired resistance may have evolved or arised.

3.7. Conclusion

In this chapter, we have isolated *A. baumannii* from the soil, water and urban environment. Clinically relevant ST10 isolates have been found, providing a fresh perspective on the possibility of urban environment as a source for community-acquired infections. Acquired resistance against carbapenem, sulfonamide and tetracycline were observed to be plasmid-mediated. These plasmids appear to be capable of inter-species dissemination with similar plasmids being reported around the world. Plasmids also contribute to the pangenome and acquired resistance of *A. seifertii*, a species that could potentially be studied as a model organism to understand emerging clones in the future. Other *Acinetobacter* species outside of Acb complex can harbour clinically relevant ARG and should be further studied to understand the complete resistome of *Acinetobacter*. The non-hospital environment may be a potential reservoir for clinically relevant *Acinetobacter* species and their ARG at large.

Chapter 4. Clinical *Acinetobacter calcoaceticus-baumannii* complex

Having examined environmental *Acinetobacter* isolates in <u>Chapter 3</u>, we sought to understand the resistome and population structure of local clinical *Acinetobacter* species. Chapter 4 describes the sequencing of a clinical *A. baumannii* reference strain, as well as the genomic epidemiology and resistome of Acb complex isolates in Singapore. The local clinical isolates provided insights into the population structure and resistome of Acb complex present in the Singapore hospital.

4.1. Complete genome of *A. baumannii* Canada BC-5

Prior to sequencing the local clinical Acb complex isolates, we applied our whole genome sequencing protocol and bioinformatics analysis pipeline on *A. baumannii* Canada BC-5. The reference strain is a clinical isolate that belonged to International Clone 1 with Pasteur Scheme ST1 and has been described to harbour antibiotic genomic island AbaR29 (Holt et al., 2016).

A. baumannii Canada BC-5 is a reference multi-drug resistant clinical strain (BEI Resources NR-17783) that was isolated in 2007 from a soldier in a civilian hospital in British Columbia, Canada. The patient was evacuated from a US military hospital, Landstuhl Regional Medical Center in Germany (Chan et al., 2015). Currently, its draft genome assembly is available in three contigs (GenBank accession number AFDN00000000.1). Two of the contigs belonged to its chromosome while the third contig that was 8,731 bp in length belonged to its plasmid pCanadaBC5-8.7. Despite this isolate being a reference strain, the gaps between the two chromosomal contigs have not yet been closed. Having a fully closed complete genome will allow the isolate to be used as a genomic reference and be helpful in future downstream gene synteny and comparative genomics analysis. A hybrid assembly strategy of combining both ONT MinION long read sequencing and Illumina HiSeq short read sequencing was used to complete the genome. Here, we report the complete genome assembly of *A. baumannii* Canada BC-5 strain.

The hybrid assembly of *A. baumannii* Canada BC-5 yielded a complete chromosome which is 4,010,093 bp in size with a GC content of 39.20%, and one plasmid consisting of 8,731 bp (GC content of 34.37%). The genome had a sequencing overage of 330X.

The genome has 3,750 predicted CDS, 6 copies of 16S-23S-5S rRNA operons, and 75 tRNA genes based on Prokka annotation (Seemann, 2014). Pairwise comparison using BLASTN showed that the completed plasmid sequence shared 100% similarity and identity to pCanadaBC5-8.7 plasmid in NCBI GenBank database, thereby confirming the presence of the plasmid. The isolate belonged to Pasteur Scheme ST1 and Oxford Scheme ST947. There were 23 AMR genes (17 ARG and 6 MRG) present in the chromosome. The genes were $bla_{ADC-176}$, bla_{OXA-23} (three copies), bla_{ADC-11}, *amvA*, *adeC*, bla_{OXA-69} ($bla_{OXA-51-like}$), *abaF*, *sul1*, *qacEdelta1*, *aadA1*, *aac(3)-Ia*, *catA1*, *tet(A)*, *sul1*, *ant(3")-IIa*. Mercury resistance operon, *merR*, *merT*, *merA*, *merD*, *merE*, and nickel resistance gene, *nreB*, were detected in the chromosome. No antimicrobial resistance genes were detected in the plasmid. CRISPR-Cas Type I-F was found with 6 cas genes located between 1,093,554 bp to 1,101,694 bp position and 52 spacers between 1,101,826 bp to 1,104,975 bp position. Prophage prediction by PHASTER revealed one intact, one questionable and five incomplete prophage regions in the chromosome. The 61.4 kb intact prophage region with GC content of 39.02% encoded 59 phage proteins and 21 hypothetical proteins. These proteins include head protein, phage-like proteins, coat protein, fiber protein, tail shaft, portal protein, integrase and attachment site. The intact region shared 40% of proteins with PHAGE_Acinet_YMC11/11/R3177 (GenBank accession: NC_041866)

The whole genome sequencing and bioinformatics protocols were subsequently adopted for the sequencing of local clinical isolates in Chapter 4.2.

4.2. Clinical profile of Acb complex isolates

Acb complex isolates (n = 117) cultured from blood samples in a public tertiary hospital, Tan Tock Seng Hospital, Singapore, were previously collected from February 2006 to December 2010.

We performed microbiological characterization using MALDI-TOF MS and colony PCR to identify the bacterial species. Due to the close genetic similarity between Acb complex species, MALDI-TOF MS Bruker BioTyper was unable to resolve species identification with high confidence. Further PCR of species-specific genes or 16S sequencing were required to confirm the species. The clinical isolates have been identified to be part of the *Acinetobacter calcoaceticus-baumannii complex* (Acb complex) with 107 *A. baumannii*, 8 *A. nosocomialis* and 2 *A. pittii* species.

Majority (80.3%, 86/107) of *A. baumannii* isolates were of nosocomial origins, with the remaining 21/107 to be healthcare associated and 1/107 to be community associated (Table 7). For *A. nosocomialis*, 4/8 were nosocomial, 2/8 were healthcare-associated and 2 were community-associated. Lastly, one *A. pittii* isolate was nosocomial while the other *A. pittii* isolate was community-associated. Healthcare associated sources may include prior admission to hospitals within the past 90 days or from nursing home previously.

Table 7. Clinical context of Acb complex in this study.

	A. baumannii	***A. nosocomialis***	***A. pittii***	**Total**
Community	1	2	1	4
Healthcare associated (admission within past 90 days)	13	1	0	14
Healthcare associated (nursing home)	6	1	0	7
Healthcare associated	1	0	0	1
Nosocomial	86	4	1	91
Total	107	8	2	117

4.2.1. High carbapenem resistance phenotype found in *A. baumannii*

All clinical Acb complex isolates showed resistance rates of 100% (117/117) to amoxicillin and cefuroxime. In *A. baumannii*, the isolates showed resistance rate of 99.1% (1/106) to ceftriaxone, 96.2% (100/104) to amoxicillin/clavulanic acid, 88.8% (95/107) to piperacillin/tazobactam, 86.9% (93/107) to ciprofloxacin, 84.1% (90/107) to imipenem and meropenem, 83.2% (89/107) to ceftazidime, 82.7% (86/104) to cefepime, 73.8% (79/107) to gentamicin and cotrimoxazole, 69.2% (74/107) to amikacin. 35.0% (41/117) of Acb complex isolates were classified to be multi-drug resistant (MDR) being resistant to three or more classes of antibiotics, while 50.4% (59/117) were extensively-drug resistant (XDR), being resistant to at least one agent in all except for one or two categories (Table 8). All isolates were susceptible to polymyxin B (Figure 15). *A. nosocomialis and A. pittii* (n = 10) isolates were largely non-MDR (80%), with 20% being MDR.

Table 8. Summary of multi-drug resistant (MDR) and extensively-drug resistant (XDR) Acb complex clinical isolates.

Species	Non-AB	MDR-AB	XDR-AB	Total
A. baumannii	9	39	59	107
A. nosocomialis	7	1	0	8
A. pittii	1	1	0	2
Total	17	41	59	117

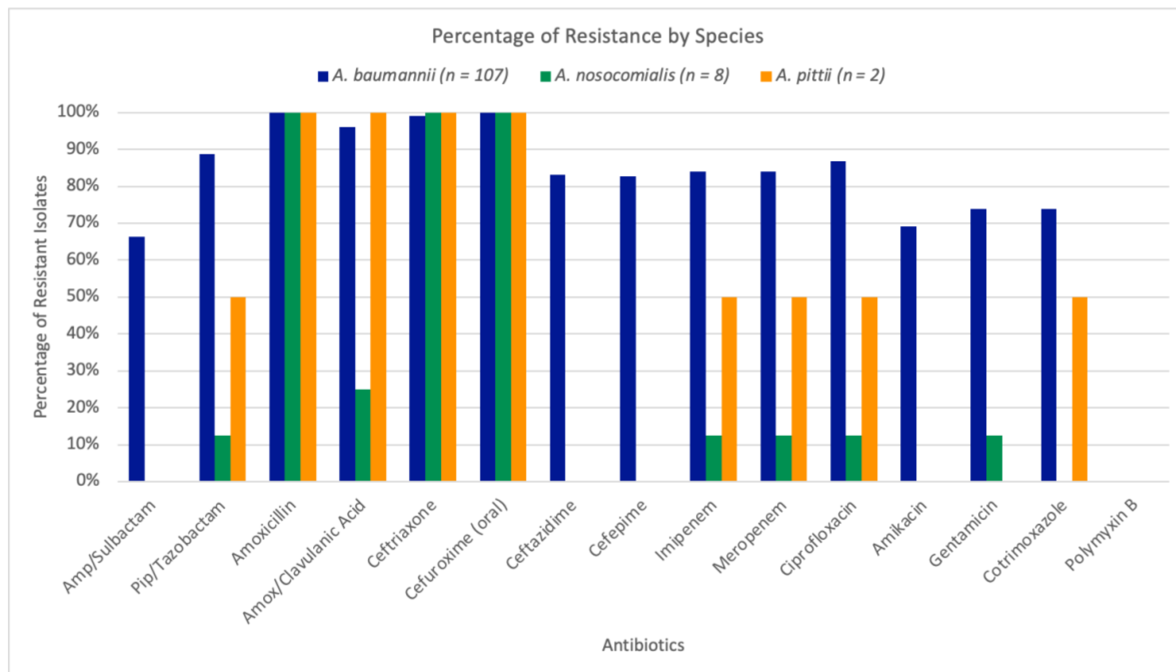

Figure 15. Antibiotics susceptibility testing profile of local clinical Acb complex isolates (n = 117) against tested panel of antibiotics.

4.3. Genomic epidemiology

To study the genetic relatedness between these clinical Acb complex isolates, whole genome sequencing was carried out on Illumina HiSeq platform which yielded an average sequencing coverage of 190X ± 4.55X per isolate.

4.3.1 MLST

Multi locus sequence typing (MLST) using Pasteur scheme was carried out to understand the clonal relationships between the isolates. A total of 11 different STs were identified. Majority of the *A. baumannii* (66.3%, 71/107) were identified to be Pasteur Scheme ST2, belonging to the International Clone 2, while 14.9% (16/107) of *A. baumannii* belonged to International Clone 1 as Pasteur Scheme ST1. There were 5.6% (6/107) of *A. baumannii* assigned to Pasteur Scheme ST10, a sequence type that has been reported to be associated with community-acquired infections (Meumann et al., 2019). ST10 has also been described as Clonal Complex 10 (CC10) (Diancourt et al., 2010). The other ST assigned include ST40 (n = 2), ST142 (n = 1), ST150 (n = 1), ST221 (n = 1), ST331 (n = 1), ST374 (n = 1), ST499 (n = 1), ST575 (n

= 2). Novel STs were detected in four of the *A. baumannii* isolates. ST142 was isolated from community-associated *A. baumannii*.

4.3.2. Core SNP Phylogeny

SNP Clustering using CSI Phylogeny had 2,351,867 SNPs covering 59.14% of the reference genome *A. baumannii* ATCC 17978 (CP000521.1) by all isolates (Figure 16). 18.8% (22/117) are non-*Acinetobacter baumannii* and non-clonal. There were 4 community-acquired infections (CAI) with 2 caused by *A. nosocomialis* and 1 by *A. pittii* and the *A. baumannii* isolate do not belong to the clonal groups by ST1, ST2 or ST10. This group of non-*A. baumannii* and non-ST1/2/10 *A. baumannii* are non-clonal and varied with long tree branches observed, supporting the evidence of possible CAI or extra-hospital origins of infection. The phylogenetic analysis revealed 3 clonal groups largely associated with ST1, ST2 and ST10. The non-ST1/ST2 *A. baumannii* (n = 20) were 45% non-MDR and 55% MDR. ST1 *A. baumannii* (n = 16) were 87.5% MDR and 12.5% XDR. ST2 *A. baumannii* (n = 71) were 19.8% MDR and 80.2% XDR.

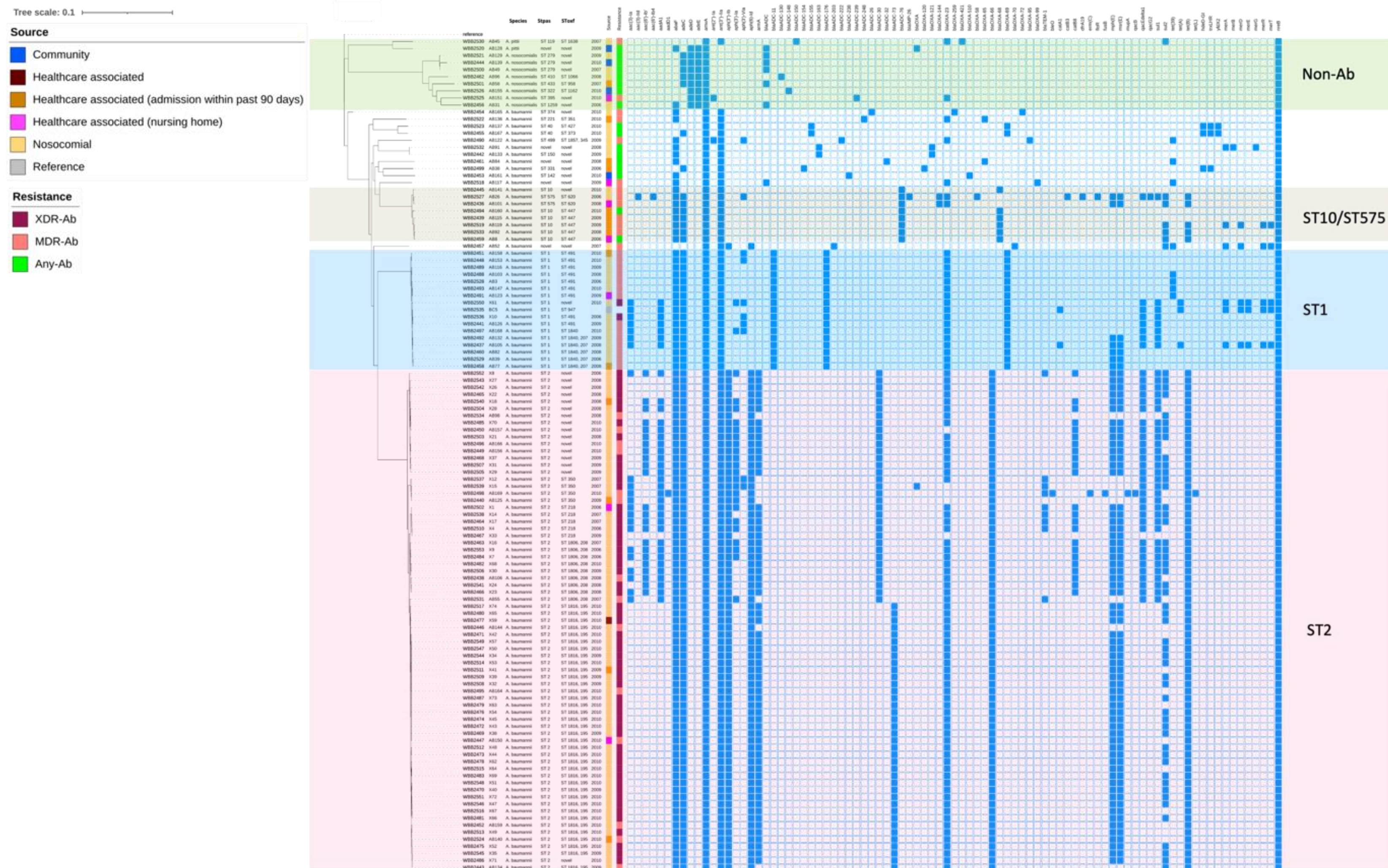

Figure 16. SNP Cluster tree of clinical Acb complex isolates in this study with their AMR genes. The isolate clades were colour coded based on MLST Pasteur Scheme highlighting ST1, ST2 and ST10/575. Each row represents an isolate plotted with their ST, collection year, source, ARG and MRG. The shaded blue box indicates the presence of the ARG and MRG in a binary plot. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).

4.3.3. Other Singapore isolates in NCBI public database

NCBI Pathogen Detection database was accessed on 4 August 2021 to understand the sequenced *Acinetobacter* isolates in Singapore. We found that there were 30 other *A. baumannii* clinical isolates from Singapore with most of them being from Singapore General Hospital. We performed SNP Clustering of these isolates using CSI Phylogeny with *A. baumannii* SGH0905 as a reference to understand their phylogenetic relationship (Figure 17). The isolates were primarily from International Clone 1 and International Clone 2 (Pasteur Scheme ST1 and ST2) with varied antimicrobial resistance genes (Figure 17). All isolates harboured *bla*$_{OXA-23}$ carbapenemase resistance gene. Mercury resistance genes were found in two ST1 isolates. This supported that both International Clone 1 and 2 have been the primary circulating clones of *A. baumannii* in Singapore.

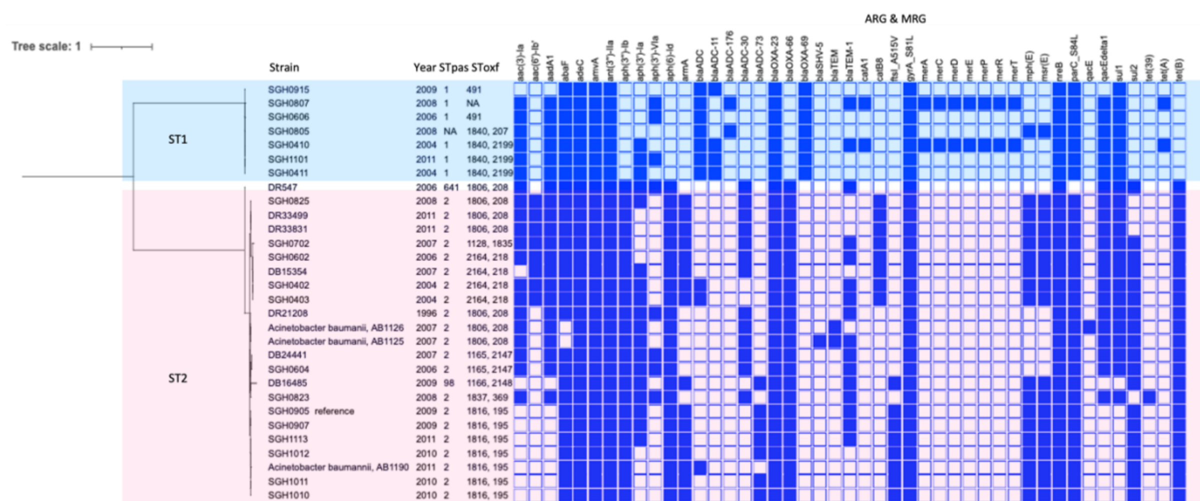

Figure 17. SNP Cluster Tree of 30 *A. baumannii* clinical isolates from Singapore in the NCBI Pathogen Detection database accessed on 4 August 2021. SNP Clustering was performed with *A. baumannii* SGH0905 as a reference. ARG and MRG data from NCBI MicroBIGG-E were plotted in the binary plot. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).

