UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Genomic analysis of carbapenem-resistant environmental Gram-Negative Bacteria

by Sopheak Hem

Thesis submitted in fulfilment of the requirements for the degree of **Doctor of Philosophy**

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Certificate of original authorship

I, **Sopheak Hem**, declare that this thesis, is submitted in fulfilment of the requirements for the award of **Doctor of Philosophy** degree at the **School of Life Science**, **University of Technology Sydney**, specifically at the **Australian Institute for Microbiology & Infection (AIMI)**.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. Furthermore, I confirm that this document has not been presented for qualification purposes at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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Statement

This thesis is presented by compilation. Chapters 4, and 5 constitute the results chapters and each has been published while Chapter 6 constitute the results chapter submitted for publication, all are in a peer reviewed journal. The figures, tables, and sections within these chapters have been appropriately revised to ensure consistency and coherence with the overall thesis.

List of publications

Title: **Genomic analysis of *Elizabethkingia* species from aquatic environments: evidence for potential clinical transmission**

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Conference Presentations

- ***Elizabethkingia* species from aquatic environmental settings: Genomic analysis of phylogeny, antimicrobial resistance, and mobile genetic elements**
  Australian Society for Microbiology (ASM) Annual Scientific Meeting 2021
  Poster presentation, 31 may – 03 June 2021 by **Sopheak Hem**
  Melbourne Convention and Exhibition Centre, Victoria, Australia

- **Genomic analysis of Carbapenem-resistant *Comamonas* in water matrices: implications for public health and wastewater treatments**
  Australian Society for Infectious Diseases (ASID) Annual Scientific Meeting 2022
  Poster presentation, 16 – 18 June 2022 by **Sopheak Hem**
  Perth Convention & Exhibition Centre, WA, Australia

- **Shared research experiences through *Elizabethkingia* spp. genomic analysis**
  Science Talk, 31 August 2022 by Sopheak Hem
  The Loft, University of Technology Sydney

- **Genomic analysis of *Elizabethkingia* species from aquatic environments: Evidence for potential clinical transmission**
  The Adelaide JAMS and Flinders Accelerator for Microbiome Exploration (FAME) team
  Oral Presentation, 20 October 2022 by **Sopheak Hem**
  Main Entrance Bradley Building, University of South Australia

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Abbreviations

AmpC	Type C ampicillinase
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BRIG	BLAST Ring Image Generator
CARD	Comprehensive antimicrobial resistance database
CC	Clonal complex
CIA	Critically Important Antimicrobial
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem-Resistant *Enterobacteriaceae*
CRR	Complex resistance region
DDH	DNA-DNA hybridization
DNA	Desoxyribonucleic acid
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GES	Guiana extended-spectrum ß-lactamase
GGDC	Genome-to-Genome Distance Calculator
GI	Genomic Islands
GIM	German imipenemase
GNB	Gram Negative Bacteria
HGT	Horizontal gene transfer
ICE	Integrative conjugative elements

IMI	Imipenem-hydrolyzing ß-lactamase
IMP	Imipenem-resistant *Pseudomonas* carbapenemase
Inc	Incompatibility
IS	Insertion Sequence
iTOL	Interactive tree of life
Kb	Kilobases
KPC	*Klebsiella pneumoniae* carbapenemase
LB	Lysogeny broth
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time of Flight
Mb	Megabases
MBLs	Metallo-ß-lactamase
MDR	Multi-drug resistant
MDS	Multidimensional scaling
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence type
NCBI	National Centre for Biotechnology Information
NDM	New Delhi metallo-ß-lactamase
NSW	New South Wales
ORF	Open reading frame
OXA	oxacillinase
PCA	Plate Counting Agar
PCR	Polymerase chain reaction
PK-PD	Pharmacokinetic-Pharmacodynamic
pMLST	Plasmid multi-locus sequence typing

RST	Replicon sequence type
SA	South Australia
SDR	Short-chain Dehydrogenase/Reductase
SIM	Seoul imipenemase
SMP	Sao Paulo metallo-ß-lactamase
SNP	Single nucleotide polymorphism
ST	Sequence type
Tn	Transposon
UTI	Urinary tract infection
VAG	Virulence-associated gene
VFDB	Virulence factor database
VIM	Verona integron-borne metallo-ß-lactamase
WGS	Whole genome sequencing
WHO	World Health Organisation
WWTPs	Wastewater treatment plants

Abstract

The contamination of water bodies with antibiotics, biocides/pesticides, metals, pharmaceuticals, as well as antibiotic-resistant bacteria creates constant selection pressures that promote horizontal gene transfer, a major driver of microbial evolution. Consequently, contaminated water may introduce multiple drug resistant pathogens into healthcare facilities, food production environments, wildlife and agriculture. There is also a growing concern that healthy human and food animal microbial flora harbour considerable antibiotic resistance and virulence gene loads. These developments enhance reliance on carbapenem and other last line antibiotics, which play a crucial role in treating severe Gram-negative bacterial infections This study aimed to investigate the issue using whole genome sequencing (WGS) on carbapenem-resistant Gram-negative bacterial isolates from environmental water sources. The investigation focused on three genera: *Elizabethkingia* (n=94), *Comamonas* (n=39), and *Citrobacter* (n=77). Comprehensive genomic analyses were performed to explore phylogenetics, species typing, SNP distances, and to characterise mobile genetic elements (MGEs), virulence-associated genes, and antibiotic resistance genes (ARGs).

The study revealed intriguing findings concerning carbapenem-resistant bacteria. In *Elizabethkingia,* environmental isolates closely related to clinical strains causing sepsis were identified, indicating the significance of natural water bodies as potential sources for pathogenic *Elizabethkingia*. Furthermore, a novel species, *E. umeracha*, was identified, carrying novel metallo β-lactamase and extended-spectrum cephalosporin gene alleles. The investigations into *Comamonas* revealed bla_{GES-5} and bla_{OXA} present on unique putative genomic islands. Additionally, one *C. denitrificans* isolate carried an IncP-1 plasmid with metal resistant genes involved in xenobiotic degradation. Regarding *Citrobacter*, it was observed that isolates from wastewater exhibited a substantial burden of ARGs, and several novel multidrug-resistant lineages were identified. Notably, *C. freundii* isolates carried two carbapenemase genes, bla_{IMP-4} located within a class 1 integron on IncM plasmids, and bla_{KPC-2} on IncP plasmids. Additionally, our study identified a *C. portucalensis* isolate carrying bla_{NDM-1}, bla_{SHV-12}, and *mcr-9* genes.

Our study provided valuable insights into the presence of carbapenem-resistant species in environmental waters. The identification of new species and novel ARG alleles enriched our understanding of the genetic diversity within these bacterial genera. Moreover, the investigation shed light on the MGEs that harbour and presumably

transmit these genes. We also identified phylogenetic and genotypic connections between environmental isolates and other sources, unveiling potential origins and transfer mechanisms of MGEs. These findings contribute to our knowledge of the spread of antibiotic resistance in water environments which is crucial for implementing effective measures to mitigate the impact of this growing public health concern.

Chapter 1. Thesis Overview

1.1 Project Background and Research Question

Understanding the genomic characteristics of resistant Gram-negative bacterial populations in natural environments is of paramount importance due to the widespread release of antimicrobial resistance (AMR) factors and selection pressures resulting from human activities. Anthropogenic activities such as wastewater management [1,2], healthcare and pharmaceutical waste [3,4], stormwater runoff [5] , agricultural practices [6,7], and aquaculture [8] all contribute to the release of antibiotic resistant bacteria, antibiotic residues, heavy metals, disinfectants, pharmaceuticals, and other chemicals. These factors create a persistent selection pressure for bacteria to develop resistance mechanisms and proliferate.

Despite mounting evidence highlighting the environment as a crucial reservoir of AMR, there is a significant knowledge gap regarding the prevalence of resistant Gram-negative bacteria (GNB) in natural environments, both in Australia and globally. In this thesis, we use the term “environment” to refer to entities that are neither human nor animal. Consequently, we define “environmental isolates” as those derived from non-human and non-animal sources. These sources include soil, water, air, plants and inanimate objects. We aimed to address this gap, at least partially, by asking the following research question: what are the genomic characteristics and AMR profiles of GNB in Australian aquatic environments?

As part of the One Health surveillance initiative OUTBREAK, water samples were collected from various sources such as wastewater, dams, and wetlands. These samples were cultured on carbapenem selective media, allowing for the isolation and subsequent whole genome sequencing (WGS) of 665 isolates. The collection of isolates represented approximately 40 genera and hundreds of species (Figure 1). However, in order to conduct in-depth genomic analyses, the study focused on three key genera: *Elizabethkingia* spp., *Comamonas* spp., and *Citrobacter* spp. These genera were chosen based on their prominence in the sample collection and/or their global relevance, as supported by a comprehensive literature review (Chapter 2).

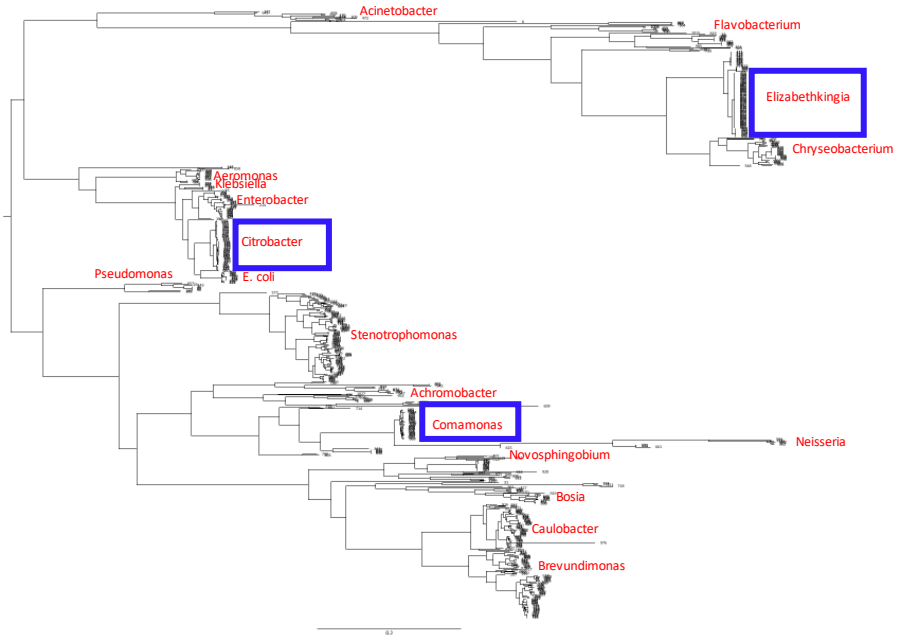

Figure 1: A phylogenetic tree which is midpoint rooted generated using Phylosift of the focal collections in the study, showcasing the genomes of all 665 isolates grown on carbapenem plates. Isolates studied in this thesis are boxed in blue.