4.4. Resistome

A wide variety of ARG were detected in the clinical isolates. An average of 12.69 ± 3.96 ARG was found in *A. baumannii* ST1 and 17 ± 3.28 ARG was found in *A. baumannii* ST2 isolates. All *A. baumannii* ST1 genomes carried the intrinsic *bla*$_{ADC-176}$

and bla_{OXA-69} ($bla_{OXA-51-like}$), as well as bla_{OXA-23} that is capable of conferring carbapenem resistance. *A. baumannii* ST2 genomes were noted to be mostly nosocomial (90.1%, 64/71), and harboured either bla_{ADC-30} or bla_{ADC-73} variant, and bla_{OXA-66} ($bla_{OXA-51-like}$) intrinsic resistance genes. Majority of the genomes 93% (66/71) harboured bla_{OXA-23}. Other core ARGs in this clade included *abaF*, *adeC*, *amvA*, *ant(3")-IIa*, *aph(3")-Ib*, *aph(6)-Id*, *tet(B). A. baumannii* isolates (n = 6) belonging to ST10 harboured bla_{ADC-76} and bla_{OXA-68} ($bla_{OXA-51-like}$) intrinsic resistance genes. Mercury resistance operon (*mer*) were found in some of the *A. baumannii* ST1 and ST10 isolates.

4.4.1 Aminoglycoside resistance

Aminoglycoside resistance gene, *ant(3"-IIa)* was found in majority (108/117) of the isolates. Other genes *aph(3")-Ib*, *aph(6)-Id*, *armA*, *aadA1*, *aph(3')-Ia*, *aac(6')-Ib'*, *aac(3)-Ia*, *aph(3')-VIa* were found in 72/117, 72/117, 63/117, 34/117, 24/117, 23/117, 21/117, 10/117 of isolates respectively. *ant(2")-Ia*, *aac(3)-IId*, *aac(6')-Ib4*, *aadD1* were also found in 2/117, 1/117, 1/117 and 1/117 respectively.

4.4.2. Macrolide, sulfonamide, tetracycline and other antibiotics resistance

Efflux pump amvA was found in all isolates, while *adeC*, *adeD* an*d adeE* was found in 101/117, 9/117 and 9/117 isolates respectively. Macrolide resistance genes, *msr(E)* and *mph(E)*, were found in 70/117 of the isolates. *ermC* was found in 1/117 isolate. Sulfonamide resistance genes, *sul1* and *sul2*, were found in 38/117 and 57/117 of the isolates respectively. Tetracycline resistance genes, *tet(39)*, *tet(A)*, *tet(B)*, *tet(L)* were found in 8/117, 3/117, 76/117 and 1/117 respectively. Quaternary ammonium resistance genes *qacEdelta1*, *qacB*, *qacG2* was found in 38/117, 1/117, 1/117 isolates respectively. Trimethoprim resistance gene *dfrA19* was found in 1/117 isolate. Phenicol resistance genes *catB8*, *catB3*, *catA1*, *floR* was found in 23/117, 1/117, 1/117 and 1/117 isolates respectively.

4.4.3. Beta-lactam resistance

Common intrinsic $bla_{OXA-51-like}$ genes, bla_{OXA-66}, bla_{OXA-69} and bla_{OXA-68} were found in 71/117, 18/117, 6/117 isolates respectively. Other $bla_{OXA-121}$ (2), $bla_{OXA-144}$ (2), bla_{OXA-65} (2), $bla_{OXA-120}$, $bla_{OXA-259}$, $bla_{OXA-421}$, $bla_{OXA-510}$, bla_{OXA-70}, bla_{OXA-95}, bla_{OXA-99} were found in the isolates too. bla_{OXA-23} was found in 89/117 isolates. Other acquired beta-

lactamase, *bla*$_{IMP-26}$, *bla*$_{OXA-58}$ and *bla*$_{OXA-72}$ were found in one isolate each. Cephalosporinase resistance genes *bla*$_{ADC-73}$, *bla*$_{ADC-30}$, *bla*$_{ADC-11}$, *bla*$_{ADC-176}$, *bla*$_{ADC-76}$, *bla*$_{ADC}$, *bla*$_{ADC-155}$, *bla*$_{ADC-163}$ were found in 38/117, 33/117, 16/117, 16/117, 8/117, 7/117, 2/117, 2/117 respectively. Other resistance genes *bla*$_{ADC-130}$, *bla*$_{ADC-148}$, *bla*$_{ADC-150}$, *bla*$_{ADC-154}$, *bla*$_{ADC-203}$, *bla*$_{ADC-222}$, *bla*$_{ADC-238}$, *bla*$_{ADC-239}$, *bla*$_{ADC-248}$, *bla*$_{ADC-26}$, *bla*$_{ADC-32}$ were found in one isolate each.

4.5. Role of plasmids in carbapenem resistance

We hypothesized that plasmids could play a role in conferring acquired resistance in *A. baumannii*. We further investigated the genetic context of several Class D beta-lactamases conferring carbapenem resistance by performing long read sequencing for the generation of hybrid assembly combining long read and short read sequencing data.

4.5.1. *bla*$_{OXA-72}$

Role of small plasmid in conferring bla$_{OXA-72}$ *carbapenem resistance*

In this study, *bla*$_{OXA-72}$ was found to be a 9,501 bp circular replicon in a non-clonal ST374 nosocomial MDR *A. baumannii strain* AB165 isolated in 2010. This is a small plasmid with 10 hypothetical proteins, *bla*$_{OXA-72}$, DNA-binding protein, *relE* toxin and XRE family transcriptional regulator. A search against NCBI plasmids revealed that it shared high similarity of 100% query cover, 99.98% identity to *A. pittii* strain XJ88 plasmid unnamed1 (CP018910.1) of similar size that was isolated from a clinical sputum sample at China in 2013. The plasmids exhibited similar *bla*$_{OXA-72}$ genetic context with XerC-XerD recombination sites flanking the ARG. They shared similarity to the environmental pK09-14 plasmid described earlier in Chapter 3.2.5. This suggests evidence of cross-species transfer of *bla*$_{OXA-72}$ plasmid within the Acb complex as highly identical plasmids have been observed in both *A. baumannii* and *A. pittii* isolates from different parts of the world.

4.5.2. *bla*$_{OXA-23}$

Role of large plasmid in conferring bla$_{OXA-23}$ *carbapenem resistance*

All *A. baumannii* ST1 isolates (16/16), 93% of *A. baumannii* ST2 isolates (66/71), one *A. pittii* and one *A. nosocomialis* harboured *bla*$_{OXA-23}$ carbapenemase gene. We further investigated the genetic context of *bla*$_{OXA-23}$ using *A. baumannii* X61, an isolate belonging to GC1. We found *bla*$_{OXA-23}$ on its chromosome and on a large plasmid that is 96,204 bp in size (Figure 18). The carbapenemase gene in both locations was flanked by IS*Aba1* and was part of Tn*2006* transposon previously described to harbour *bla*$_{OXA-23}$ (Nigro and Hall, 2016b). The plasmid was found to be similar with 97% query cover, 99.99% identity against *A. baumannii* strain VB2486 plasmid pVB2486_1 (NZ_CP050404.1) that is 99,090 bp in size when compared against the NCBI Plasmids database. Plasmid pVB2486_1 was isolated in Christian Medical College, India, from a pneumonia sputum sample in 2019 and belonged to a host from GC1 (Pasteur Scheme ST1, Oxford Scheme ST1604, 231). The large plasmid has been possibly circulating in International Clone 1 isolates in the clinical setting.

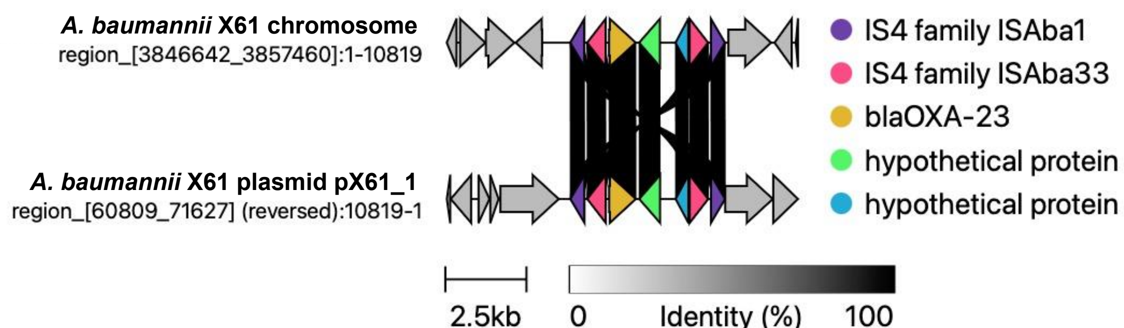

Figure 18. Presence of *bla*$_{OXA-23}$ flanked by IS*Aba* in Tn*2006* context in the chromosome and plasmid of *A. baumannii* X61. The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

Using *A. baumannii* AB98, a clinical isolate that belonged to GC2 in our study, we found the *bla*$_{OXA-23}$ gene to be plasmid mediated on a large plasmid pAB98_1 that is 86,344 bp in size. Other resistance genes present in this isolate were found to be

chromosomally encoded and were not found on this plasmid. The plasmid was identical to *A. baumannii* plasmid pA85-3 (NC_025109.2), that is 86,334 bp in size, with 100% query cover and 99.91% identity when compared using BLAST on Complete Plasmids database. Plasmid pA85-3 was from a GC1 isolate from Australia isolated in 2003. On both plasmids, *bla*$_{OXA-23}$ was flanked by IS*Aba1* as part of Tn*2006* transposon.

This suggests that *bla*$_{OXA-23}$ could be circulating as a large plasmid between *A. baumannii* GC1 and GC2 isolates. Interestingly, one of the BLAST hits was against *A. baumannii* SGH0905 plasmid pS32-2 (NZ_MG954379) with a query cover of 80% and identity of 99.87%. The plasmid was slightly smaller than pAB98_1 at 70,833 bp in size and did not harbour the *bla*$_{OXA-23}$ gene (Figure 19). *A. baumannii* SGH0905 was isolated from a blood specimen in 2009 from Singapore General Hospital (Blackwell et al., 2017). The pS32-2 plasmid could possibly present as a plasmid backbone without the *bla*$_{OXA-23}$ carbapenemase resistance gene in Singapore (Figure 20). In Chapter 3.3, we have also briefly described the plasmid that was similar to pS32-2 plasmid harbouring *bla*$_{OXA-82}$ (*bla*$_{OXA-51-like}$) in clinical *A. seifertii*. The plasmid should be further investigated given the potential of *bla*$_{OXA}$ carbapenemase to be introduced and maintained in the plasmid. We speculate that the plasmid could carry other machineries to aid in its dissemination, persistence, or could have a role to play to confer additional fitness through other virulence factors to help its survival in a clinical host environment. Mobile genetic elements IS*Aba1* and Tn*2006* transposons have also been described to play a role in the mobilization of carbapenemase genes between plasmids and chromosomes (Nigro and Hall, 2016b; Siguier et al., 2014).

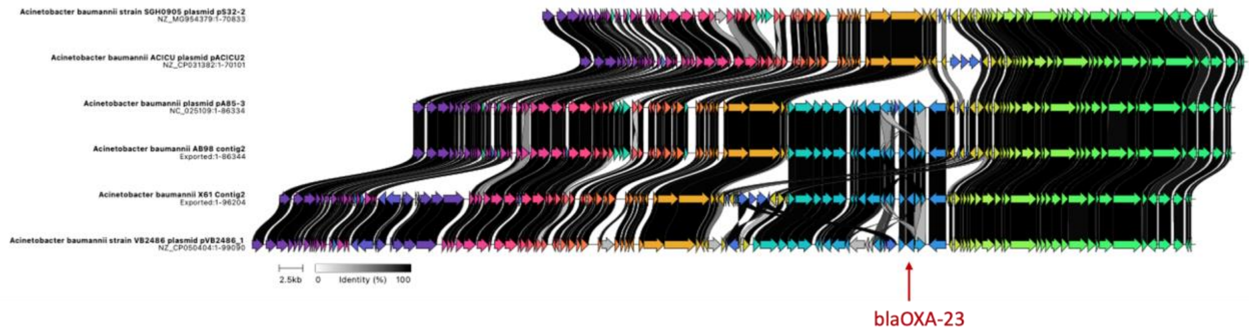

Figure 19. Gene synteny plot of large plasmids harbouring *bla*$_{\mathrm{OXA-23}}$ (gene indicated by red arrow) with suggested plasmid backbone without *bla*$_{\mathrm{OXA-23}}$. *bla*$_{\mathrm{OXA-23}}$ was not found in plasmid pS32-2 (top row) and pACICU2 (second row). The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

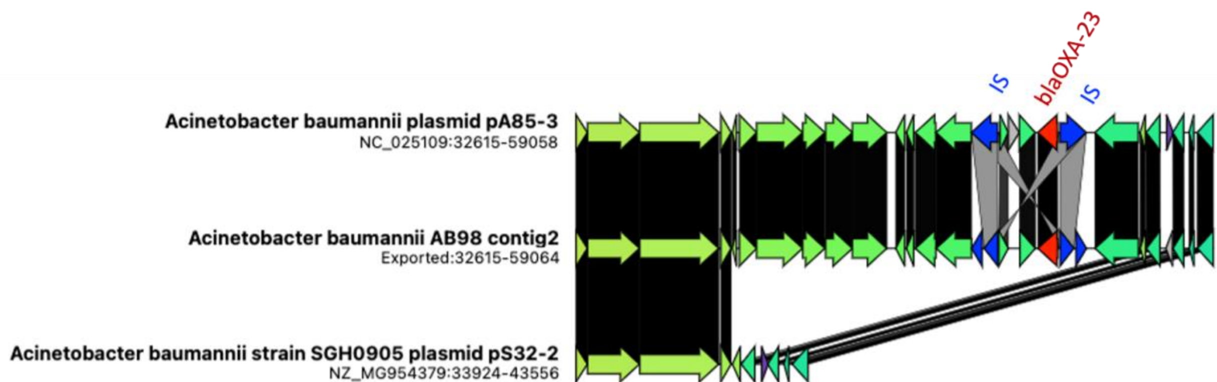

Figure 20. Gene synteny plot highlighting the *bla*$_{\mathrm{OXA-23}}$ (in red) and IS elements (in blue) in Tn*2006* genetic structure present in *A. baumannii* plasmid pA85-3 and plasmid AB98_1. Tn*2006* structure was absent in plasmid pS32-2 (bottom row). The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

The *bla*$_{\mathrm{OXA-23}}$ gene found in *A. pittii* AB45 and A*. nosocomialis* AB151 of our isolates were located in contigs of 2,709 bp and 2,657 bp in length respectively. As the genome assemblies were in contig levels, it was difficult to determine the genetic context of *bla*$_{\mathrm{OXA-23}}$ on whether it could be chromosomal or plasmid-mediated. Using NCBI Pathogen Detection database, one of the *A. nosocomialis* harbouring *bla*$_{\mathrm{OXA-23}}$ had a complete genome for us to further understand the possible genetic context of the carbapenemase gene. *A. nosocomialis* SSA3 (PDT000201413.1), a blood isolate

collected in South Korea in 2017, had three occurrences of *bla*$_{OXA-23}$ in the chromosome. Each *bla*$_{OXA-23}$ was flanked by IS*Aba1* with the similar structure to Tn*2006*, suggesting the potential role of MGE in the mobilisation and integration of ARG in the chromosome.

4.5.3. *bla*$_{OXA-58}$

bla$_{OXA-58}$ *in megaplasmids*

Our data demonstrated the role of megaplasmid in contributing drug resistance to non-clonal *A. baumannii* isolates. We first drew our attention to *A. baumannii* AB26, belonging to Pasteur Scheme ST575, as it had significantly much higher number of ARG with an extra of 13 ARG compared to its neighbouring isolates that share the same ST in the phylogenetic tree (Figure 16). This clinical isolate was also briefly mentioned in Chapter 3.1.5.

The hybrid assembly revealed that *bla*$_{OXA-58}$ was carried on a 247,526 bp megaplasmid pAB26_1. There were 14 ARGs, namely *dfrA19*, *blaIMP-26*, *qacG2*, *aac(6')-Ib4*, *catB3*, *qacEdelta1*, *sul1*, *aph(3')-Via*, *aac(3)-Iid*, *bla*$_{OXA-58}$, *sul2*, *msr(E)*, *mph(E)*, *floR*, found on this multi-drug resistant megaplasmid. The 247 kb plasmid was identical to *A. pittii* strain C54 plasmid pC54_001 (NZ_CP042365.1, 256,887 bp) with 100% query cover and 99.99% identity. *A. pittii* C54 was isolated in 2014 from Sydney, Australia. The pAB26_1 plasmid also shared a query cover greater than 85% with other *Acinetobacter* species plasmids, including *A. haemolyticus* strain TJR01 plasmid pAHTJR1, *A. lwoffii* strain H7 plasmid pH7-250, *A. johnsonii* XBB1 plasmid pXBB1-9 and *A. ursingii* strain RIVM0061 plasmid pRIVM0061_IMP-4_171109_B01. These suggested that the megaplasmid was likely to be inter-species as it has been disseminating between the different *Acinetobacter* species beyond the closely related Acb complex.

Comparison against other large plasmids and megaplasmids harbouring *bla*$_{OXA-58}$ revealed that *bla*$_{OXA-58}$ was flanked by IS*Aba3* and shared similar gene synteny with other similar plasmids (Figure 21). This prompted further investigation of *bla*$_{OXA-58}$ and potential *bla*$_{OXA-58-like}$ variants to understand their evolution in Chapter 5.3.1.