1.2 Project aims

- Characterise the population structures of the three chosen bacterial genera through phylogenetic studies, genotyping, and single nucleotide polymorphism (SNP) analysis.
- Characterise antimicrobial resistance genes (ARGs) and virulence associated genes (VAGs), plasmid replicon sequence types and critical mobile genetic elements (MGEs) using bioinformatic methods to highlight any potential threat from environmental isolates in our study and compare these gene profiles to sequences in public databases.
- Identify possible novel species or rare species from the environmental water sourced and contribute these genomes to public database repositories.
- Compare the dataset generated by this project with WGS data accumulated in public database repositories, sourced from humans, swine, poultry, and wild bird populations to identify evidence of the mobility of dominant clonal lineages and mobile genetic elements.

1.3 Contribution to the literature

. The primary findings of the study were as follows:

- The study identified environmental *Elizabethkingia anophelis* that were closely related to sepsis-causing clinical isolates. This discovery highlights water bodies as an important source for pathogenic *Elizabethkingia* spp., shedding light on potential transmission dynamics.
- The study determined the genetic context and mobilization potential of carbapenemase-encoding genes in the three species under investigation. Notably, it revealed evidence of the horizontal acquisition of carbapenemase bla_{GES-5} via genomic islands in *Comamonas* and the presence of carbapenemases bla_{IMP-4} and bla_{KPC-2} on IncM2 and IncP6 plasmids, respectively, in *Citrobacter* species. These findings contribute to our understanding of the mechanisms underlying the spread of AMR in these bacteria.
- The study identified a novel species, *Elizabethkingia umeracha*, expanding the known diversity within the *Elizabethkingia* genus.
- By adding 33 *Comamonas denitrificans* genomes to public databases, this study significantly enriched our knowledge of the genetic diversity of this species. Prior to this study, only one *Comamonas denitrificans* genome was available, underscoring the importance of this contribution.
- The study discovered diverse Australian environmental populations of *Citrobacter*, including the identification of 17 novel sequence types (STs) and several novel multidrug-resistant (MDR) lineages.
- The study identified the presence of a recently reclassified species, *Citrobacter portucalensis*, in wastewater. Furthermore, one *C. portucalensis* isolate was found to carry genes conferring resistance to nine different classes of antibiotics. The arrangement of genes in this isolate's genome was investigated in greater detail.

In summary, these findings provide valuable insights into the genetic characteristics, epidemiology, and potential public health implications of carbapenem-resistant Gram-negative bacteria in aquatic environments. They expand our understanding of the diversity, evolution, and transmission dynamics of these bacteria,

providing a foundation for further research and potential interventions to mitigate their impact on human health and sheds light on the genetic features of mobile genetic elements carried by these bacterial lineages that harbour genes encoding resistance to clinically important antibiotics.

Chapter 2. Literature review

2.1 Antimicrobial resistance: A global public health threat

Antimicrobial resistance (AMR) poses a major threat to human health around the world. It refers to the ability of microorganisms, such as bacteria, viruses, fungi, and parasites, to develop resistance to the drugs used to treat infections caused by them, resulting in prolonged illnesses, higher healthcare costs, and increased mortality rates [9].

A study conducted in 2019 across 204 countries and territories revealed that 4.95 million deaths were associated with bacterial AMR, with 1.27 million deaths directly attributable to it [10]. This death toll is projected to steadily rise, imposing an estimated economic burden of around 86 trillion dollars by 2050 [11]. This clearly leads to a profound global impact on economics and healthcare management [12].

The rise of AMR is influenced by several factors. Misuse and overuse of antimicrobial drugs, both in human medicine and agriculture, have played a major role in accelerating the development and spread of resistance. Inappropriate prescribing practices, patients not completing full courses of antibiotics, and the use of antibiotics as growth promoters in livestock over many years have contributed to the emergence and dissemination of resistant strains [13]. Resistant bacteria can easily spread between countries through international travel and trade, further exacerbating the problem. Developing countries are particularly vulnerable due to inadequate healthcare infrastructure, limited access to quality antimicrobials, and poor infection prevention and control practices [11].

The most widely consumed antibiotics worldwide are β-lactams, which include penicillins and cephalosporins, and are used to treat infections caused by both Gram-negative and Gram-positive bacteria [14]. However, the overuse of β-lactams results in resistance, necessitating the use of "last-resort antibiotics", such as carbapenems, for the treatment of severe infections in situations where both Gram-negative and Gram-positive bacteria are resistant to penicillins, cephalosporins, and extended-spectrum β-lactams (ESBLs) [15]. The development of carbapenem resistance results in complete resistance to all β-lactams [14, 15,16].

The selective pressure exerted by an antibiotic lead to a scenario where populations of predominantly susceptible bacteria are killed providing an opportunity

where resistant bacteria survive and proliferate. Consequently, the population becomes predominantly composed of resistant bacteria [17] (Figure 2) enhancing opportunities for mobile elements that carry many of the resistance genes to spread to other bacterial populations that are simultaneously experiencing the same selection pressure. These scenarios are exacerbated by co-selection, a process that arises because resistance genes co-evolve, often within close proximity to one another on the same mobile genetic element. As such any one of a number of different selection pressures (biocide, metal or pharmaceutical agents) can promote resistance to a clinically important antibiotic.