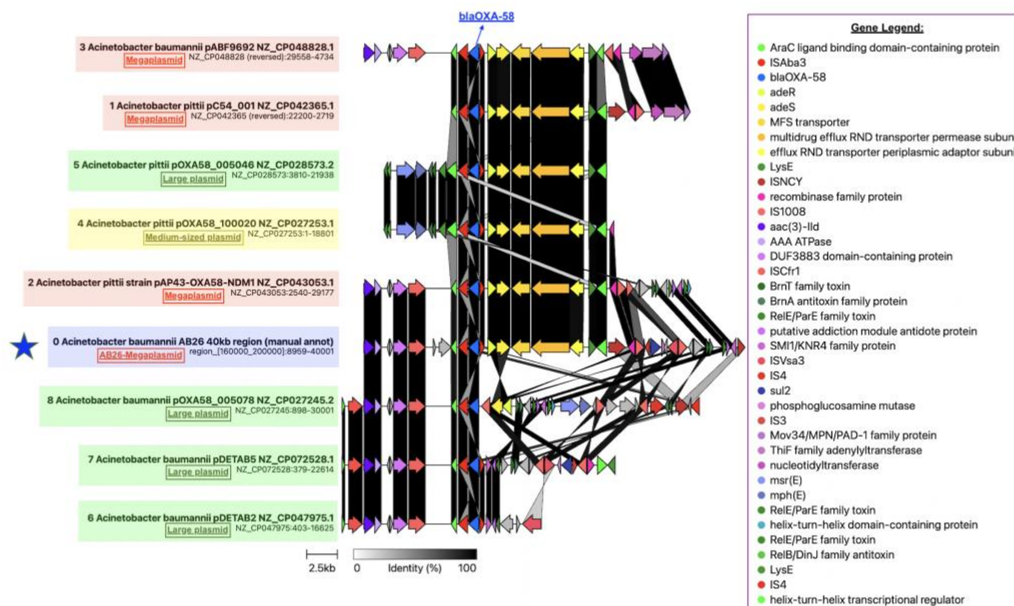

Figure 21. Genetic context of bla_{OXA-58} in large plasmids and megaplasmids. Plasmid pAB26_1 isolated in this study was indicated in blue star. The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

Bla_{OXA-58} in Singapore

To our best of knowledge, *A. baumannii* AB26 is the second report of sequenced bla_{OXA-58} harbouring *Acinetobacter* species in Singapore. The other sequenced isolate available on NCBI database was strain WPB103 (CP034427.1) originally deposited as *Acinetobacter baumannii*, isolated from Singapore hospital wastewater in 2017. Bla_{OXA-58} was found to be flanked by truncated IS*1* family transposase on a gapped scaffold in a contig of 1,167 bp. As the genome was in contig level, and the gene was found on a short contig, we were unable to discern if this was part of a plasmid sequence. Upon further investigation, we also found that strain WPB103 may be a novel *Acinetobacter* species as it did not share high similarity within the proposed cut-off values when compared using dDDH and ANI against known *Acinetobacter* species. Beyond sequenced isolates, there have been earlier reports of bla_{OXA-58} in *A. baumannii* isolates from Singapore (Koh et al., 2007; Koh et al., 2012).

4.6. Discussion

4.6.1. International Clones and ST

The clinical *A. baumannii* population is diverse, but two clones dominate in Singapore, *International* Clone 1 (ST1) (15.0%) and International Clone 2 (ST2) (66.4%). These are global clones are of concern as they are generally multi-drug resistant (MDR) and could also be extensively drug resistant (XDR) (Antunes et al., 2014; Hamidian and Hall, 2018; Holt et al., 2016). Other clinically relevant species from the Acb complex, namely *A. nosocomialis* and *A. pittii* have been found to be responsible for a number of community associated infections in this study. They highlight the importance of studying Acb complex to better understand their manifestation in community infections.

Community-associated sequence types ST10/ST575 have been isolated in our study suggesting potential environmental circulation of these clinically relevant isolates. ST10 has been described to be associated with community-acquired infections (Meumann et al., 2019). Other diverse ST sequence types, including novel ones, have also been isolated locally, suggesting the large diversity of *A. baumannii* capable of causing infections. There is a need for future epidemiological study in the non-hospital environmental to determine potential extra-hospital reservoirs. It is a worrying concern that one such non-clonal clinical isolate *A. baumannii* AB26 was found to be harbouring a megaplasmid containing 14 ARG that conferred antimicrobial resistance against multiple classes of antibiotics. The megaplasmid was analysed to be likely capable of inter-species transfer given that it was also found in *A. pittii*.

Prior to this study, there were 30 sequenced clinical *Acinetobacter* isolates from Singapore retrieved from NCBI Pathogen Detection database on 4 August 2021, with primarily ST1 and ST2 reported. Our study has more than quadrupled the number of clinical *A. baumannii* and other Acb complex isolates in the database, allowing additional genomic epidemiological study to examine the transmission of *A. baumannii*.

4.6.2. Carbapenem Resistance

We have described the high carbapenem resistance (84.1%, 90/107) of clinical *A. baumannii* isolates from Singapore, which has been consistent with previous reports of high carbapenem resistance in Singapore (Cai et al., 2017; Hsu et al., 2017). Carbapenem resistance are found to be mediated by oxacillinase beta-lactamases, such as *bla*$_{OXA-23}$. This is a worrying trend as carbapenem resistance remains high locally and regionally (Hsu et al., 2017). The nosocomial origins of these isolates indicate that carbapenem resistance is likely circulating in the hospital environment.

We found evidence of acquired carbapenem resistance through inter-species plasmids that may be circulating within clinical Acb complex and beyond. Plasmids have been previously described to play a critical role in the dissemination of antimicrobial resistance genes in *Acinetobacter* species (Lean and Yeo, 2017; Salgado-Camargo et al., 2020; Salto et al., 2018). Other reports have also supported the finding of small plasmids involved in the dissemination of *bla*$_{OXA-72}$ (Brasiliense et al., 2019; Jia et al., 2019). Here, we have found large plasmids and megaplasmids from local clinical *A. baumannii* that were involved in the dissemination of *bla*$_{OXA-58}$ and *bla*$_{OXA-23}$. We have managed to find a large plasmid with the absence of beta-lactamase gene and its variants that were capable of harbouring *bla*$_{OXA-23}$ and *bla*$_{OXA-58}$ genes. In addition, we had also found instances where the genome may be attributed to an incorrect species based on the time of deposition or by the submitting group. We found that *Acinetobacter* spp. strain WPB103 (CP034427.1) was originally deposited as *A. baumannii* but should belong to a novel *Acinetobacter* species. The plasmids were found to be circulating across different geography and time, suggesting a potential worldwide dissemination of these plasmids. Apart from conferring carbapenem resistance, plasmids have been shown to confer aminoglycoside resistance to International Clone 2 (Pasteur Scheme ST2) isolates (Nigro and Hall, 2016a). The increasing use of long read sequencing technology has enabled these plasmids to be identified, completed and analysed in greater detail.

The presence of megaplasmid harbouring large number of antimicrobial resistance genes capable of conferring multi-drug resistance is worrying. Similar plasmids to *A. baumannii* AB26 plasmid pAB26_1 have been found in different *Acinetobacter* species. Megaplasmids in *Acinetobacter* have been described to be able to disseminate

globally and harbour accessory genes that are specific for their ecological niche (Ghaly et al., 2020). Future studies can seek to understand the accessory genes carried on this megaplasmid as well as on the fitness cost required to harbour and maintain it in the clinical setting. Apart from ARG and MRG, there could be other survival or persistence genes in the plasmid to maintain plasmid stability.

4.6.3. Role of MGE in AMR

MGE, such as transposases, IS elements and recombinases may also play a role in the recombination of antimicrobial resistance genes and genome diversification (Touchon et al., 2014). Transposon Tn*2006* harbouring *bla*$_{OXA-23}$ could have been involved in the mobilization of the carbapenemase gene out of the plasmids and into the chromosome or vice versa (Nigro and Hall, 2016b). *bla*$_{OXA-23}$ was found in the similar genetic context of Tn*2006* on the chromosome and plasmid of *A. baumannii* X61. XerC-XerD recombinase sites were found flanking *bla*$_{OXA-72}$ in a small plasmid, consistent with a similar plasmid isolated from the environment in this study. Such genetic structures present an ongoing risk of further mobilisation of the ARG onto other plasmids.

4.7 Conclusion

In summary, our study has shown that there were diverse sequence types as well as known international clones of *A. baumannii* circulating in the local clinical settings of Singapore. The community-acquired origins of Acb complex are worrying, suggesting a potential environmental or community reservoir for these opportunistic pathogens. We observed high carbapenem resistance and found that plasmids could play an important role in the inter-species dissemination and evolution of antimicrobial resistance genes. Large plasmids and megaplasmids could be a potential source of resistome to confer multi-drug resistance to these isolates.

Chapter 5. Genomic Epidemiology of global *A. baumannii*

In this chapter, publicly available databases were accessed to study the resistome of *A. baumannii* isolated and sequenced worldwide. This chapter expands and builds upon the understanding of environmental and clinical *A. baumannii* in Singapore that have been explored in Chapter 3 and Chapter 4 respectively.

Genome databases provide an open collection of sequenced genomes that were submitted by users alongside with its associated metadata, such as the collection year, geographical location of isolation, sample source and in some instances antibiotic susceptibility testing phenotypes. WGS technologies have enabled routine sequencing of isolates as part of food and agricultural surveillance, public health surveillance, clinical laboratory investigations as well as from research studies. The use of publicly available databases may provide a broader global perspective on the available dataset and understanding of *A. baumannii*. Here, we report the analysis of the *A. baumannii* genome, plasmidome, and resistome from one of the largest datasets in the public domain, the Pathogen Detection (PD) and Microbial Browser for Identification of Genetic and Genomic Elements (MicroBIGG-E) databases recently established by US National Center for Biotechnology Information (NCBI) (Sayers et al., 2020).

5.1. Genomic Epidemiology

The NCBI Pathogen Detection database collates and centralises data of clinically important bacterial and fungal pathogens from various datasets in NCBI as well as specifically submitted data from routine surveillance programs. It had a total of 15,262 isolates under the "*Acinetobacter baumannii*" group category as of 31 December 2021. The majority (94.1%, 14,355/15,262) of the isolates belonged to *A. baumannii* species, with the others being from Acb complex and other *Acinetobacter* spp. (Table 9), as these close relatives of *A. baumannii* also cause similar clinical infections. With increasing use of next generation sequencers, there has been a rapid rise in WGS data submissions in PD since its inception in 2010, especially over the recent 5 years with 4,638 genome submission in 2021 (Figure 22). These assemblies are in various

level of completeness with 63.7% in contig level, 13.0% in scaffold level, and minority of 2.44% and 0.20% in complete genome and chromosome level respectively (
Table 10). The low number of complete genomes available poses a challenge in understanding the genetic context of ARG to understand if they are chromosomally or plasmid encoded, and for applications where high-quality sequence data and long contigs are required.

Data in NCBI PD database included submissions of Short Read Archive (SRA) dataset which currently accounted for 20.7% of the total number of isolates in PD
Table 10). The inclusion of SRA dataset is helpful for researchers as it saves computation time and resources to perform a separate assembly or prediction of AMR genes from raw sequencing FASTQ reads. This combined data in PD contained more data than NCBI's main Genome database that had only 5,776 genome assemblies as of 6 January 2022. However, NCBI Genome (but not PD) has a Genome Tree report that provided a dendrogram to demonstrate the clonal relationship of *A. baumannii* isolates. We observe that at least half of the genomes belong to the two International Clones IC1 and IC2 that correspond to ST1 and ST2 using MLST Pasteur Scheme (Figure 23). This indicates that the vast majority of *A. baumannii* sequenced to date are clonal and clinical in origin.

Table 9. Number of *Acinetobacter* isolates in NCBI Pathogen Detection database. Acb complex species are indicated by asterisk.

Species	Number of isolates	Percentage
*Acinetobacter baumannii**	14,355	94.06%
*Acinetobacter calcoaceticus**	29	0.19%
*Acinetobacter lactucae**	16	0.10%
*Acinetobacter nosocomialis**	223	1.46%
Acinetobacter oleivorans	36	0.24%
*Acinetobacter pittii**	486	3.18%
*Acinetobacter seifertii**	58	0.38%
Acinetobacter sp.	59	0.39%
Total	**15262**	**100.00%**

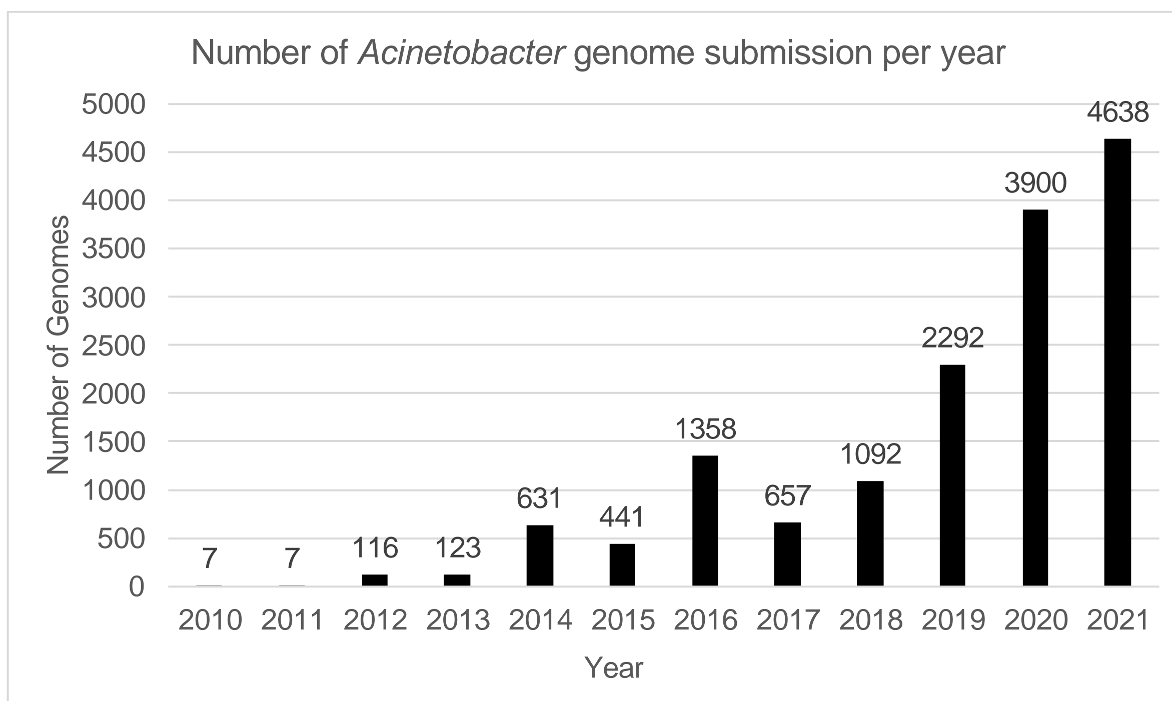

Figure 22. Rise of *Acinetobacter* genomes submission into NCBI Pathogen Detection database over the years.

Table 10. Number of *Acinetobacter* genome assemblies and their level of completeness in NCBI Pathogen Detection database.

Level of Completeness	Number of Assemblies	Percentage
Chromosome	31	0.20%
Complete Genome	372	2.44%
Scaffold	1,987	13.02%
Contig	9,716	63.66%
Short Reads Archive (SRA)	3,156	20.68%
Total	15,262	100.00%

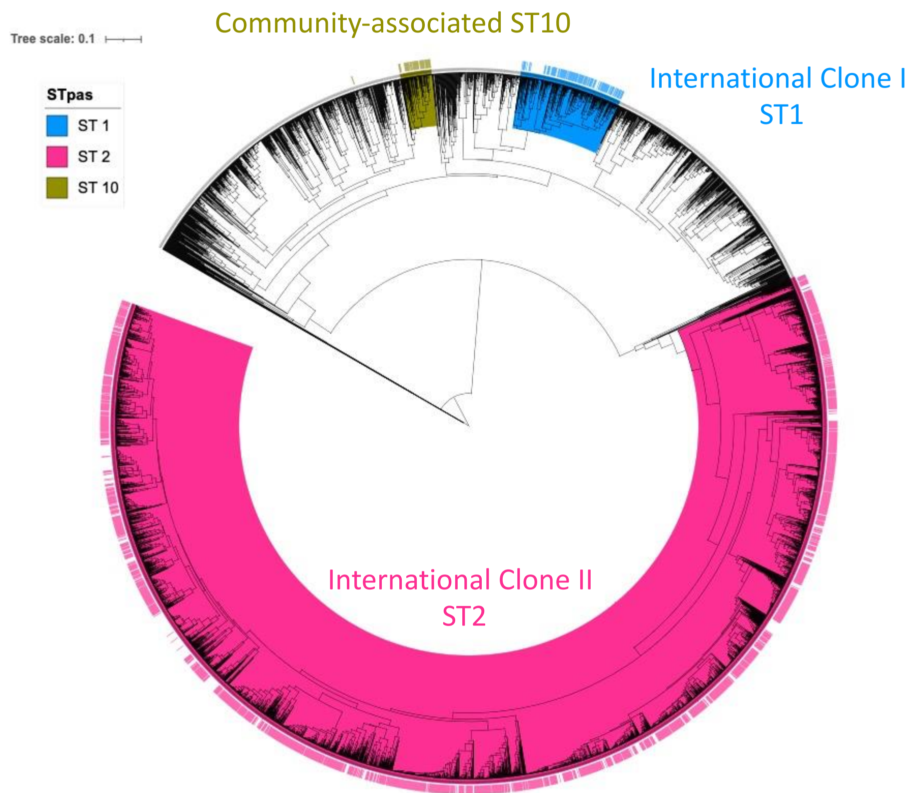

Figure 23. NCBI Genome Tree of *A. baumannii* (n = 4,302) downloaded on 31 March 2021. International Clone 1 and 2 belonging to Pasteur Scheme ST1 and ST2 respectively, as well as community associated ST10 have been highlighted. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).

5.1.1 Environmental and animal isolates

To understand the source of isolates deposited, we assessed and manually curated the provenance metadata to check the isolation type and source against NCBI BioSample entries and published literature. Majority of the isolates deposited remained to be of human clinical origin. Out of 11,424 *Acinetobacter* isolates accessed on 19 March 2021, we found 127 non-hospital environmental isolates, from 87 animals, 10 plants, 17 soil and 13 water sources (Table 11).

Table 11. Summary of environmental *Acinetobacter* isolates from NCBI Pathogen Detection database accessed on 19 March 2021. ^ denotes species not from Acb complex.

Species	Animal	Soil	Water	Total
Acinetobacter baumannii	61	8	10	79
Acinetobacter calcoaceticus	0	8	1	9
Acinetobacter lactucae	4	4	0	8
Acinetobacter nosocomialis	0	0	1	1
Acinetobacter oleivorans^	6	2	0	8
Acinetobacter pittii	11	3	0	14
Acinetobacter seifertii	5	2	1	8
Total	87	27	13	127

We found clonal relationships between whole genomes of environment and clinical isolates. For instance, using high resolution whole genome SNP clusters from NCBI PD database, a mangrove soil *A. baumannii* MSP4-16 isolate (PDT000011385.2) harbouring *sul2* ARG from India was related to various clinical strains from India, China and US with a maximum distance of 12 SNPs and average distance of 7 SNPs between the isolates (Figure 24). All isolates shared similar ARG and MRG including *sul2* ARG against sulfonamide and *mer* mercury resistance operon. These isolates were collected across different years, with *A. baumannii* DSM 30007 (also known as reference type strain ATCC 19606) recovered in US prior to 1948 (Hamidian et al., 2020).

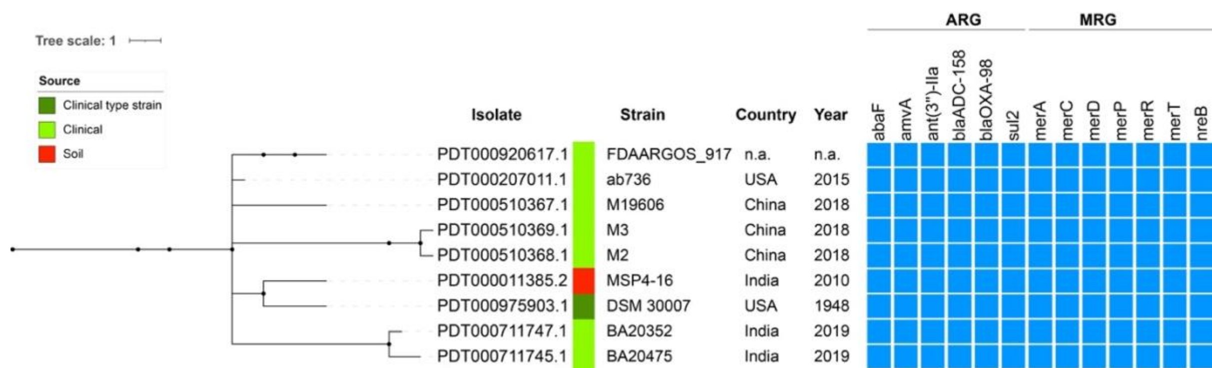

Figure 24. NCBI Pathogen Detection SNP Cluster Tree PDS000005000.17 of mangrove soil *A. baumannii* isolate PDT000011385.2 (source coloured in red) against other clinical strains, including DSM 30007 (also known as ATCC 19606) reference type strain of clinical urine origin. There is a maximum distance of 12 SNPs and average distance of 7 SNPs between the isolates. Partial ARG found on contigs that are less than 750 bp in length have been excluded. The ARG and MRG were plotted with data obtained from NCBI MicroBIGG-E database. Tree scale indicates SNP distance. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).

An animal *A. baumannii* isolate from duck faeces in China was closely related to two clinical strains from Myanmar and Thailand isolated between 2015 to 2017 by whole genome SNP clustering (Figure 25). The different isolates had a maximum distance of 45 SNPs and average distance of 35 SNPs. Despite their clonal relationship, they share different ARG genes though the intrinsic resistance genes *bla*$_{ADC-169}$ and *bla*$_{OXA-402}$ were similar.

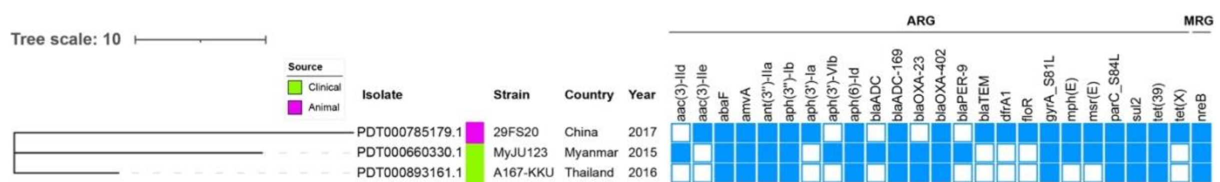

Figure 25. NCBI Pathogen Detection SNP Cluster Tree PDS000076303.1 of duck faecal *A. baumannii* isolate PDT000785179.1 (source coloured in pink) with two other clinical strains. There is a maximum distance of 45 SNPs and average distance of 35 SNPs between the isolates. The presence of ARG and MRG were plotted with data

obtained from NCBI MicroBIGG-E database. Tree scale indicates SNP distance. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).

5.1.2 *A. baumannii* ST10

Results presented in earlier Chapters indicated the potential diversity and relatedness of the *A. baumannii* ST10 clade commonly associated with human community acquired infection (CAI). In Chapter 3.1.1, *A. baumannii* isolated from local urban water samples shared the same ST10 (but different Oxford ST scheme) with a neighbouring clinical isolate in the phylogenetic tree. In Chapter 4.2, local clinical *A. baumannii* ST10 isolates were found to be of nosocomial origin. In this Chapter, genomes of the ST10 clade were found to be non-clonal with isolates having relatively long and varying branch lengths. In addition, ST10 isolates have also been found in animal samples according to genome submission records.

We examined the researcher-reported sources of global ST10 isolates submitted to the public database. We discovered two dog isolates with ST10 (Pasteur scheme) but with ARG profiles different from human isolates (Figure 26). These ST10 animal isolates were the first non-human isolates sharing CAI ST10 to the best of our knowledge. The ST10 clade has been observed to have disseminated across different countries over 25 years from 1994 to 2019. The diverse ARG profiles between these isolates suggest that they are likely to be horizontally transferred through plasmids, rather than vertically transferred. Different ARGs were found on different branches of the tree with clonal subclades harbouring similar ARG profile. Although the direction of transmission cannot be established in these molecular epidemiological surveys, these implicate the environment and community as potential reservoirs of ARG and chromosomes prevalent in human isolates.

Figure 27. NCBI Genome Tree showing the clade of *A. baumannii* Pasteur ST10 isolates (n = 64) with animal isolates highlighted in red. The presence of antimicrobial resistance genes (ARG) and metal resistance genes (MRG) were plotted with data obtained from NCBI MicroBIGG-E database. Country and year metadata of the isolates were retrieved from NCBI Pathogen Detection database. Data not available indicated as “n.a.”. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).

5.2. Plasmidome of *A. baumannii*

To understand the prevalence of plasmids in *A. baumannii*, complete *A. baumannii* genomes in NCBI database were analysed. Plasmids were found in 77.6% (288 out of 371), with a median of 2 plasmids per genome (range of 1 to 11 plasmids) for those containing plasmids. Among complete plasmid sequences of *A. baumannii* deposited in NCBI Genome (n = 682, accessed on 3 Jan 2022), plasmid sizes ranged from 359 bp to a 1,024,410 bp megaplasmid, with a median of 15 kb length (Figure 28). The GC content ranged from 29.3% to 59.4% with a median of 35.9%. The anomalies observed at the extreme ends of the spectrum, such as contigs less than 2 kb in size, were possibly incomplete due to sequencing or assembly artefacts. Based on size and frequency distribution *A baumannii* plasmids could be arbitrarily classified into five broad groups based on their sizes and GC content (Table 12).

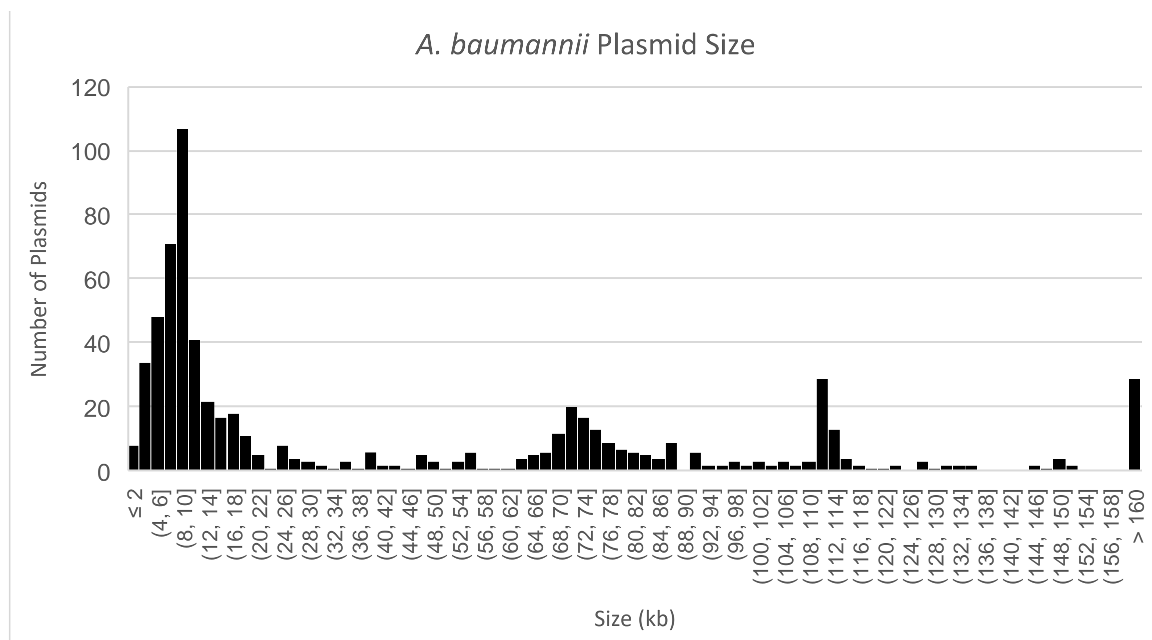

Figure 28. Frequency distribution histogram of *A. baumannii* plasmids (n = 682) based on their size (kb).

Table 12. Classification of *A. baumannii* plasmids by size.

Class	Range	Mode Size	Number of plasmids	Percentage of plasmids
Small	2-30 kb	8	398	58%
Intermediate	30-60kb	40	38	6%
Medium	60-100 kb	70	133	20%
Large	100-140 kb	110	84	12%
Mega	>160 kb	220	29	4%
Total			682	100%

Close to a third (35.3%, 241/682) of these plasmids harboured at least one antimicrobial resistance gene or metal resistance gene. Of these plasmids, 198 plasmids harboured only ARG, 8 plasmids harboured only MRG and 8 plasmids harboured both ARG and MRG. There was a mean of 3.16 ± 3.70 and median of 1 (range of 1 to 11) ARG per plasmid, and a mean of 2.44 ± 1.52 and a median of 2 MRG per plasmid. AMR genes have been found in plasmids of different sizes from small, medium, large to megaplasmids, with a median length of 68,225 bp in size. Notably plasmids with ARG tended to be longer than plasmids not carrying any ARG (Figure 29). The top 10 ARG carried by these plasmids are *sul2* (8.08%), *aph(3')-Via* (6.06%), *sul1* (5.94%), *mph(E)* (5.82%), *msr(E)* (5.82%), *aph(3")-Ib* (4.99%), *aph(6)-Id* (4.87%), *bla*$_{OXA-23}$ (4.28%), *qacEdelta1* (3.68%) and *ant(2")-Ia* (3.44%) (Figure 30). In terms of ARG classes, there were highest number of resistance genes for aminoglycoside, beta-lactam, sulfonamide, macrolide, phenicol and tetracycline in a descending order (Figure 31).

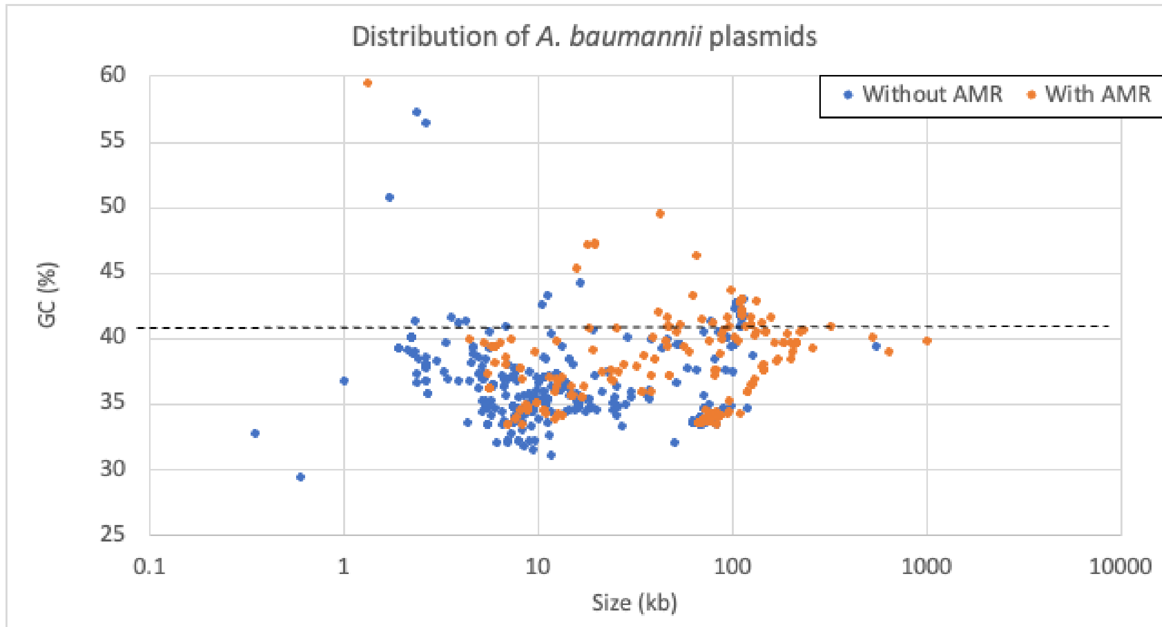

Figure 29. Distribution of *A. baumannii* plasmids (n = 682) of their GC% against size (kb) with (in orange) and without (in blue) antimicrobial resistance (AMR) genes. Each dot represents an *A. baumannii* plasmid. The median GC% for *A. baumannii* chromosomes is indicated by the dotted line.

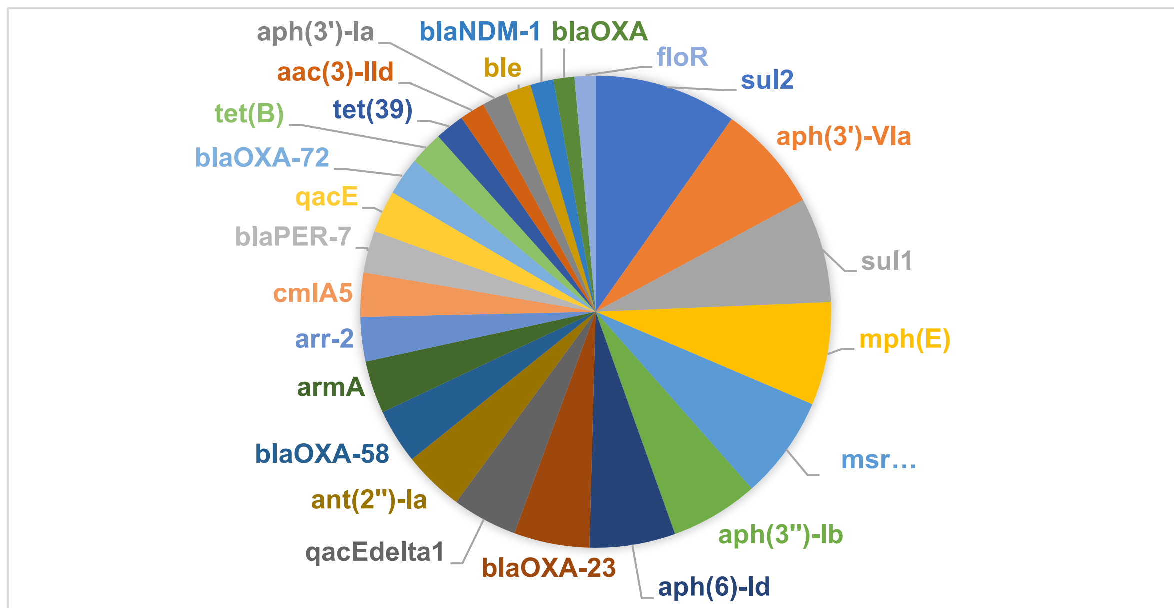

Figure 30. Pie chart of top 25 ARG (top 82.45%) found in *A. baumannii* plasmids.

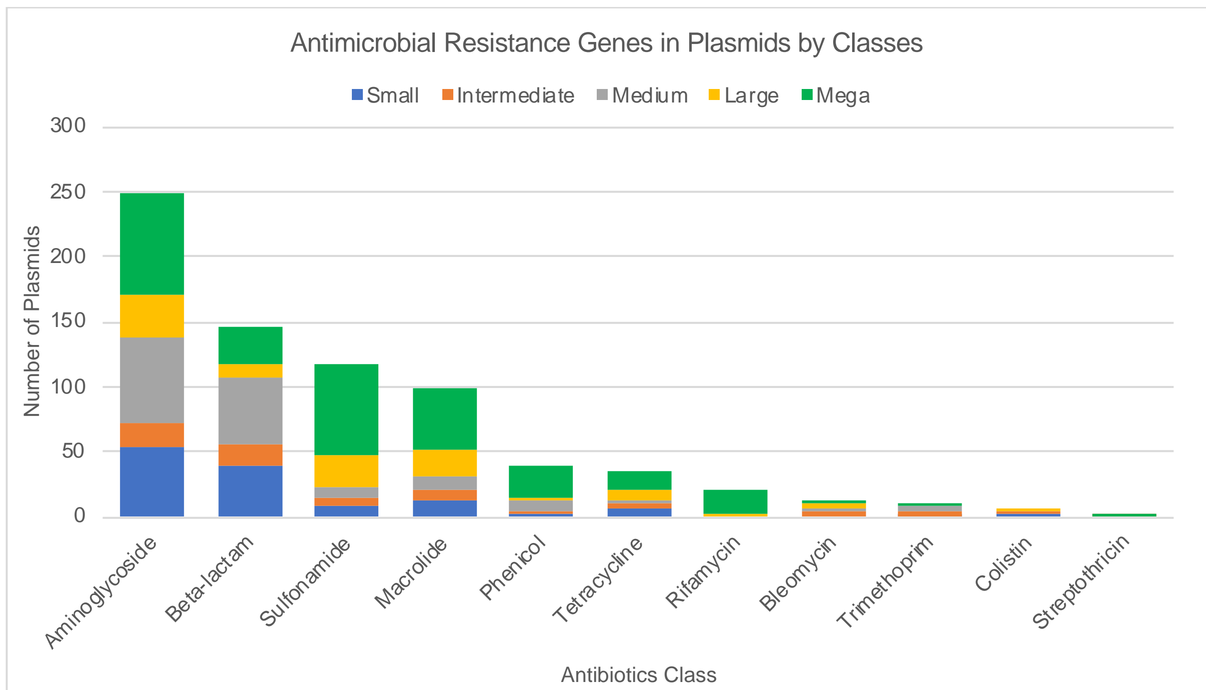

Figure 31. Number of antimicrobial resistance genes by antibiotics classes in *Acinetobacter* plasmids.

5.3. Resistome of *A. baumannii*

The resistome of *A. baumannii* in its genome (both chromosomal and plasmid) was studied using NCBI MicroBIGG-E data accessed on 31 December 2021. A total of 263,242 ARG and MRG from 27 classes were found in 12,106 *Acinetobacter* spp. isolates. Aminoglycoside resistance are the most prevalent, followed resistance against beta-lactam, efflux pumps, quinolone, macrolide, sulfonamide, nickel, fosfomycin, tetracycline and quaternary ammonium, phenicol and mercury in decreasing order (Figure 32).

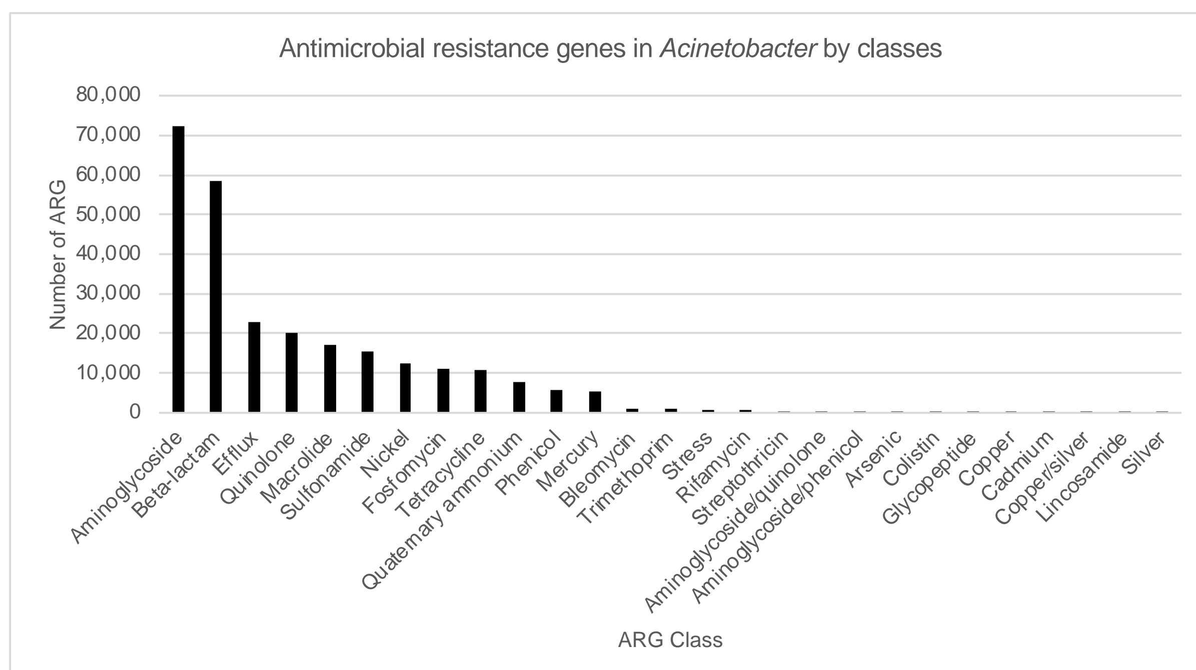

Figure 32. Count of ARG in *Acinetobacter* by class, using all available genomes (completed and not completed).