Addressing AMR requires a multi-pronged approach that includes measured and informed antimicrobial use, a greater emphasis on genomic surveillance, improved communication, and data sharing. While there is pressing need for the development of new antimicrobial drugs [18] unless there are substantial improvements to how we manage antimicrobial selection pressures, these new drugs will likely experience comparatively short-lived success [13].

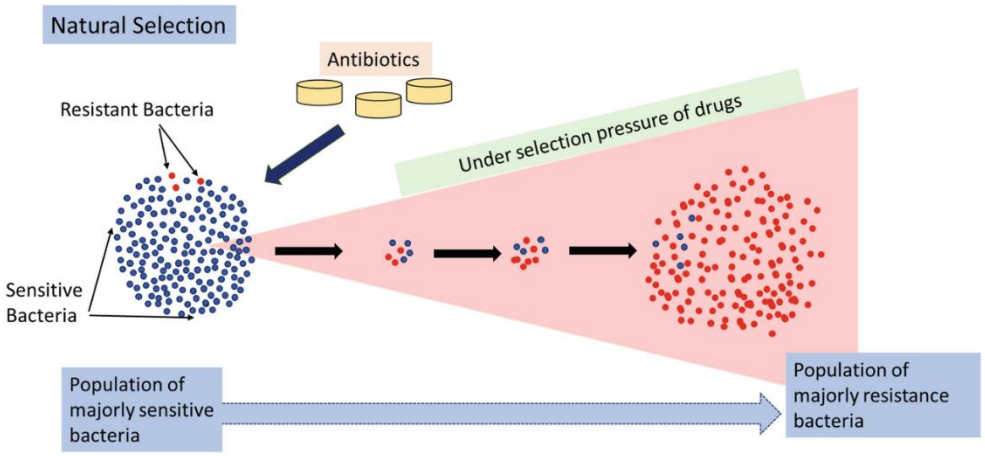

Figure 2: Diagram illustrating the development of antibiotic resistance depicted through selective pressure by killing susceptible bacteria, allowing antibiotic-resistant bacteria to survive and multiply. Figure obtained from [19].

2.2 Emergence of AMR in Gram-negative bacteria

Gram-negative bacteria (GNB) cause a plethora of diseases, including pneumonia, gastrointestinal diseases, urinary tract infections UTIs), and bloodstream infections. Therefore, emergence of AMR in GNB is a critical concern for public health, particularly in hospital and healthcare settings [19], as common and previously easy-to-

treat infections become harder to treat and potentially lethal [9]. Gram-negative bacteria, such as *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Escherichia coli* are all important members of the ESKAPE-E pathogen group (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter* spp. and *E. coli*), which account for the majority of AMR bacterial infections worldwide [20], and are associated with the highest risk of mortality and increased healthcare costs [21]. The literature is full of reports of ESKAPE-E strains exhibiting multidrug-resistance (MDR),defined as acquired resistance to a minimum of one drug within three or more categories or classes of antimicrobial agents [22]. As such, the World Health Organization (WHO) recently listed ESKAPE-E pathogens as a priority for the development of new antibiotics [23].

MDR Gram-negative infections are a major global health challenge due to the inadequate response of these pathogens to antimicrobials [24]. The unique structure of Gram-negative bacterial cell wall which comprises two lipid membranes contributes to their ability to acquire and transfer resistance mechanisms. The outer membrane acts as a selective chemical barrier, restricting the entry of antibiotics rendering them unable to exert their therapeutic potential [25,26]. Additionally, GNB can develop various resistance mechanisms, including the production of enzymes to inactivate antibiotics, efflux pumps to expel drugs from the cell, and alterations in drug targets [27].

The consequences of AMR in GNB are significant. Infections caused by these resistant pathogens are associated with higher morbidity and mortality rates compared to susceptible strains [9]. Furthermore, the limited treatment options for MDR Gram-negative infections often necessitate the use of last-resort antibiotics, such as carbapenems and colistin. However, as mentioned previously, even these options are increasingly being compromised by the emergence of carbapenem-resistant and colistin-resistant strains, leaving clinicians with few effective options [28]. Resistance to carbapenems emerged shortly after their introduction into the clinical environment, similar to other antibiotics used to treat Gram-negative bacteria (Figure 3) [29]. Colistin is currently an antibiotic of last resort for the treatment for life-threatening infections caused by carbapenem-resistant GNB. However, bacteria resistant to colistin have been detected in several countries, leading to infections that currently have no effective antibiotic treatment [30].

Bacteraemia caused by GNB is a pressing public health issue, leading to severe disease and billions of dollars in global economic costs [31] . Between 1997 to 2013, the

global proportion of bacteraemia cases attributed to GNB including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*, increased from 33% to 43% [32]. UTIs caused by GNB such as *E. coli*, *K. pneumoniae*, *P. mirabilis*, and *Citrobacter* species are another severe public health problem, affecting 150 million people worldwide each year [33]. Nosocomial infections caused by these microorganisms pose a significant challenge in patient management, particularly in lower respiratory tract infections where they exhibit high resistance rates and non-responsiveness to antibiotic therapy [34].