Examining further into the beta-lactam class revealed 29,668 records of beta-lactamases against carbapenem in the isolates. A total 165 beta-lactamases against carbapenem with 144 from the *bla*$_{OXA}$ family. There were 98 intrinsic *bla*$_{OXA-51}$ family carbapenemase with *bla*$_{OXA-66}$ as the most prevalent. *bla*$_{OXA-66}$ has been associated as the intrinsic *bla*$_{OXA-51-like}$ gene for the International Clone 2 isolates. This finding is in concordance given that the majority of *A. baumannii* deposited belonged to International Clone 2. There were also 575 *bla*$_{OXA}$ entries that were identified to be part of *bla*$_{OXA-51}$ family with a mean coverage of 91.4% ± 14.7% with a median of 100%. These *bla*$_{OXA}$ variants have a mean identity of 99.3% ± 2.19% with a median of 99.6%.

The high identity indicates that these are likely to be novel *bla*$_{OXA-51-like}$ variants that have yet to be characterised and assigned to a *bla*$_{OXA}$ allele. Apart from the intrinsic *bla*$_{OXA-51-like}$ beta-lactamase, acquired *bla*$_{OXA}$ alleles *bla*$_{OXA-72}$, *bla*$_{OXA-24}$ and *bla*$_{OXA-58}$ were one of the most prevalent types identified. Other beta-lactamases *bla*$_{NDM-1}$ and *bla*$_{NDM}$ were also found in the database.

ARG in the complete genomes were analysed to better understand their genetic context on whether they're chromosomal or plasmid-borne. ARG conferring aminoglycoside resistance were the most prevalent in completed genomes, followed resistance against beta-lactam, efflux pumps, quinolone, macrolide, sulfonamide, nickel, fosfomycin, tetracycline and, phenicol in decreasing order (Figure 33A). Efflux pumps and fosfomycin resistance were found to be chromosomally encoded. Aminoglycoside nucleotidyltransferases, *ant(3")* was found on the chromosome but not on plasmids (Figure 33B). Other aminoglycoside resistance genes were found on both chromosome and plasmids. About 30% of both macrolide resistance genes, *msr(E) and mph(E)*, were found on plasmids.

In terms of classes of beta-lactamases, *bla*$_{OXA}$ was the most prevalent, followed by *bla*$_{ADC}$ and *bla*$_{TEM}$ (Figure 33C). In the *bla*$_{OXA}$ family, 83% of *bla*$_{OXA-23}$ was found in chromosomes and the remaining 17% in plasmids (Figure 33D). The high prevalence in *A. baumannii* chromosome is remarkable considering *bla*$_{OXA-23}$ ARG to be originally mobilised from the chromosomes of *A. radioresistens*. Next, *bla*$_{OXA-66}$ was found to be entirely chromosomal. This is a variant of the intrinsic *bla*$_{OXA-51-like}$ gene that has been associated with the International Clone 2 (ST2) isolates. Other intrinsic *bla*$_{OXA-51-like}$ ARGs, *bla*$_{OXA-69}$*, bla*$_{OXA-64}$*, bla*$_{OXA-65}$*, bla*$_{OXA-259}$*, bla*$_{OXA-68}$*, bla*$_{OXA-104}$ were found on chromosomes too. *bla*$_{OXA-235}$ and *bla*$_{OXA-237}$ (*bla*$_{OXA-235-like}$) ARGs were found on both chromosomes and plasmids. All *bla*$_{OXA-58}$ and *bla*$_{OXA-72}$ carbapenemase genes were found to be plasmid encoded, suggesting that they have yet to be introduced into the chromosomes unlike *bla*$_{OXA-23}$.

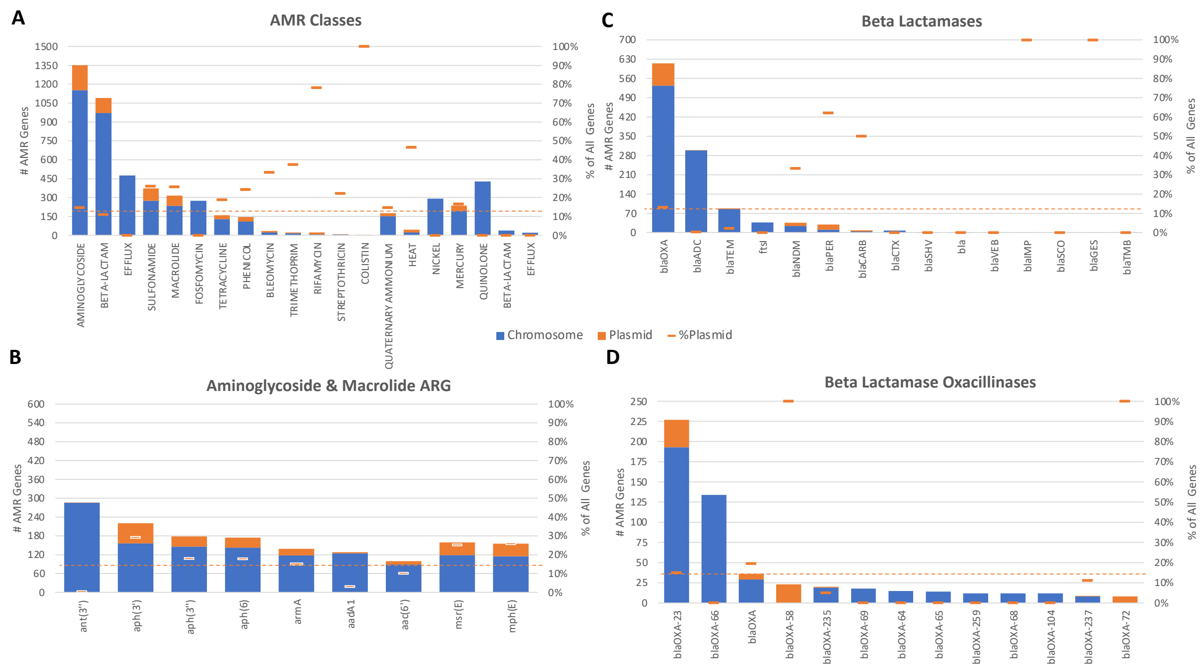

Figure 33. Number of AMR genes in *A. baumannii* chromosomes and plasmids in completed genomes. The ARG were separated by (A) AMR classes, (B) aminoglycosides and macrolide, (C) beta-lactamases and (D) beta-lactamase oxacillinases. The graph indicates the number of genes found in chromosomes (blue) and plasmids (orange). The percentage of the ARG found in plasmids is indicated by the orange solid line within the chart (annotated as %Plasmids) with the axis on the right. The dotted line indicates the percentage of *bla*$_{OXA-23}$ found in plasmids.

Next, we sought to understand the presence of ARG in different environmental sources, particularly in extra-hospital environment. ARG were found in non-host associated environments, with gene content in water, soil and plants lower than in animals and human isolates (Figure 34). Human and hospital-associated clinical isolates have the highest number of ARG gene count.

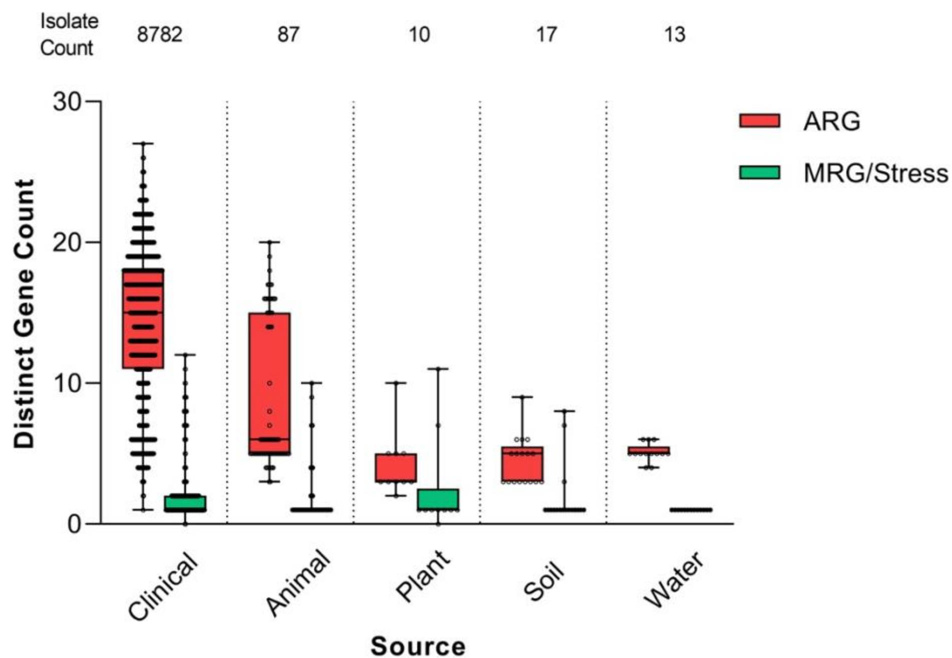

Figure 34. Box and whisker plot of distinct antimicrobial resistance genes (ARG), metal resistance genes and stress genes (MRG/Stress) count in *Acinetobacter* isolates (n = 8909, downloaded from NCBI MicroBIGG-E database on 19 Match 2021) from clinical (includes human and hospital-associated isolates), animal, plant, soil and water sources.

Clinically relevant ARG such as the carbapenemase-producing bla_{OXA-72} gene were found in water and soil isolates across continents. Conserved arrays comprising ARG, toxin-antitoxin and replicase genes were also present and syntenic in circular contigs from animal and clinical isolates, suggesting that genetically selfish plasmids mediated dissemination and horizontal transfer of ARG between ecologically diverse *A. baumannii* strains (Figure 35) (Jia et al., 2019). Toxin-antitoxin systems could play an important role in the maintenance of plasmids in the various environment without antibiotics pressure. These results suggest that the soil and water environment

present a potential reservoir for ARG and that antimicrobial usage in veterinary and clinical settings exerts selection pressure for ARG in these host environments.

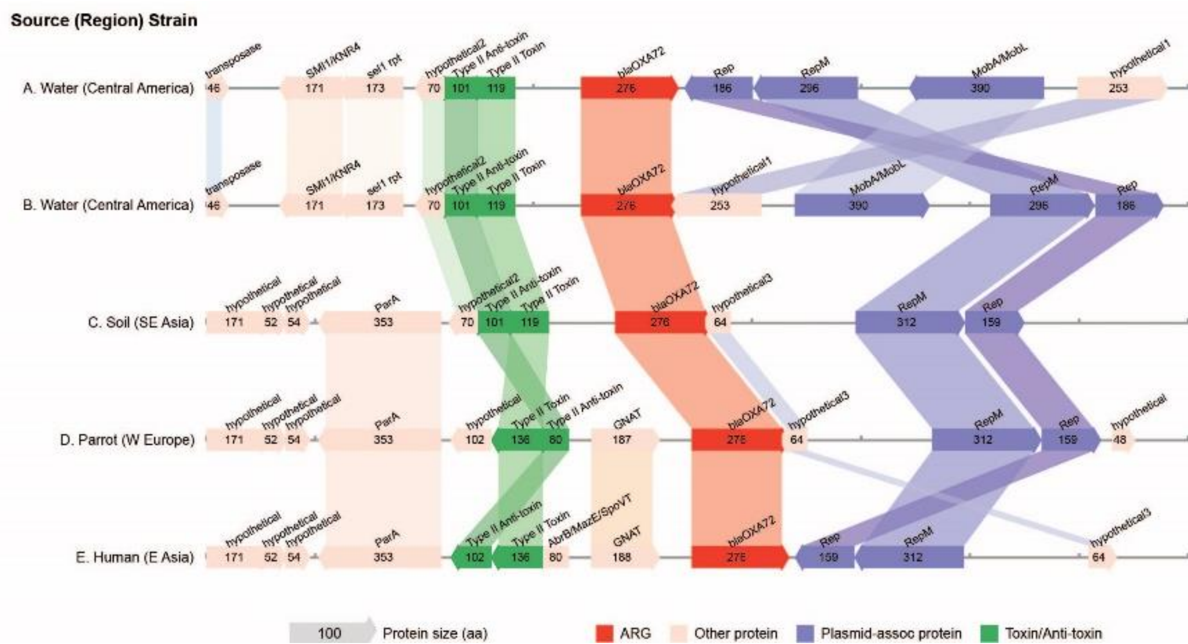

Figure 35. Gene synteny plot highlighting the dissemination of *bla*OXA-72 carbapenemase gene (coloured in red) in contigs NZ_JACZEE010000032.1 and NZ_JACZEI010000032.1 from environmental water isolates INTEC_AI10 and INTEC_BI15, soil isolate plasmid pK09-14 (KY704308.1), animal isolate plasmid pIHIT32296 (NZ_CP033871.1) and clinical human isolate plasmid pA52-OXA-72 (NZ_CP034097.1).

5.3.1. *bla*OXA-58-like variants

Earlier in Chapter 3.2.6 and Chapter 4, we have isolated an environmental megaplasmid pE88-42_1 and a clinical megaplasmid pAB26_1 harbouring *blaOXA-58*, which is one of the main *bla*OXA family circulating in *Acinetobacter* species.

*bla*OXA-58-like carbapenemases have been an under-studied group with 3 variants excluding *bla*OXA-58 reported in a review on OXA beta-lactamases in *Acinetobacter* species (Evans and Amyes, 2014). Given the potential of possible evolution of *bla*OXA alleles, we seek to understand if there have been new variants of *bla*OXA-58. To understand the diversity of OXA-58, we constructed a maximum likelihood tree based on protein alignment of 923 OXA family reference proteins from NCBI Reference Gene Catalog. We found 6 OXA-58-like variants, namely OXA-96, OXA-97, OXA-164, OXA-397, OXA-420 and OXA-512. These variants have one to four amino acid substitutions compared to OXA-58 (Figure 36). To understand the genetic context of these *bla*OXA-58-like variants, we accessed NCBI Pathogen Detection database of *A. baumannii* on 8 Oct 2021 to study complete genomes that harboured these variants. We found *bla*OXA-96, *bla*OXA-164 and *bla*OXA-420 in four different *A. baumannii* plasmids.

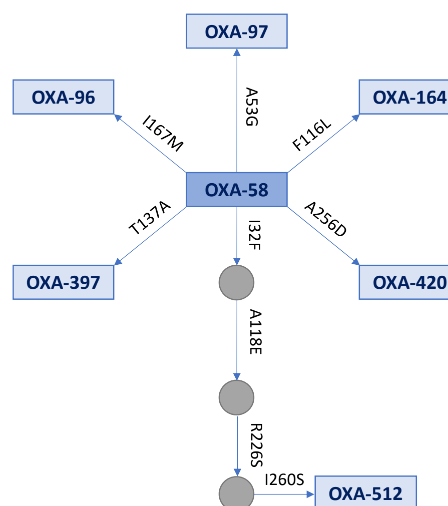

Figure 36. OXA-58-like variants (n = 6) and their amino acid substitutions from OXA-58. The grey circles represent hypothetical intermediate proteins in no specific order that would have accumulated the mutations to result in the final variant.

Comparing the different plasmids harbouring *bla*$_{\text{OXA-58-like}}$ variants, the plasmids did not share great similarity between them. However, the flanks of *bla*$_{\text{OXA-58-like}}$ gene were observed to be IS elements (Figure 37). Given the plasmid-mediated context of *bla*$_{\text{OXA-58-like}}$ gene with MGE, it prompted the potential idea that the variants could have evolved in plasmids. This was further supported by the earlier finding that all *bla*$_{\text{OXA-58}}$ ARGs were plasmid-mediated in *A. baumannii*.

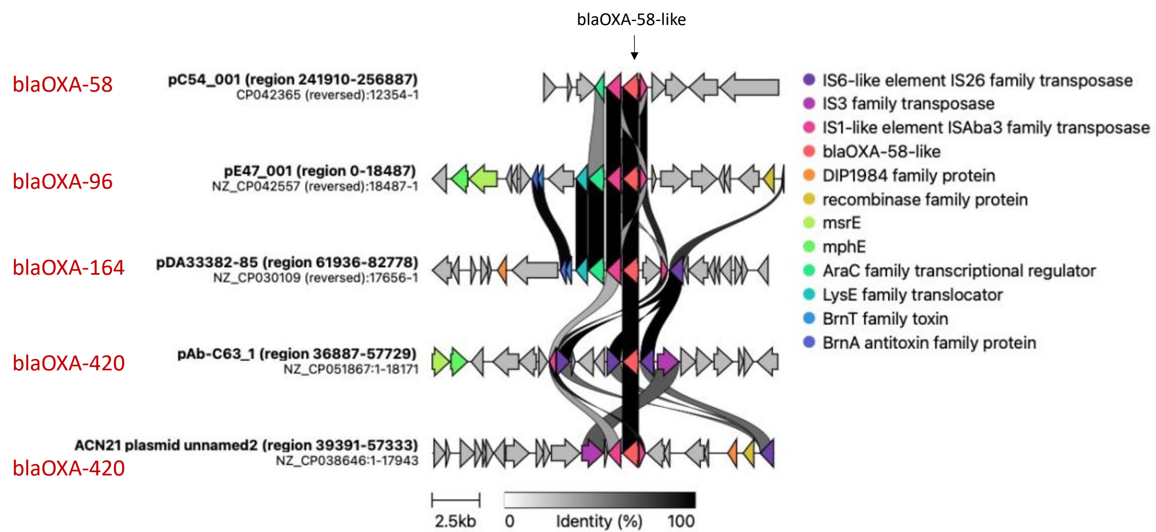

Figure 37. Gene synteny plot of *bla*$_{\text{OXA-58-like}}$ genes and their flanks in *Acinetobacter* plasmids. The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

5.3.2. ***bla*$_{\text{OXA-96}}$**

bla$_{\text{OXA-96}}$ was found in megaplasmid pE47_001 (NZ_CP042557.1, 327,867 bp) in *A. baumannii* E47 isolated from Australia in 2013. It was flanked by IS*3* and IS*Aba3* transposases which were similar to the flanks of *bla*$_{\text{OXA-58}}$ in pAB26_1. More than half (69%) of the plasmid were similar to pAB26-1 that harboured *bla*$_{\text{OXA-58}}$. It also had a hit against *A. johnsonii* XBB1 plasmid pXBB1-9 (NZ_CP010351.1) with a query cover of 81% and 98.92% identity, suggesting the inter-species dissemination of similar plasmids across into different *Acinetobacter* spp. that is not part of the Acb complex.

We found that 10% of the plasmid pE47_001 were 100% identical with part of plasmid p201330-IMP (NZ_MN961671.1) that was 168 kb in size from *P. aeruginosa* strain 201330 (Figure 38). Another *P. aeruginosa* strain PA34 plasmid pMKPA34-2 (NZ_MH547561.1) that was 26,862 bp in size also had an entire match to this region with 99.96% identity. This suggests that part of the pE47_001 plasmid harbouring $bla_{\text{OXA-96}}$ could be tracked to *P. aeruginosa* plasmid with the remaining from *Acinetobacter* species plasmid. We found the presence of IS*1182*-like element IS*Cfr1* family transposase, vicinal oxygen chelate (VOC) proteins, recombinase and Tn*7* like transposition proteins from the *P. aeruginosa* plasmid. An aminoglycoside resistance gene, *Aac(3)-IId*, was also found to be mobilised from the *P. aeruginosa* plasmid. Given the shared gene content and synteny, pE47_001 is likely a mosaic plasmid comprising of *Acinetobacter* spp. and *P. aeruginosa* plasmids.

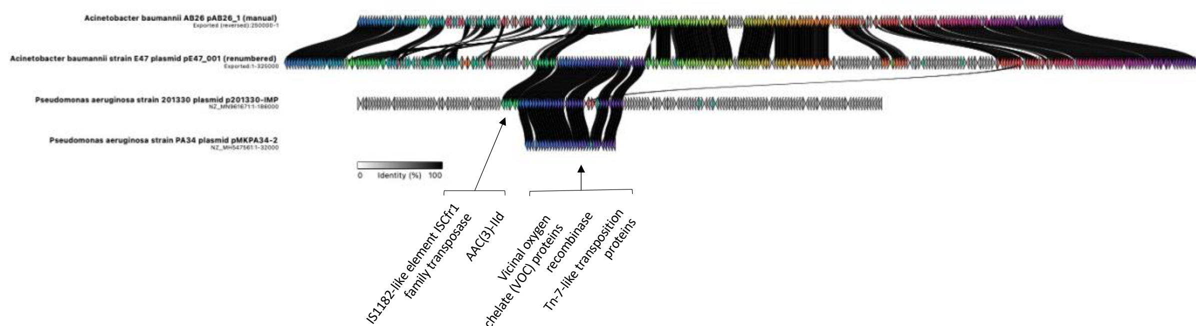

Figure 38. Gene synteny plot of *A. baumannii* E47 mosaic plasmid pE47_001 (second row) against *A. baumannii* AB26 plasmid pAB26_1 (top row) and *P. aeruginosa* plasmids p201330-IMP (second last row) and pMKPA34-2 (last row). Regions of *P. aeruginosa* plasmids were found in mosaic plasmid pE7_001. The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

5.3.3. *bla*$_{OXA-164}$

bla$_{OXA-164}$ was found in *A. baumannii* strain DA33382 plasmid pDA33382-85 (NZ_CP030109.1, 84,678 bp). The 84 kb large plasmid was isolated from a respiratory tracheal secretion sample in Germany in an unknown year. The plasmid shared little similarity (11%) with megaplasmid pAB26_1 isolated in this study. We found that the plasmid pDA33382-85 harbouring *bla*$_{OXA-164}$ had similar backbone as the other *bla*$_{OXA-23}$ large plasmids described earlier in Chapter 4.5.2. It shared higher query cover of 78% to *A. baumannii* plasmids harbouring *bla*$_{OXA-23}$, such as pAB98_contig2 in this study and pA85-3 (Figure 39). It was also similar with a query cover of 77% to *A. baumannii* SGH0905 plasmid pS32-2 which did not have *bla*$_{OXA-23}$ or any other *bla*$_{OXA}$ variants. According to gene synteny analysis, the additional region harbouring *bla*$_{OXA-164}$ has IS*30*-like transposase that could have resulted in the integration or recombination of this region into the large plasmid backbone. The *A. baumannii* SGH0905 plasmid pS32-2 backbone has also been described in Chapter 3 on the *A. seifertii* plasmid harbouring *bla*$_{OXA-82}$ (*bla*$_{OXA-51-like}$) and in Chapter 4 on the clinical *A. baumannii* harbouring plasmid-mediated *bla*$_{OXA-23}$.

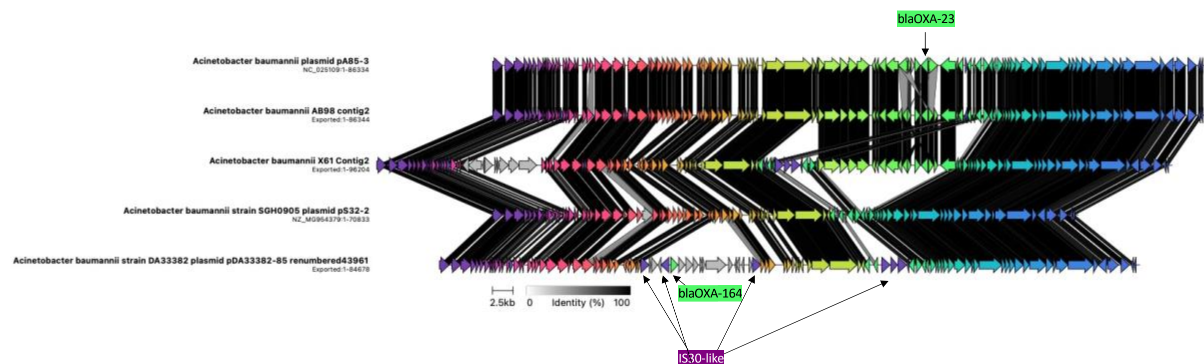

Figure 39. Gene synteny plot of large plasmid pS32-2 (second last row) and other similar plasmids capable of harbouring *bla*$_{OXA-23}$ and *bla*$_{OXA-164}$. Local clinical *A. baumannii* plasmids pAB98_contig2 and pX61_contig2 contained an additional regional harbouring *bla*$_{OXA-23}$. Plasmid pDA33382-85 harboured *bla*$_{OXA-164}$ (*bla*$_{OXA-58-like}$) gene and did not have the *bla*$_{OXA-23}$ region. The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

5.3.4. ***bla*$_{\text{OXA-420}}$**

bla$_{\text{OXA-420}}$ was found in *A. baumannii* ACN21 plasmid unnamed (NZ_CP038646.1, 57,333 bp) isolated from a bacteriaemia blood sample in India in 2018. The plasmid contained additional antimicrobial resistance genes, such as *mphR*, *msrE*, *armA*, *sul1*, *qacE*, *bla*$_{\text{CARB-2}}$. This plasmid was closely similar and syntenic to a human faecal isolate collected in Japan in 2012, *A. variabilis* strain RYU24 plasmid pRYU24 (NZ_LC591943.1, 68,069 bp) with a query cover of 97% and identity of 99.86% (Figure 40). In addition to *bla*$_{\text{OXA-420}}$ and the other ARGs, pRYU24 contained an additional region harbouring *bla*$_{\text{NDM-1}}$ and *aph(3')-6* resistance genes that may be likely to be obtained from *A. lwoffii* plasmid pNDM-BJ02 (NC_019281.1, 46,165 bp) possibly through recombination mediated by IS elements. This highlighted the role of inter-species plasmids circulating in *Acinetobacter* spp. population conferring diversity and drug resistance.

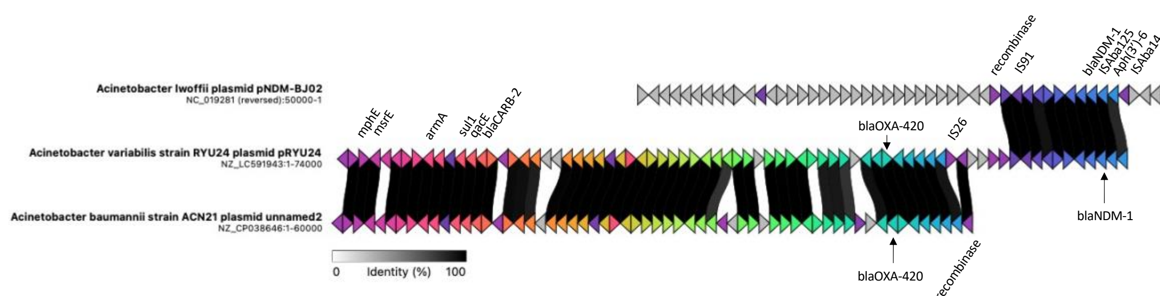

Figure 40. Gene synteny plot of *Acinetobacter variabilis* plasmid pRYU24 (middle row) harbouring *bla*$_{\text{OXA-420}}$, *bla*$_{\text{NDM-1}}$ and other ARG. *A. baumannii* ACN21 plasmid unnamed2 (middle row) harbouring *bla*$_{\text{OXA-420}}$ shared similar genes and synteny with plasmid pRYU24. *A. lwoffii* plasmid pNDM-BJ01 (top row) harboured similar *bla*$_{\text{NDM-1}}$ region that was flanked by IS elements. The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

bla$_{\text{OXA-420}}$ was likely to be related to *Acinetobacter sp.* WCHA45 pNDM1_010045 (NZ_CP028560.1, 190,170 bp) which had two occurrences of *bla*$_{\text{OXA-58}}$ and one

bla$_{\text{NDM-1}}$ (Figure 41). While the query cover and synteny between these two plasmids was low with query cover of 32% and identity of 98.4%, the *bla*$_{\text{OXA-58-like}}$ genes were observed to be flanked by similar IS elements.

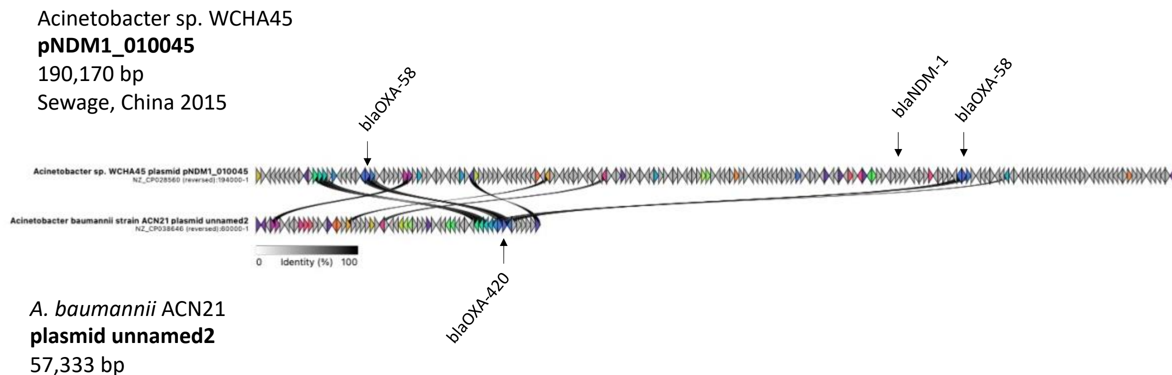

Figure 41. Gene synteny plot of *Acinetobacter* plasmids harbouring *bla*$_{\text{OXA-58}}$ (top row) and *bla*$_{\text{OXA-420}}$ (*bla*$_{\text{OXA-58-like}}$; bottom row). The shaded region between the sequences represents the percentage of amino acid identity. Alignment and visulisation were performed using Clinker (Gilchrist and Chooi, 2021).

bla$_{\text{OXA-420}}$ was also found in *A. baumannii* Ab-C63 plasmid pAb-C63_1 (NZ_CP051867.1, 81,353 bp) that was isolated from a sputum sample in Ghana, West Africa in 2016. This plasmid shared higher similarity of 61% query cover and 98.4% identity to *Acinetobacter sp.* WCHA45 plasmid pNDM1_010045 that was isolated from sewage in China in 2015. As described above, large plasmid pNDM1_010045 contained two copies of *bla*$_{\text{OXA-58}}$ and one copy of *bla*$_{\text{NDM-1}}$. This provides another evidence on our hypothesis that *bla*$_{\text{OXA-420}}$ could have evolved from *bla*$_{\text{OXA-58}}$ through these large plasmids.

5.4. Discussion

5.4.1. Genomic Epidemiology

The increasing use of whole genome sequencing has significantly expanded public databases facilitating continual monitoring for antimicrobial resistance. During the course of this project, the number of contributed genomes grew substantially as a consequence of international surveillance efforts and large genome centres (Sanger Centre UK, US DOE Sequencing Centers) that performed WGS and had open data-sharing policies. In the recent years, we had observed an increase in the number of *A. baumannii* genome sequences deposited (Figure 22). However, these genomes were largely of human clinical origins with only 0.35% (40/11,424) isolated from soil and water environment. Using the SNP Clusters identified from NCBI PD database, we found an environmental soil *A. baumannii* isolate that was related to clinical isolates from the same country and other parts of the world with similar ARG content (Figure 24). This demonstrated that environmental isolates could potentially be linked to clinical strains. Similarly, animal *A. baumannii* isolates were observed to be related to clinical strains and were also found in the context of ST10.

In this work, we have used NCBI Pathogen Detection database that included GenBank assemblies and short read archive data to provide a summary of isolates and their antimicrobial resistance genes. While the database is invaluable, it is limited by the completeness, quality and accuracy of data and metadata information provided by data submitters. These metadata included the isolation date, source, and antibiotics susceptibility testing phenotypic data. While NCBI has implemented several checks on the genomic data, such as taxonomic checks to ensure that the data submitted is reliable, it is important for researchers to perform their own quality check and curation to ensure that the data is accurate. We observed genomic data that deviated too far from the expected sizes or GC content to be biologically feasible (e.g. Plasmids in Figure 29). Such sequences could be a result of erroneous sequencing or a sequencing artefact. Currently, the database does not flag out these entries or provide a confidence scoring to assess its quality. Therefore, data curation and careful interpretation would be required when using public data. We excluded data that did not meet in-house quality criteria (e.g. assemblies with more than 500 contigs). It should also be noted that less than 3% of genome assemblies were close to finished

status, i.e. chromosomal or complete (complete genomes included the plasmids). Unfinished genomes in many contigs and scaffolds generally provide sufficient data for species identification, sequence typing (by MLST, ribosomal MLST), phylogenetic analysis (using ribosomal and core SNPs) and determination of virulence and resistance genes and genotypes. However complete genomes are required for other types of analyses and applications such as analysis of synteny, plasmid versus chromosome locations, upstream and flanking promoter elements, genomic pathogenicity islands, and resolution of copy number and repeats. Hence in our study, only a small subset (372 of 15,262) of all assemblies could be used for such high resolution analysis of plasmid versus chromosomal locations of ARG. Resolving genomic repeats such as rRNA operon, transposons and insertion sequences to finish a genome would require some form of long read sequencing or hybrid long/short read sequencing such as what we had performed in Chapter 3 and 4. While large scale projects for public health, agricultural and food surveillance depend on the higher throughput short-read sequencing, it is envisaged that availability of long read sequencers (e.g. Pacific Biosciences, Oxford Nanopore) could increase the submission of more complete and finished genomes.

At present, there is also a lack of metadata standardisation. For instance, some isolates annotated as from an "environmental" source were found that they were isolated from the hospital environment including catheters and wound dressings. This would affect the interpretation and classification of these isolates of clinical or hospital-associated origin. Isolates from human samples were also annotated variably, such as "*Homo sapiens*", "Human" or "human". The use of scientific names, common names, singular terms, plural terms, and lack of case sensitivity were some of the more prevalent issues observed in the metadata fields. These would require additional data clean-up and curation to ensure that they were annotated correctly for downstream analysis. Hence it is critical for researchers to share accurate metadata in a standardised manner together with deposition of high quality sequencing data in public databases as recommended by the Global Microbial Identifier initiative and others (Wielinga et al., 2017). This would enable the research community to build comprehensive visualisation dashboards for real-time surveillance and tracking of emerging outbreaks. Genomic epidemiological meta-analysis across clinical, veterinary, and environmental microbiology disciplines would provide fresh

multisectoral insights. The timely deposition, sharing and curation of whole genome sequences and comprehensive metadata are to be further encouraged.

Another limitation of using these databases is that the majority of the isolates were of clinical origin. These tend to be hospital-associated where non-serious community acquired infections may not be studied as often. They were either being isolated from the patient or from the hospital-associated environment. Within this population of bacteria isolated from humans, there is also a lack of isolates from healthy human population, such as from commensals or microbiome metagenomics studies. Missing metadata would also hinder the characterisation of these genomic data into analysis subgroups. There is a need to expand the database to include non-hospital environmental isolates. Together, these extra-hospital groups of isolates would be beneficial to better understand the intrinsic characteristic of *A. baumannii* for comparison against successful circulating clones.

As we have illustrated here, clinical *A. baumannii* could be potentially linked to environmental and animal sources. Further analysis of ST10 clade revealed that this clade is non-clonal, consisting of diverse isolates with different branch lengths. The diverse ARG profiles further suggest that these ARG could be introduced horizontally through plasmids. The predominance of clinical isolates and lack of non-hospital environmental isolates in the global dataset may be a bias as certain clades might be overrepresented. With limited environmental data, the environmental origins and reservoir of ST10 isolates remain unclear. Environmental surveillance, particularly in non-hospital or non-veterinary settings, is therefore important for the understanding of ARG transmission. To these ends, the (97) genomes of environmental Acb isolates sequenced in Chapter 3 would constitute a significant addition to the current global data. There is also a need for One Heath approach for potential extra-hospital reservoirs, including soil and water, to better understand the dissemination and transmission of community-acquired *A. baumannii* infections and antimicrobial resistance.

5.4.2. Plasmidome

A. baumannii plasmids are diverse, with a wide range of sizes and are capable of acquiring different ARGs. Plasmids were found in 78% of *A. baumannii* isolates with a median of two plasmids per genome. This suggests that *A. baumannii* is plasmid rich. We found that almost a third of the plasmids harboured ARG, consistent with a previous study on *A. baumannii* plasmids (Salgado-Camargo et al., 2020). The remaining plasmids without ARG may be responsible for other functions that have yet to be characterised. These plasmids might harbour genes associated with plasmid maintenance and stability, conferring evolutionary advantages, so that they can persist in and across bacterial populations. In addition, the possession of genetic modules, such as TA systems, partitioning proteins, and conjugation systems, would allow the plasmids to be maintained in the host. Taken together, plasmids can potentially serve as a backbone for the introduction of ARG mediated by MGE.

Plasmids were found to harbour different ARG classes such as aminoglycoside, beta-lactam, sulfonamide, macrolide and tetracycline. Interestingly, bla_{OXA-58} and bla_{OXA-72} were exclusively found on plasmids, suggesting that they have yet to be introduced into the chromosome. On the other hand, 17% of bla_{OXA-23} were found in plasmids, with the remainder in chromosomes. This could be due to the high number of IC1 and IC2 clones which could harbour AbaR GRI containing bla_{OXA-23}. The classes of ARGs harboured on plasmids also differed according to plasmid size. ARG against aminoglycoside and beta-lactam were found across all plasmid sizes while ARG against sulfonamide and macrolides were more commonly found in large and megaplasmids. Earlier in Chapter 3, we observed that different ARGs were disseminated through distinct plasmids in *A. seifertii.* This further suggests that different types of plasmids (grouped by their sizes) may contribute to the spread of different ARGs. Plasmid sizes are known to be inversely correlated with plasmid copy number and these plasmid properties would impact its evolution, segregation, partitioning, vertical transmission and modulation of horizontal gene transfer (Carroll and Wong, 2018; Ilhan et al., 2018).

Despite the large number of isolates sequenced (more than 15,000), there were relatively few complete plasmid sequences available (less than 700). Given the increasing evidence of plasmids with broad host range, it would be relevant to include

the analysis of Acb complex and *Acinetobacter* spp. plasmids. By expanding the analysis to include these close neighbours, the role and extent of plasmids in horizontal gene transfer of plasmids could be better elucidated. Using plasmids harbouring *bla*$_{OXA-58-like}$ ARGs as examples, we found that MGE and mosaic plasmids could play an important role in the plasticity of plasmids as well as contributing to the plasmidome of *Acinetobacter* species.