Lack of effective antibiotics are affecting healthcare systems globally, regardless of the country's development level. Ineffectiveness due to the spread of drug resistance has led to more difficult-to-treat infections and increased mortality rates [35]. There is an urgent need for new antibiotics, particularly to treat carbapenem-resistant and colistin-resistant Gram-negative bacterial infections. However, if effective tools for the prevention and adequate treatment of drug-resistant infections are not found and access to existing and new quality-assured antimicrobials is not improved, these new antibiotics will face the same fate as current ones, resulting in increased treatment failure [36].

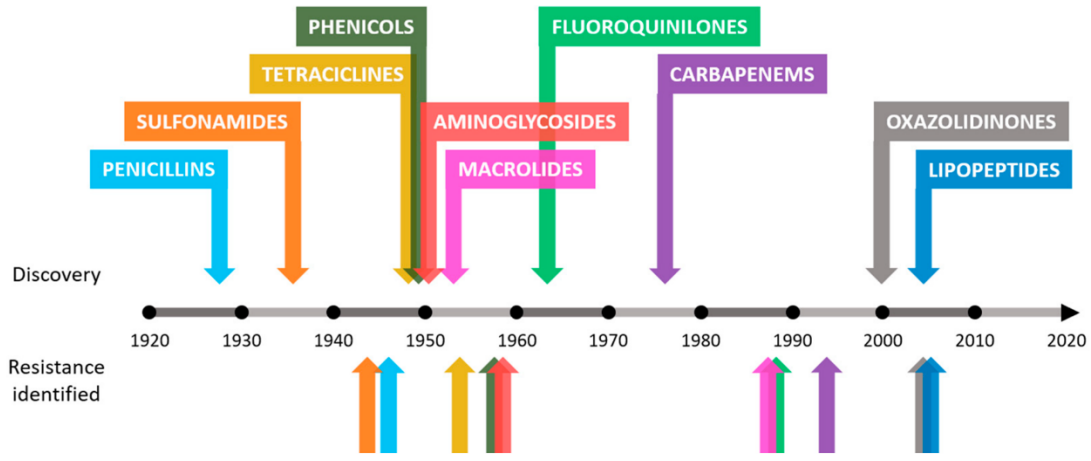

Figure 3: Emergence of antibiotic resistance following introduction into clinical use. This graph shows the time lag between an antibiotic being introduced to clinical use and the first appearance of resistance. Figure obtained from [29].

2.2.1 AMR in environmental GNB

The environment serves as a reservoir for diverse GNB populations [37]. These bacteria can be exposed to antimicrobial agents through various sources such as agricultural runoff (antimicrobials, biocides, and metals), wastewater discharge, and the

release of pharmaceuticals and antimicrobial residues from hospitals and the general community, into the environment. Consequently, GNB in the environment may encounter selective pressures favouring the development and persistence of AMR [38].

Humans can become colonised with resistant bacteria following exposure to contaminated medical equipment, water (consumable and recreational), and companion animals [39–41]. Particularly problematic are highly contaminated environments, such as wastewater treatment plants and hospital drainage systems, which can act as hubs for pathogen accumulation, facilitating the exchange and acquisition of genetic elements, including ARGs [41,42].

Many antibiotics are partially absorbed and unmetabolised antimicrobial components are subsequently released into the environment through sewage treatment plants, waterways, and food animal and horticultural practices [43]. Therefore, the environment, especially water and soil, serves as a dynamic ecosystem that harbours a diverse pool of ARGs [44,45]. Collectively, the environment likely represents the largest reservoir of known and latent antimicrobial resistance genes and has an abundance of mobile genetic elements to capture these genes. As such environmental bacteria play a crucial role as a source of new ARGs [46]. Latent ARGs, defined as genes encoding antimicrobial activity that are yet to reach clinical significance and remain poorly or undefined in AMR gene repositories. However, a recent study analysing more than 10,000 metagenomic samples found that latent genes are abundant in wastewater and in environmental microbial communities [47]. Latent ARGs are widely distributed and often mobilised and associated with MGEs [47]. Latent ARGs are also evident in human and animal microbiomes and are found in ESKAPE pathogens [48]. This suggests that latent ARGs may pose significant risks to human health [46,49,50] but are poorly defined and remain undetected.

Latent ARGs likely to contribute to poor concordance in phenotype/genotype associations in some pathogens (48) and present a major knowledge gap in efforts to implement mitigations strategies to better control the flow of resistance genes from a one health perspective. These knowledge gaps also hamper efforts to extend the efficacious half-life of existing and new antibiotics.

The importance of investigating known and novel ARGs carried by environmental bacteria should not be underestimated. Prominent ESBLs ($bla_{\text{CTX-M}}$), quinolone resistance genes (*qnrA),* fosfomycin resistance genes (*fosA8)* and carbapenemases

(*bla*$_{OXA-48}$, and *bla*$_{NDM-1}$) all originated from marine and soil bacterium and have subsequently entered clinical isolates through plasmids [51]. The presence of these genes in both environmental and pathogenic bacteria further highlights the interconnectedness of resistance gene dissemination [52].

2.2.2 AMR in environmental *Elizabethkingia*, *Comamonas* and *Citrobacter* species

It is known that many environmental bacteria are intrinsically resistant to carbapenems. For example, *Elizabethkingia* species are environmental GNB that display intrinsic resistance to carbapenems [53]. Another genus of GNB , *Comamonas* spp., is abundant in aquatic and soil environments, including wastewater, harbouring genes encoding carbapenemases and other clinically important antibiotic resistance genes [54,55]. Similarly, *Citrobacter* spp., which are ubiquitous in soil, food, and water bodies, and are part of the commensal intestinal microbiota of humans and animals are intrinsically resistant to broad and extended-spectrum beta-lactamases due to the presence of chromosomal *bla*$_{CMY}$ genes [56–58]. However, recent reports have also identified carbapenemase-encoding genes in *Citrobacter* populations [59–61].

Understanding the resistance patterns of environmental bacteria can help predict the regional resistance situation [62] and potentially provide indications of historical antibiotic use [63]. Several recent studies have used sewage monitoring as a complement to clinical surveillance of resistance, phenotypic analyses, and ARG analysis [64,65].

2.2.2.1 AMR in *Elizabethkingia* species

Elizabethkingia is a genus of Gram-negative bacteria that belongs to the *Weeksellaceae* family, commonly found in the environment, including soil, water, and plants, and can also be present in healthcare settings [66]. This genus was named after Elizabeth O. King, an American microbiologist who made significant contributions to clinical microbiology.

Elizabethkingia species are opportunistic pathogens that can cause severe infections in humans, particularly in immunocompromised individuals or those with underlying health conditions. There are six species identified: *E. meningoseptica, E. anophelis, E. miricola, E. occulta, E. ursingii*, and *E. bruuniana* [53]. Among these species, *E. meningoseptica* and *E. anophelis* are the two known human pathogens causing bloodstream infections, pneumonia, meningitis, and UTIs [53,67].

Elizabethkingia spp. carry genes that confer resistance to clinically important antibiotics. These unique genes include the chromosomal metallo-beta-lactamase genes bla_{B} and bla_{GOB}, which encode resistance to carbapenems and bla_{CME}, which confers resistance to all cephalosporins [68].

Treating *Elizabethkingia* infections is challenging, and case fatality rates are high. Added to carbapenem and cephalosporin resistant, these bacteria have also been reported to produce simultaneous resistance to many other antimicrobial agents, including most β-lactams and inhibitors, aminoglycosides, macrolides, tetracycline, vancomycin, fluoroquinolones, and sulfamethoxazole-trimethoprim. In addition to bla_{CME}, bla_{B} and bla_{GOB}, *Elizabehkingia* isolates have been reported carrying other β-lactamase genes, such as bla_{OXA} and bla_{TEM} [69]. Other resistance genes found in *Elizabethkingia* species include the aminoglycoside resistance genes *rnaA*/*rnaB* and the chloramphenicol resistance gene *catB11*. Aminoglycoside resistance genes such as *aadS*, *aac(3)-IVb*, and *aac(3)-IIIc* have also been detected [69]. Fluoroquinolone resistance is associated with mutations in the *GyrA* gene, specifically a single amino acid substitution (Ser83Ile or Ser83Arg) in *GyrA* [70].

To date, most studies on *Elizabethkingia* have been performed on clinical isolates. For example, a study performed in Saudi Arabia in 2019 reported diverse resistance genes in clinical *E. meningoseptica* strains, including different lactamases, fluoroquinolone resistance mutations in *gyrB*, (tetracycline resistance genes *tetA*, *tetO* and *tetB*) and numerous multidrug resistance efflux pumps but it did not delve into the specific mechanisms by which these genes confer resistance [71]. A recent Australian study reported that some Australian *E. anophelis* isolates are genetically closely related to strains from the United States, England, and Asia [72].

Comparative genomics of clinical and environmental strains of *Elizabethkingia* spp. identified evidence of nosocomial transmission in patients, indicating probable infection from a hospital reservoir [72] but little has been done to extend the research to investigate isolates from natural environments. Another study in 2019 at Taiwan [73] utilized comparative genomics to analyze the genomes of six species within the *Elizabethkingia* genus, including *E. meningoseptica*, *E. anophelis, E. miricola, E. bruuniana, E. ursingii*, and *E. occulta*.

The study identified clusters of orthologous groups and observed an expansion of the pan genome, indicating adaptive evolution in response to environmental changes.

Genomic analyses of these isolates showed evidence of various antimicrobial resistance mechanisms, including multidrug resistance efflux pumps, β-lactamases, proteins linked to vancomycin resistance, and quinolone-resistance determining regions (related to DNA gyrase and topoisomerase IV). While the study provides information about the presence of various antimicrobial resistance mechanisms in the studied species, it does not elaborate on their specific impact or significance. It is unclear how these resistance factors contribute to the overall antimicrobial resistance profile of the species or their potential implications for clinical treatment. Further exploration of the functional aspects and clinical relevance of these antimicrobial resistance mechanisms would help bridge this knowledge gap.

2.2.2.2 AMR in *Comamonas* species

Comamonas spp. are non-fermenting Gram-negative bacilli, consisting of 24 species identified to date [74]. They are found in various environmental settings such as soil, wastewater, and freshwater bodies including ponds and rivers. They have also been isolated from industrial environments like activated sludge and polluted soil, as well as from the hospital environment and clinical samples such as urine, pus, blood, faeces, and kidneys [75].

While *Comamonas* species have diverse catabolic abilities, some species can also act as opportunistic pathogens causing serious infection such as septicaemia and endocarditis in immunocompetent hosts [76–78].