It is an ongoing challenge to identify plasmids from short read analysis (SRA) data. It can be difficult to reconstruct plasmids, especially larger plasmids over 50 kb, from short read sequencing data or from fragmented assemblies (Arredondo-Alonso et al., 2017). In addition to the presence of repeats that may pose a challenge to assemble longer contigs, plasmid sequences may also integrate into chromosomes, further complicating the determination of plasmid contigs. The prediction of plasmid sequences is critical to understanding the context of ARG in the bacterial genome.

There are limited strategies and tools for the *in silico* typing of *Acinetobacter* plasmids. For instance, *Enterobacteriaceae* plasmids can be characterised based on their incompatibility (Inc) groups. Online tools such as PlasmidFinder and pMLST allow *in silico* detection and typing of these Inc groups from sequencing data and can aid the prediction of plasmids in *Enterobacteriaceae* isolates and selected Gram-positive species (Carattoli et al., 2014). However, these tools are not applicable for the analysis of *Acinetobacter* plasmids. An early study had proposed the use of PCR-based replicase (*rep*) gene typing for the characterisation of *Acinetobacter* plasmids (Bertini et al., 2010). Broadly, plasmid typing approaches for *Acinetobacter* plasmids can be divided into replicon typing and plasmid mobility (MOB) typing which were based on plasmid replication (replicase) and mobilisation function (relaxase) respectively (Orlek et al., 2017a; Orlek et al., 2017b). A more recent study has proposed the classification of *Acinetobacter* plasmids based on a combination of factors including size, replicase (*rep*) gene and mobilisation (*mob*) gene (Mindlin et al., 2020). However, there are no web or bioinformatics tools designed for the typing of *Acinetobacter* plasmids at present.

There are emerging tools that explore the use of machine learning to computationally predict the presence of plasmids. One such example is PlasForest which uses homology-based machine learning classifier to identity plasmids from contig and

scaffold level genome assemblies with high sensitivity to detect plasmid contigs over 50 kb with 2.2% of false positives (Pradier et al., 2021). Another tool, Deeplasmid, uses deep learning approach to predict plasmids from assembled contigs by considering the sequences and features such as GC content, contig gene density and hits against genes and protein domains specific to chromosomes or plasmids (Andreopoulos et al., 2021). While these tools do not require complete assemblies, it is important to understand that they may yield false positives and can only be used as an indicative prediction of the putative plasmids. These new tools could be further explored for the identification of plasmids in other large genomic datasets, such as in metagenomic data. Long read sequencing remains to be the gold standard to identify plasmids in bacterial isolates. As more complete genomes are sequenced, there would be more plasmids deposited in the public database to better understand plasmid epidemiology and evolution.

Another public web resource is the Plasmid Database PLSDB (Galata et al., 2018). It is an interactive platform that has integrated the plasmid records and associated metadata information from NCBI Nucleotide database. The web server allows users to browse the plasmid records through filtering the various parameters or searching using the Mash search of plasmids or BLASTN search of genes. Though it includes plasmid typing data using PlasmidFinder and pMLST, this additional information is relevant for *Enterobacteriaceae* but not for *Acinetobacter*. Unfortunately, the PLSDB is not being updated real-time to reflect the live database. As of 10 Jan 2022, the PLSDB database was using a version dated 23 June 2021 that contained information of 518 *A. baumannii* plasmids which was less than the current 682 plasmids available in NCBI. Having a real time database would ensure up-to-date analysis and surveillance of potential clusters and plasmids of concerns.

5.4.3. Resistome

Large diversity of acquired and intrinsic *bla*$_{OXA}$ were observed in *A. baumannii* genomes. We investigated the genetic context of *bla*$_{OXA\text{-}58\text{-}like}$ carbapenemase genes and revealed that plasmids could be a potential source for the evolution of *bla*$_{OXA\text{-}58}$ variants. The analysis tapped upon the use of NCBI Pathogen Detection and MicroBIGG-E database consisting of publicly available genomes and their submitted metadata to understand its epidemiology and transmission. *bla*$_{OXA\text{-}58}$ carbapenemase genes were found to occur exclusively on plasmids. The presence of *bla*$_{OXA\text{-}58}$ variants on large plasmids were investigated and found to share similar IS*Aba* gene flanks when compared with other known plasmids in NCBI Plasmids database. While our analysis is limited by the number of completed genomes harbouring *bla*$_{OXA\text{-}58\text{-}like}$ genes, our findings suggest that these plasmids could have played a role in the evolution of these variants. The presence of IS element flanking *bla*$_{OXA\text{-}58}$ could recapitulate the emergence of *bla*$_{OXA\text{-}23}$ ARG which was mobilised out of *A. radioresistens* chromosome into plasmids. *bla*$_{OXA\text{-}23}$ from these plasmids eventually got integrated into *A. baumannii* chromosomes. The conserved gene segments could allow site-specific homologous recombination of *bla*$_{OXA\text{-}58}$ to exchange between plasmids or be mobilised into a chromosome. We have found closely related plasmids to these variants that either share high similarity or gene flanks surrounding the ARG.

We found evidence of a mosaic 328 kb megaplasmid pE47_001 comprising sequences from both *A. baumannii* and *P. aeruginosa* plasmids, suggesting that some of its plasmid gene content may originate from a broad host range between orders in the *Gammaproteobacteria* class of Gram-negative bacteria. While we were unable to determine the possible mechanisms of how the mosaic plasmid was formed, we observed that the regions of the mosaic plasmids were flanked by MGEs. Transposons have been described to be responsible for possible recombination events contributing to mosaic plasmids (Pesesky et al., 2019). This highlights the importance of identifying and studying the presence of MGE in the plasmids to better understand their role in contributing to plasmid plasticity. The mosaic plasmid further indicates that the *Acinetobacter* plasmidome could be influenced with the influx of genes through plasmid regions from other families and classes of bacteria.

We also discovered a plasmid that harbours the *bla*$_{OXA-58-like}$ *variant, bla*$_{OXA-420}$ from *A. variabilis*, a species isolated from animals and humans, including from blood, urine and wound sites (Krizova et al., 2015). This relationship was detected using BLAST search against *Acinetobacter* plasmid database, rather than the NCBI Pathogen Detection database, which comprehensively covers Acb complex genomes, but not other *Acinetobacters*. This finding further demonstrates the role of inter-species plasmids in the dissemination and evolution of ARG from an *Acinetobacter* species outside the Acb complex.

5.5. Conclusion

Public genome databases are an emerging resource with the increasing use of whole genome sequencing in routine clinical and research settings. Majority of *A. baumannii* isolates in NCBI Pathogen Detection database were of clinical origins with diverse ARG. Using the limited number of complete genomes available, plasmids were found in 77.6% of *A. baumannii*, with 35.3% of plasmids containing ARG. *A. baumannii* plasmids have distinct size groups and the potential to harbour a wide variety of ARG to confer multi-drug resistance. The inter-species dissemination of these plasmids demonstrated that other species outside of Acb complex could be a potential resistome reservoir. Carbapenem resistance genes *bla*$_{OXA-58}$ and *bla*$_{OXA-72}$ were found exclusively on plasmids. Furthermore the presence of MGE flanking *bla*$_{OXA-58}$ and its variants suggests that ARG could evolve in plasmids and are at risk of being mobilised by IS elements. With the expansion of genomes available and open data availability, there is a need to scale towards having more automated analysis tools and interactive platforms for better data visualisation. The development of microbial genomics dataverse will be beneficial for genomic epidemiology studies.

6. Conclusion and Future Work

6.1. Conclusion

In this work, *A. baumannii* and their antimicrobial resistance determinants in the non-hospital environment were characterised and compared against local clinical isolates as well as those in public databases to assess their genetic relatedness. Diverse *A. baumannii*, together with other members of Acb complex and *Acinetobacter* spp., were isolated from the urban roads, soil and water environment. Interestingly, the prevalence of *A. baumannii* was higher in urban road than soil samples. The ability to survive on inanimate surfaces suggests the dynamic metabolic ability of *A. baumannii*, and its ability to survive in other unidentified ecological niches. Environmental ST10 isolates from urban water canals shared genetic relatedness with local ST10 clinical isolates, suggesting that the aquatic environment could be a potential source of CAI. While we have discovered and characterised two novel species from the water environment, there are likely other novel *Acinetobacter* spp. have yet to be isolated and characterised. We found a lower prevalence of acquired ARG in environmental and animal isolates, indicating that ARG burden is highest in the clinical setting. However plasmid-borne ARG are found in environmental *A. baumannii* that are not hospital-associated. In addition to conferring AMR, plasmids could also contribute to the repertoire of accessory genes as seen in the pangenome analysis of *A. seifertii*. The natural and urban environment remains to be an understudied reservoir for *A. baumannii* and the Acb complex species.

The genomics study of clinical Acb complex in Singapore indicated that *A. baumannii* remains as the primary species isolated from bloodstream infection samples in the hospital. Clinical *A. baumannii* were largely from International Clone 2 and International Clone 1, though other diverse ST, including ST10 were found too. The genomic epidemiology of clinical *A. baumannii* isolates in Singapore conforms with the current understanding that IC1 and IC2 are the main circulating clones in the hospitals. Multi-drug resistance was found mostly in IC2 and IC1 isolates with *bla*$_{OXA-23}$ as the main source of carbapenem resistance, again in keeping with other international reports. In our study, we found one ST10 clinical isolate to be phylogenetically related to *A. baumannii* with similar intrinsic ARG isolated from environmental water. This suggests that clinical disease, particularly community-acquired infections (CAI), could

arise from environmental sources, and that environmental isolates are potentially virulent. More environmental studies are needed to better understand the natural environmental reservoirs of *A. baumannii* such as the urban terrain, both biotic and abiotic. Other Acb complex species, *A. nosocomialis* and *A. pittii,* were present among nosocomial and community-acquired infections, and these are targets for future study of ARG transmission.

Plasmids play an important role in the dissemination of ARG across the Acb complex (Figure 42B). ARGs against carbapenem, sulfonamide and tetracycline were found in our environmental *A. baumannii* isolates and they are plasmid borne. These plasmids consisted of different sizes and shared similarities to plasmids reported in other Acb complex isolates. This suggests that apart from clinical isolates, ARGs were also circulating within the environmental isolates of the Acb complex. For example, carbapenem resistance ARG bla_{OXA-72} was identified in the small plasmid of an environmental *A. baumannii* that we isolated from a soil sample far from human settlement. In clinical isolates, we found large plasmids containing another carbapenem resistance gene bla_{OXA-23} in the genetic context of the transposon Tn*2006*. The same transposon with bla_{OXA-23} has shown to be integrated in *A. baumannii* chromosome (Figure 42C). We have also sequenced a megaplasmid that conferred MDR to a clinical *A. baumannii* strain. An analysis of the *A. baumannii* plasmidome from global strains revealed a large diversity of plasmids and their ability to harbour many different classes of ARG.

The evolution of ARG and its flanking genes are mediated by MGE. The carbapenem resistance gene, bla_{OXA-58}, and its $bla_{OXA-58-like}$ variants were found in different large plasmids flanked by IS elements (Figure 42D). These genes could be mobilised onto a suitable plasmid via MGE. They could then persist by capitalising on other elements in the plasmid for plasmid maintenance and stability in its ecological niche, such as the toxin-antitoxin systems. Alternatively, they could be maintained by conferring a fitness advantage to the host in the presence of antibiotic or nutrient selection pressure. One of the consistent observations across our studies is that similar plasmids were often found in different *Acinetobacter* species suggesting that they could have a broad host range and transfer horizontally between different strains and clades (Figure 42E). This would also mean that other *Acinetobacter* spp. could be a reservoir of ARG for *A. baumannii.* The evidence of a mosaic plasmid with sequences from both *A. baumannii*

and *P. aeruginosa* suggests the possibility of shared gene content between bacteria from two different genera. Inter-species dissemination of plasmids poses a risk of introducing antimicrobial resistance between Acb complex as well as other *Acinetobacter* species.

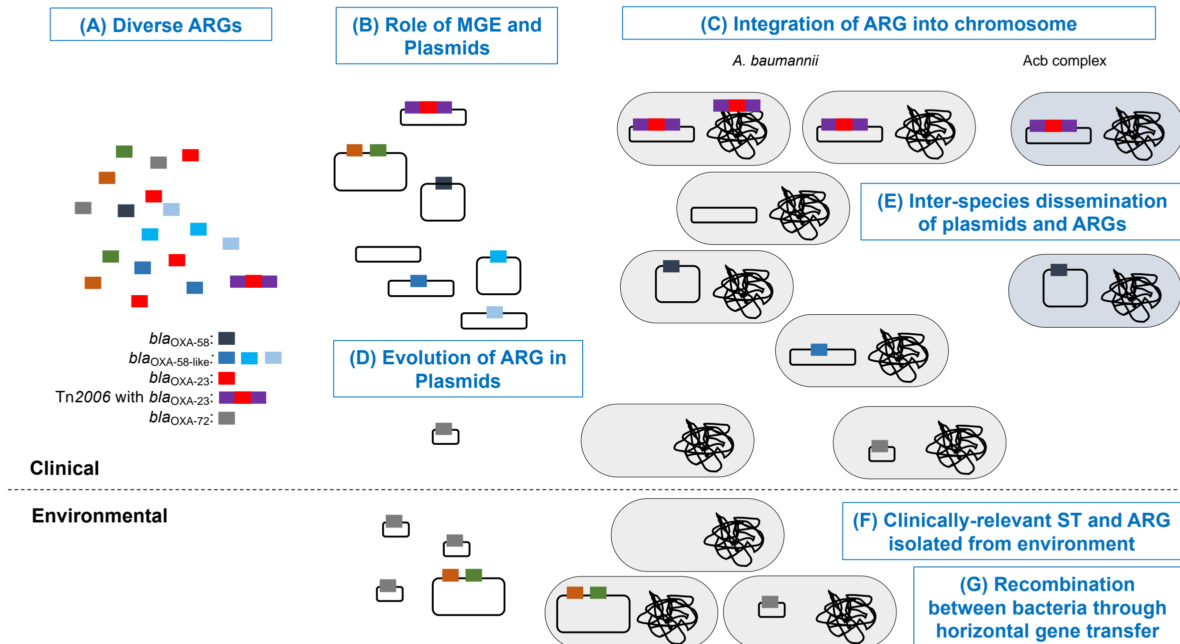

Figure 42. Schematic overview of ARG transmission mechanisms identified in this study. Diverse ARGs were observed in the resistome of *A. baumannii*. (B) Some ARGs were found to be plasmid-borne with the presence of MGE. (C) *bla*$_{\text{OXA-23}}$ in Tn*2006* could be integrated in both the chromosome and plasmid. (D) *bla*$_{\text{OXA-58}}$ have been found circulating in plasmids where *bla*$_{\text{OXA-58-like}}$ could have evolved and arised. (E) The plasmids with their clinically-relevant ARG were observed to disseminate across Acb complex and beyond. (F) The environment is a potential reservoir for clinically relevant ST and ARG. (G) The environmental reservoir presents an opportunity for genetic recombination between bacteria through horizontal gene transfer of plasmids and ARG between different hosts and niches.

We hypothesised that *A. baumannii* ARG originate from the environment. In this work, environmental *Acinetobacter* spp., including Acb complex and novel species, were isolated from novel non-hospital and urban environments which were non-human related. Environmental A. b*aumannii* were diverse and had shared ST with clinically

relevant isolates. We discovered a plasmid harbouring the *bla*$_{OXA-72}$ carbapenemase resistance gene in *A. baumannii* isolated from a pristine environment where carbapenems are not used. We suggest two possible scenarios for such ARG to persist and disseminate in the environment. Firstly, *bla*$_{OXA-72}$ could have been introduced from humans to the environment without antibiotics selection pressure through plasmid persistence modulated by the TA system found on the plasmid. Alternatively, the environment could have provided some form of selection pressure from unidentified carbapenem analogs or a beta-lactam metabolic source for *bla*$_{OXA-72}$ to be maintained. Such environmental reservoirs present a risk for ARG to originate from the environment and subsequently mobilised into the bacteria hosts. MGEs involved in the mechanisms of horizontal gene transfer via plasmids were identified in the plasmids. ARGs shared flanking IS elements and transposons which would allow them to mobilise and hitchhike onto larger ARG arrays and eventually as a GRI into the chromosome. Plasmidome and synteny analyses showed this accumulation of ARG in MDR isolates. Further evolution of ARG may occur in plasmids, as seen by the point mutations of *bla*$_{OXA-58}$ carbapenemase resistance gene resulting in *bla*$_{OXA-58-like}$ variants. These variants have been observed in different plasmids where additional ARGs were accumulating. We suggest that the microbial consortium in the environment could provide further plasmid plasticity with the help of MGE. This could result in the exchange of gene segments harbouring ARG in mosaic plasmids. Eventually, ARG and GRI were mobilized into circulating clinical clones conferring them MDR and XDR statuses. These evidences suggest that ARG could have originated from the environment from a gene-centered view.

In conclusion, *A. baumannii* could be circulating in the non-hospital urban environment with the potential to acquire ARG through horizontal gene transfer. The environment could be a potential reservoir for Acb complex capable of causing nosocomial and community-acquired infections (Figure 42F). The interaction of isolates from different environmental niches could result in recombination events that play a role in dissemination of plasmids containing ARG through horizontal gene transfer (Figure 42G). The use of whole genome sequencing and comparative genomics study have highlighted the crucial role that plasmids play in the dissemination of ARG between different Acb complex and beyond. The continual surveillance of *Acinetobacter* spp.

and their resistome in the natural environment and hospital is needed to understand their origin, transmission, and evolution.

6.2. Significance

This work provides a deeper insight into the possible natural environmental reservoirs of the Acb complex, including *A. baumannii* and its possible origin in community acquired infections. The study determines the resistome of Acb complex in the non-hospital and host environments to establish the prevalence and diversity of AMR genes in these populations. By tapping upon Acb complex genomes from publicly available databases, this study provides a broader perspective to analyse the local environmental and clinical isolates. Using the One-Health approach, we demonstrate how horizontal gene transfer mechanisms and evolutionary mechanisms may be involved in the transmission and evolution of AMR genes between environmental and clinical Acb complex isolates. The openness sharing of WGS data and availability of new bioinformatics and cloud computing tools enable the analysis of a large retrospective genomic datasets to allow the study of emerging trends across geography and time. By understanding how antimicrobial resistance originate and spread from the community and into the hospital, proper quarantine and infectious disease control measures can be implemented to minimize the clonal transmission of antimicrobial resistance genes.

6.2.1. Translational Impact

One Health surveillance across different environments in Singapore would detect new sources of AMR and bacterial strains of interest. Routine public health monitoring of samples by isolation and sequencing could be carried out on an ongoing basis by the relevant authorities for soil and water (National Parks and Public Utility Board), imported food, plants, meat (Singapore Food Authorities), wildlife animals (Animal & Veterinary Service) and local cases of pneumonias (community-acquired pneumonia and ventilator-associated pneumonia), bloodstream infections and wound infections from primary and tertiary care (Ministry of Health). This would expand onto the existing work done in this study to track and monitor *A. baumannii* and its ARG in different sectors.