However, they may be underdiagnosed in routine clinical microbiology due to poor growth [79], and even when successfully cultured, biochemical identification tests often mistakenly classify them as *Pseudomonas* species. Additionally, MALDI-TOF MS identification can also lead to misidentification of *Comamonas* at the species level [80]. While WGS could be used for accurate speciation, there is currently a lack of WGS data on many *Comamonas* species. Furthermore, antimicrobial resistance (AMR) in *Comamonas* species poses a significant concern due to its potential impact on human health.

Resistance to β-lactam class antimicrobials in *Comamonas* spp. has been attributed to the presence of multiple genes. A study in China in 2017 found *C. testosteroni* carries a novel Class A β-lactamase (*CzoA*). Located upstream of *czoA* is a *LysR* family transcriptional regulator *czoR*. Studies showed that CzoR is likely to regulate the expression of *czoA* because it specifically binds to the *czoA* regulatory region [81].

Expression of *czoA* results in resistance to benzylpenicillin, ampicillin, cefalexin, cefazolin, cefuroxime, ceftriaxone, and cefepime. In *C. kerstersii*, several resistance genes were identified, including *tetA, strB, sul1*, bla_{OXA-1}, *strA, sul2, catB3*, and *floR*. Additionally, *C. thiooxydans* was found to possess bla_{IMP-8} reported from UTI in China with bla_{IMP-8} and bla_{OXA} genes (encoding resistance to β-lactams) and *aac(6')-Ib-c* gene (resistance to aminoglycosides) [55]. Antimicrobial susceptibility analysis indicated that the *C. thiooxydans* strain ZDHYF418 was susceptible to imipenem, intermediate to meropenem, and was resistant to aztreonam, fluoroquinolones, and aminoglycosides.

*T*he *bla* $_{IMP-8}$ gene found in *C. thiooxydans* ZDHYF418 [55] was chromosomally located, and was part of a Tn402-like class 1 integron characterized by the following structure: *DDE-type integrase/transposase/ recombinase-tniB-tniQ-recombinase family protein-aac(6')-Ib-cr-bla* $_{IMP-8}$*-intI1*. Another study in Japan in 2022 identified a bla_{IMP-1} gene in *C. thiooxydans* NR4028 possessed plasmid-encoded IMP-1, a common carbapenemase gene in Japan, and resistant to meropenem [82]. The IMP-1-encoding plasmid was about 30 kbp in length and did not have a locus Tra region, which is an essential region for conjugation.

Moreover, this study found that the plasmid structure of *C. thiooxydans* NR4028 with similar plasmid replicase genes and the genetic environment of resistance genes in IMP-1-encoded plasmids. The plasmid *repA* replicase gene that was present in their isolate was different from that circulating in *Enterobacterales*, but had high homology to those of plasmids in non-fermenting Gram-negative bacteria, including *Vitreoscilla filiformis* (CP022425), *Xanthomonas* spp.(CP024031), and *P.aeruginosa* (KR106190) [83]. These findings highlight the presence of various resistance mechanisms within different species of *Comamonas*, underscoring the importance of monitoring and understanding antimicrobial resistance in this bacterial genus.

Given their potential involvement in wastewater bioaugmentation and bioremediation strategies, it is also of interest to investigate whether these bacteria have the ability to transfer resistance genes to other microorganisms, thereby contributing to the spread of resistance in different ecological niches.

2.2.2.3 AMR in *Citrobacter* species

Citrobacter species are Gram-negative, opportunistic pathogens commonly found in the environment, as well as in the gastrointestinal tracts of human and animal [84]. *Citrobacter* spp. have the potential to cause a range of infections involving the

urinary and respiratory tracts, bloodstream, and intra-abdominal infections. Up to date, 16 species have been identified. *C. koseri* has been associated with cases of neonatal meningitis and brain abscess while *C. freundii* with gastroenteritis, neonatal meningitis, and septicemia [85]. *C. freundii* is a frequent cause of UTI [86]. AMR in *Citrobacter* species poses a challenge in the treatment of infections caused by these bacteria. Resistance to various classes of antimicrobial agents, including β-lactams, aminoglycosides, fluoroquinolones, and sulfonamides, has been reported [87].

Citrobacter species are intrinsically resistant to broad and ESBL due to the presence of chromosomal bla_{CMY} genes [88,89]. However, recent reports have identified the presence of carbapenemase encoding genes in *Citrobacter* populations. Metallo-β-lactamases (MBLs), encoded by genes such as bla_{KPC}, bla_{OXA-48}, bla_{VIM}, bla_{IMP}, and bla_{NDM}, confer resistance to carbapenems and hydrolyze almost all β-lactams, making the treatment of MBL-carrying pathogens challenging [90]. AMR encoding genes are reported with increasing frequency in *C. freundii* in recent years [91]. MDR *C. freundii* has been reported in numerous hosts including chickens, in petting zoo animals, turtles, foxes and ducks [87,92–95].

The increased prevalence of AMR in *Citrobacter* species emphasizes the need for effective surveillance and improved infection control measures, and appropriate antimicrobial stewardship. The focus needs to shift from having a clinical imperative to one that adopts a one health approach: human-animal-environment [96] and apply genomic methodologies to examine lineages of *Citrobacter* spp. This data underpins efforts to optimize infection prevention and control practices to minimize the transmission of resistant *Citrobacter* strains.