Antibiotic stewardship would continue to play an important role in preventing the rise of AMR. Curtailing of high-risk non-clinical antibiotics use would be necessary, especially in agricultural, aquacultural and veterinary use. This would entail a global effort to reduce and eliminate antibiotics use in farms and agricultural produce. The

finding of *bla*$_{OXA-72}$ carbapenemase gene on a plasmid isolated from a pet parrot further highlights the need for antibiotics stewardship in veterinary settings (Prenger-Berninghoff et al., 2017). Domestic animals such as dogs may present a potential reservoir for clinically relevant ST as observed in *A. baumannii* ST10. Clinically, more targeted antibiotics prescription using precision medicine would be required. Prescription of antibiotics should ideally be made after WGS and genotyping of clinical isolates with first-line antibiotics treatment for non-MDR strains. These multi-sectoral efforts would lead to more appropriate use of antibiotics and limit the selection pressure of ARG in these settings.

Clinical WGS for clinical microbiology can be implemented for the routine sequencing of all strains in addition to current microbiological culture and AST. This would provide epidemiological surveillance for the tracking of nosocomial infections and early identification of outbreak clones. The ARG genotypes would complement the AST phenotypes to better inform antibiotics prescriptions based on the prevalence of AMR. This would enable timely infectious disease control measures to be implemented to limit further hospital-acquired transmission.

The global genome repository of *A. baumannii* would increase significantly from these WGS efforts. At present, there are less than 50 *A. baumannii* genomes from Singapore out of over 15,000 genomes worldwide. Having additional local genomes from different environments would enable further investigation into the evolution of *A. baumannii* and ARG in Singapore. For instance, variants of AbGRI3 carrying the armA aminoglycoside resistance gene were found in Singapore (Blackwell et al., 2017).

Lastly, the urban environment could be a possible a risk factor for *A. baumannii* CAI. Further environmental sampling and epidemiological screening would be beneficial to identify residential and occupational exposure (e.g. drivers and street workers) to *A. baumannii*. This would yield One Health insights on whether these environments may present a higher risk of infection.

6.3. Limitations of Study

We have examined *A. baumannii* from the environment and clinical setting as well as those deposited in the public available database to better understand its genetic relatedness between the isolates and their resistome. Environmental sampling was limited to a convenient random sampling of different non-hospital environment to determine the prevalence of *A. baumannii.* On the other hand, clinical isolates were limited to bloodstream infection isolates that were mostly of nosocomial or healthcare-associated origins. There is a lack of systematic environmental sampling and community-acquired infection isolates for source tracking and determination of the role of environmental reservoir for community-acquired infections. However, this study has provided a foundational understanding of this research gap for follow-up work to be conducted in this area.

Genomic epidemiology has yielded rich data to support the possibility of an urban environmental reservoir containing clinically relevant ST *A. baumannii*. It has also provided evidence of inter-species plasmids containing ARG and its dissemination into different *Acinetobacter* species globally. However, one of the limitations of using such an approach is that genomic epidemiology itself can suggest but cannot prove horizontal gene transfer, plasticity, selection and evolution with absolute certainty. These would require further functional and mechanistic studies with the available environmental and clinical isolates. Nevertheless, data from comparative genomics are useful to develop hypothesis to aid the interpretation of the genomic diversity and patterns observed and guide future functional studies. Genomic epidemiology allows the identification of trends, anomalies and deviations from the norm which would inform further investigations. Global datasets value-add onto individual case studies by including a greater representation of the entire population structure. This approach provides a more comprehensive understanding of the genomic landscape.

One of the key limitations of using publicly available data from NCBI Pathogen Detection and MicroBIGG-E database is that apart from ARG and MRG gene, it does not contain other genomic features that would be helpful in the analysis and interpretation of AMR. It would be ideal if the coding sequences from GenBank annotation data files could be integrated in the analysis. Additional genomic features such as the presence of mobile genetic elements, virulence factors, CRISPR array

and prophages, would yield richer information about the genome. Transposons and insertion sequences found along the flanks upstream or downstream of ARG would provide an indication on the potential mobilisation of the gene (Partridge et al., 2018). Another feature, such as plasmid-*dif* (*pdif*) modules that could be involved in XerCD-*dif* site-specific recombination, could also be included when analysing the plasmids (Balalovski and Grainge, 2020). These inverted repeats modules could mediate the mobilisation of genes that they flanked, and have been found to be flanking certain ARG, such as bla_{OXA-72} (Andrea et al., 2009; Brasiliense et al., 2019; Fonseca et al., 2020). There are other elements of the plasmids, such as their gene density, gene function, toxin-antitoxin system, partitioning system, or conjugative elements, that were not included in these databases. Having a fully annotated genome would allow deeper analysis and inference of plasmid properties, such as predicting the possible horizontal gene transfer mechanisms that it could perform.

Although it is possible to study these additional features in individual genomes or small sample sets, it would be challenging to implement this for a large genome dataset. With Acb complex genomes expected to increase to tens of thousands of assemblies in the coming months, integrated pipelines and workflows are needed for high throughput analysis of the genomes. It is important that these databases provide the provenance of additional analyses that were conducted so that users can assess the relevance and currency of the data. The automated pipeline can perform quality checks, characterisation, typing and prediction of properties based on the genomic features present in the genome. Having a dataset rich in genomic annotations will provide insights into the relevance and importance of plasmids in the dissemination and evolution of ARG. The dataverse of microbial genomics could enable more informative comparative genomics, synteny analysis and genomic epidemiology studies.

6.4. Future Work

A follow-up systematic environmental study can be conducted to assess if *A. baumannii* can be repeatedly isolated in the same sites to show its spatial and temporal distribution. This would enhance the understanding of its prevalence in the urban environmental reservoir and provide additional isolates to compare the evolution of genomes across time. The proximity of anthropogenic activities could also be measured to better understand the impact of human activities on the soil samples collected. Using the environmental isolates obtained from this study, functional studies can be done to determine their virulence and metabolic abilities. These experiments may include assessing their ability to metabolise complex hydrocarbon and organic compounds, persist on inanimate surfaces, form biofilms, and cause infection. Future studies can seek to determine the microbial consortium on the urban environment harbouring *A. baumannii* to understand their interactions with the microbial communities outside of the host environment. These would provide insights onto the gene flux between *A. baumannii* and other bacterial species. Having a systematic characterisation will allow the determination of possible ecotypes of *A. baumannii* that can occupy different ecological niches.

The resistome of Acb complex can be further studied by including an in-depth analysis of plasmids and mobile genetic elements. Different plasmid lineages could be studied experimentally to assess its stability and persistence with and without antibiotics selection pressure. For instance, the *bla*$_{OXA-72}$ encoding plasmid isolated from the soil environment could be assessed if it would be lost upon passaging in an antibiotic-free culture condition. Apart from the known plasmid genes, such as replicases, relaxases and TA systems, there are many other hypothetical proteins in the plasmidome that have yet to be characterised. An *in silico* gene ontology study could be useful to determine the potential functions of these genes, particularly if they were associated with conjugation. The mechanism behind the horizontal gene transfer of these plasmids across different species can be further assessed through functional studies. Alternatively, an expanded comparative genomics to include other Gram-negative bacteria, such as *Enterobacteriaceae*, would be helpful to assess the shared resistome and plasmid gene content with different species. Long term *in vitro* evolution studies could assess the possibility of *bla*$_{OXA}$ family ARG evolving into new variants in plasmids under selection pressure. This might be able to elucidate if plasmids could

be responsible for the evolution of *bla*$_{OXA-58-like}$ carbapenemase variants. We have also previously observed that IS elements and transposons may have been involved in the mobilisation of ARG across plasmids. Studies that only show the presence and absence of ARG may miss value information on its increased expression by IS elements. The study of IS element insertion sites in the genomes could provide valuable insights into the potential ability of IS elements being integrated into the genomes. From a genomics perspective, the flanks of the ARG should be studied in greater detail to determine if there were MGE, IS elements or other genomic features in close proximity that could affect gene expression and function.

In AMR studies, metal resistance is often overlooked, presumably due to its lack of direct implication on infection control and treatment. However, the study of MRG may yield insights into their relationship with ARG in the genomes. Metal resistance have been described to be associated with antimicrobial resistance and sometimes to co-occur with ARG (Baker-Austin et al., 2006; Li et al., 2017; Pal et al., 2017). We have observed a gradient in ARG abundance from environmental isolates to animal and human isolates, but not for MRG abundance. A gene-co-occurrence analysis or network analysis could be performed to study the association between ARG and MRG to explore the possible ecologically driven selection of AMR. Next, functional studies could be carried out to assess if these MRGs are active. Different MRG, such as *mer* mercury resistance operon, were found in some of the plasmids in *A. baumannii* and *A. seifertii*. Similarly, intrinsic nickel resistance gene, *nreB*, was found in all isolates of these two Acb complex species. *nreB* gene could be further studied to determine its prevalence, diversity and function in Acb complex. The genetic locus of *nreB* in the chromosomes would provide information on whether the gene has been passed down vertically, or if there is an ancestral source from one of the *Acinetobacter* species. If the gene is indeed active with a functional purpose, it could also be assessed for evidence of convergent evolution.

The genomic epidemiology study can be expanded to include metagenomics and microbiome data. Metagenomics studies have been conducted across different environment, ranging from terrestrial, aquatic to extreme environments (Bashir et al., 2021; Biller et al., 2018; Corrêa et al., 2020). The Earth Microbiome Project is one of the leading soil metagenomics studies that has expanded successfully over the years

(Gilbert et al., 2014; Thompson et al., 2017). The rise of next generation sequencing and long read sequencing have enabled shotgun metagenomics studies to be conducted at much higher throughput (Quince et al., 2017). Recently, the use and generation of metagenome-assembled genomes (MAGs) from suitable datasets could provide an *in silico* based environmental sampling approach to expand the number of available genomes (Bickhart et al., 2022; Chen et al., 2020). Similarly, the inclusion of microbiome dataset can provide an understanding on *A. baumannii* as a commensal in healthy individuals. This would fill in the gap of having more non-clinical human isolates for comparison against environmental and clinical isolates. By harnessing the available metagenomics and microbiome data, new perspectives on the prevalence and diversity of *A. baumannii* in the environment and hosts can be achieved. Integrating with accurately curated and standardised metadata, a real-time visualisation of *A. baumannii* genomics database can be built for future genomic epidemiological studies.

There are many other questions on *A. baumannii* and its Acb complex that remained to be unanswered. Further genomic epidemiology and microbiological studies will be required to contribute to the understanding of the origins and evolution of Acb complex and their resistome. The non-hospital urban environment as a potential reservoir for *A. baumannii* would warrant further investigation. As antimicrobial resistance continues to be a global threat, it is important to determine the mechanisms of how ARG could disseminate and the evolutionary successes behind the circulating MDR isolates. We encourage that the sequenced genomes are continued to be shared on public databases to promote open science and sharing of genomics data so that future big data studies can benefit from the expanded database containing natural environment isolates from different ecological niches.

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8. Supplementary Data

Supplementary Table S1. Additional information on MLST sequence types found in environmental isolates. Prototypic strain information is obtained from pubMLST database.

Isolate	ST (Pasteur)	ST (Pasteur) prototypic strain information	ST (Oxford)	ST (Oxford) prototypic strain information
E20-4	350	DV 35 (Switzerland, food, 2013)	Novel	-
E209-112	372	No strain information available in database	701	No strain information available in database
E88-42	150	JX215 (China, sputum, 2009) 09-780 (Japan, sputum, 2008)	Novel	-
E88-39/40	494	MRSN7204 (USA, wound, 2003)	753	No strain information available in database
E89-47/48	1043	IHIT31932 (Germany, animal, 2015)	Novel	-
E90-55/56/58	241	AB_2007-16-25-01-7 (USA, medical environment, 2007) AB_TG27339 (USA, sputum) IHIT31963 (Germany, animal, 2015) 792950 (Brazil, blood, 2006)	613	AB_2007-16-25-01-7 (USA, medical environment, 2007) AB_TG27339 (USA, sputum)

		118362 (USA, stool/rectal swab)		
E91-61/62/63/64/66	Novel	-	1776	ACIN00151 (USA, 2016)
E90-60	412	OIFC0162 (USA, upper respiratory tract, 2012) WC-348 (Iraq, skin, 2008)	Novel	-
K08-20-1	203	LUH14280 (Croatia, lower respiratory tract, 2009) ZQ4 (Iraq, blood, 2016) 32490 (China, blood 2015)	Novel	-
K09-14	46	AB204 (China, blood, 2015) LUH7852 (Czech Republic, sputum, 1994)	Novel	-
R16-8	374	A246 (Australia, blood, 2016) KCRI-49 (Tanzania, wound, 2013)	1416	AB250 (Thailand, sputum, 2010)
K10-01 (*A. nosocomialis*)	768	75R (France, stool/rectal swab, 2014) 42988 (China, IV catheter, 2017)	1740	No strain information available in database

Supplementary Table S2. Additional information on sequenced environmental isolates (n = 36) and their ARG and MRG.

Seq_ID	EY_ID	Location_tag	Isolate	Source	Species	MLST Pas	MLST Oxf	*aadA22*	*adeC*	*adeD*	*adeE*	*ant(3'')-IIa*	*aph(3'')-Ib*	*aph(3')-IX*	*aph(6)-Id*	*blaADC*	*blaADC-106*	*blaADC-154*	*blaADC-163*	*blaADC-18*	*blaADC-26*	*blaADC-52*	*blaOXA*
								ARG															
WBB2597	0367	E20	E20-4	Soil	*Acinetobacter baumannii*	350			1			1				1							
WBB2604	0449	E209	E209-112	Water	*Acinetobacter baumannii*	372	701					1				1							
WBB2605	0454	E212	E212-120	Water	*Acinetobacter baumannii*							1				1							
WBB2593	0382	E54	E54-25	Soil	*Acinetobacter baumannii*							1				1							
WBB2573	0395	E88	E88-42	Soil	*Acinetobacter baumannii*	150	744					1	1		1				1				
WBB2574	0399	E88	E88-46	Soil	*Acinetobacter baumannii*				1			1				1							
WBB2576	0392	E88	E88-39	Soil	*Acinetobacter baumannii*	494	753					1					1						
WBB2598	0400	E89	E89-47	Soil	*Acinetobacter baumannii*	1043										1							1
WBB2575	0408	E90	E90-55	Soil	*Acinetobacter baumannii*	241	613					1										1	
WBB2582	0412	E90	E90-60	Soil	*Acinetobacter baumannii*	412			1			1				1							
WBB2583	0413	E91	E91-61	Soil	*Acinetobacter baumannii*		1776					1				1							
WBB2587	0836	K05	K05-39	Soil	*Acinetobacter baumannii*											1							1
WBB2588	0830	K06	K06-29	Soil	*Acinetobacter baumannii*							1				1							
WBB2589	0821	K07	K07-22	Soil	*Acinetobacter baumannii*							1										1	
WBB2579	0818	K08	K08-20-1	Soil	*Acinetobacter baumannii*	203						1						1					
WBB2590	0809	K09	K09-14	Soil	*Acinetobacter baumannii*	46						1									1		
WBB2606	1120	R16	R16-8	Soil	*Acinetobacter baumannii*	374	1416					1									1		
WBB2570	0435	R20	R20-2	Soil	*Acinetobacter baumannii*							1				1							
WBB2571	0366	E20	E20-3	Soil	*Acinetobacter nosocomialis*				1	1	1					1							
WBB2591	0772	K10	K10-01	Soil	*Acinetobacter nosocomialis*	768	1740		1	1	1					1							
WBB2577	0499	E88	E88-44	Soil	*Acinetobacter pittii*					1	1	1								1			
WBB2585	0417	E91	E91-65	Soil	*Acinetobacter pittii*					1	1					1							1
WBB2586	0128	C75	C75	Soil	*Acinetobacter seifertii*					1	1					1							
WBB2592	0217	S6	S6-S21	Soil	*Acinetobacter seifertii*					1	1					1							
WBB2601	0529	26	26	Water	*Acinetobacter radioresistens*																		1
WBB2568	0874	K03	K03-52	Soil	*Acinetobacter radioresistens*																		
WBB2569	0856	K04	K04-42-2	Soil	*Acinetobacter radioresistens*																		1
WBB2566	0419	E91	E91-67	Soil	*Acinetobacter schindleri*																		1
WBB2564	0146	C93	C93	Soil	*Acinetobacter berezeniae*																		
WBB2562	0389	E55	E55-35	Soil	*Acinetobacter berezeniae*																		1
WBB2603	1129	C41	C41	Water	*Acinetobacter gerneri*									1									1
WBB2610	0001	W4-4-4	W4-4-4	Water	*Acinetobacter tandoii*																		1
WBB2554	0471	R14	R14-20	Sludge	*Acinetobacter towneri*																		
WBB2558	0472	R14	R14-45	Sludge	*Acinetobacter towneri*			1															1
WBB2561	0473	R17	R17-1	Sludge	*Acinetobacter towneri*																		
WBB2555	0475	R21	R21-22	Sludge	*Acinetobacter towneri*																		

Seq_ID	*blaOXA-104*	*blaOXA-117*	*blaOXA-121*	*blaOXA-208*	*blaOXA-229*	*blaOXA-259*	*blaOXA-272*	*blaOXA-337*	*blaOXA-340*	*blaOXA-374*	*blaOXA-64*	*blaOXA-688*	*blaOXA-694*	*blaOXA-71*	*blaOXA-72*	*blaOXA-78*	*blaOXA-817*	*blaOXA-91*	*qacEdelta1*	*mph(E)*	*msr(E)*	*sul1*	*sul2*	*tet(39)*	*tet(B)*
	ARG																								
WBB2597										1															
WBB2604		1																						1	
WBB2605								1																1	
WBB2593										1															
WBB2573			1																				1		1
WBB2574				1																					
WBB2576									1																
WBB2598																									
WBB2575																		1							
WBB2582				1																					
WBB2583													1												
WBB2587																									
WBB2588												1													
WBB2589														1											
WBB2579																1									
WBB2590	1														1										
WBB2606						1																			
WBB2570											1														
WBB2571																									
WBB2591																									
WBB2577							1																		
WBB2585																									
WBB2586																									
WBB2592																									
WBB2601																									
WBB2568																	1								
WBB2569																									
WBB2566																									
WBB2564					1																				
WBB2562																									
WBB2603																									
WBB2610																									
WBB2554																				1	1				
WBB2558																			1			1		1	
WBB2561																				1	1				
WBB2555																				1	1				

Seq_ID	*nreB*	*merA*	*merD*	*merR*	*merT*	*hdeD-GI*	*shsP*	*trxLHR*	*yfdX1*	*yfdX2*	# ARGs and MRGs
	MRG					HRG					
WBB2597	1										5
WBB2604	1					1	1	1	1	1	10
WBB2605	1										5
WBB2593	1										4
WBB2573	1	1	1	1	1						12
WBB2574	1										5
WBB2576	1										4
WBB2598	1										3
WBB2575	1										4
WBB2582	1										5
WBB2583	1										4
WBB2587	1										3
WBB2588	1										4
WBB2589	1										4
WBB2579	1					1	1	1	1	1	9
WBB2590	1										5
WBB2606	1										4
WBB2570	1										4
WBB2571	1										5
WBB2591	1										5
WBB2577	1										6
WBB2585	1										5
WBB2586	1										4
WBB2592	1										4
WBB2601											1
WBB2568											1
WBB2569											1
WBB2566											1
WBB2564											1
WBB2562											1
WBB2603											2
WBB2610											1
WBB2554											2
WBB2558						1		1		1	8
WBB2561											2
WBB2555											2

Supplementary Figure S1. SNP Clustering phylogenetic tree of environmental *Acinetobacter* isolates (n = 55) with their ARG and MRG. Environmental source includes soil (coloured in brown) and water (coloured in water) Blank ST indicates novel ST profile. Phylogenetic tree was visualized using iTOL v6 software (Letunic and Bork, 2021).