2.3 Mechanisms behind AMR

AMR can arise through intrinsic and acquired resistance mechanisms, facilitated either by the capture of resistance genes through horizontal gene transfer (HGT) for the latter or via genetic modifications to molecules, resulting in altered function for the former. These adaptations enable microbes to resist the actions of antimicrobial agents. One common mechanism is the production of enzymes by bacteria that leads to resistance to important classes of antibiotics. For example, the β-lactamases inactivate or modify β-lactams. Another mechanism involves the alteration or mutation of target sites [26] in core genes needed to execute essential cellular functions. Certain bacteria may undergo genetic changes that modify the structure of their cell wall or other components, making

them less susceptible to the effects of antibiotics. This alteration can reduce the ability of antibiotics to bind to their target and inhibit the growth or kill the bacteria. Efflux pumps are also used by bacteria to resist antibiotics. These pumps actively expel the drug from inside the cell, preventing it from reaching its target and exerting its antimicrobial activity. This mechanism is particularly relevant in multidrug-resistant bacteria, as efflux pumps can confer resistance to multiple classes of antimicrobial drugs (Figure 4A).

ARGs can be found on the bacterial chromosome or mobilised by plasmids. Additionally, ARGs on the chromosome may have been obtained through HGT [97] (Figure 4B) [17]. HGT is facilitated by mobile genetic elements (MGEs) such as plasmids and phages and enables the movement of ARGs not only within the same species but also between different species and genera. This mobility is achieved through various mechanisms, including conjugation, transduction, and transformation [98].

HGT serves as the primary driver of MDR in both Gram-negative and Gram-positive bacteria. The diversity and mobility of antimicrobial resistance genes contribute to the emergence and spread of AMR [99]. Resistance that is acquired via plasmids plays a crucial role in both HGT. It is important to distinguish between the different mechanisms that influence HGT (see below). Once resistance genes are captured by the action of insertion elements and via the action of other mobile genetic elements, complex resistance regions can be assembled by facilitating the assembly of ARGs through transposition and recombination mechanisms [100].

A. B.

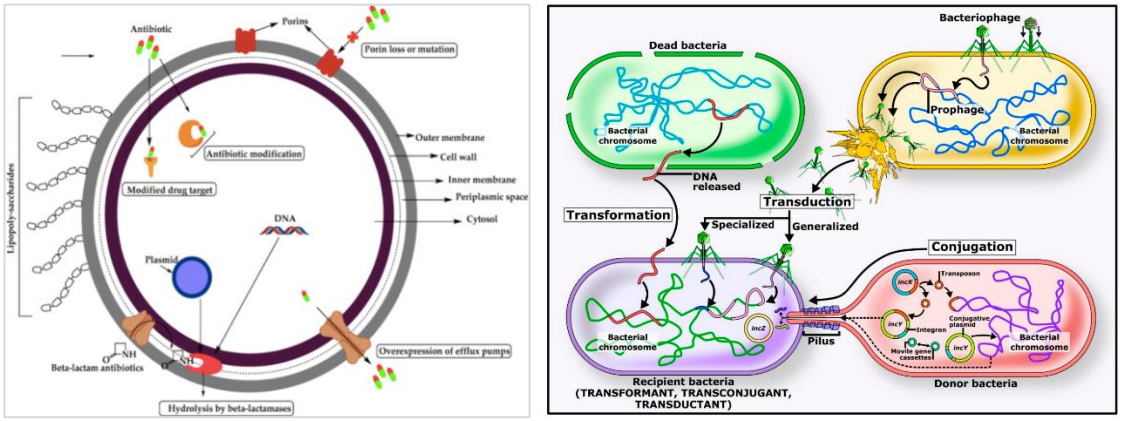

Figure 4: Mechanisms behind antimicrobial resistance in Gram–negative bacteria. (A) Expression of intrinsic resistance such as target modifications, permeability alterations by loss of outer membrane porins, mutations chromosomal genes resulting in antibiotic-inactivating enzymes and efflux pump. Involved mechanisms in horizontal gene transfer. Figure obtained from [101]. (B) Horizontal transfer of AMR via conjugation, transduction, and transformation. Figure obtained from [102].

2.3.1 Mechanisms behind carbapenem resistance

The mechanisms behind carbapenem resistance are often multifactorial, involving a combination of different resistance mechanisms in a single bacterium. However, carbapenem-resistant GNB employ three primary resistance mechanisms: i) carbapenemase-producing enzymes, ii) porin mutations, and iii) efflux pump expression [103]. Carbapenemase-producing enzymes represent a critically important mechanism which can inactivate carbapenems and other β-lactam antibiotics [24]. The capture of a novel genes encoding carbapenemase activity on a mobile genetic element poses a serious threat to the long-term effectiveness of these last-line, life-saving antibiotics.

Carbapenemases have been divided into three classes: A, B and D. Class A carbapenemases encompass penicillinases encoded by such genes as *bla*$_{GES}$, *bla*$_{IMI}$, *bla*$_{SME}$ and *bla*$_{KPC.}$ Class B are comprised of metallo-β-lactamases encoded by such genes as *bla*$_{NDM}$, *bla*$_{VIM}$ and *bla*$_{IMP}$, and Class D contains oxacillinases represented by a series of *bla*$_{OXA}$ genes [104] (Figure 5). For Carbapenem resistant *Enterobacteriaceae* (CRE) are generally divided into two main subgroups: carbapenemase-producing CRE (CP-CRE) and non-carbapenemase-producing CRE (non-CP-CRE) (Figure 5B) [105].

A. B.

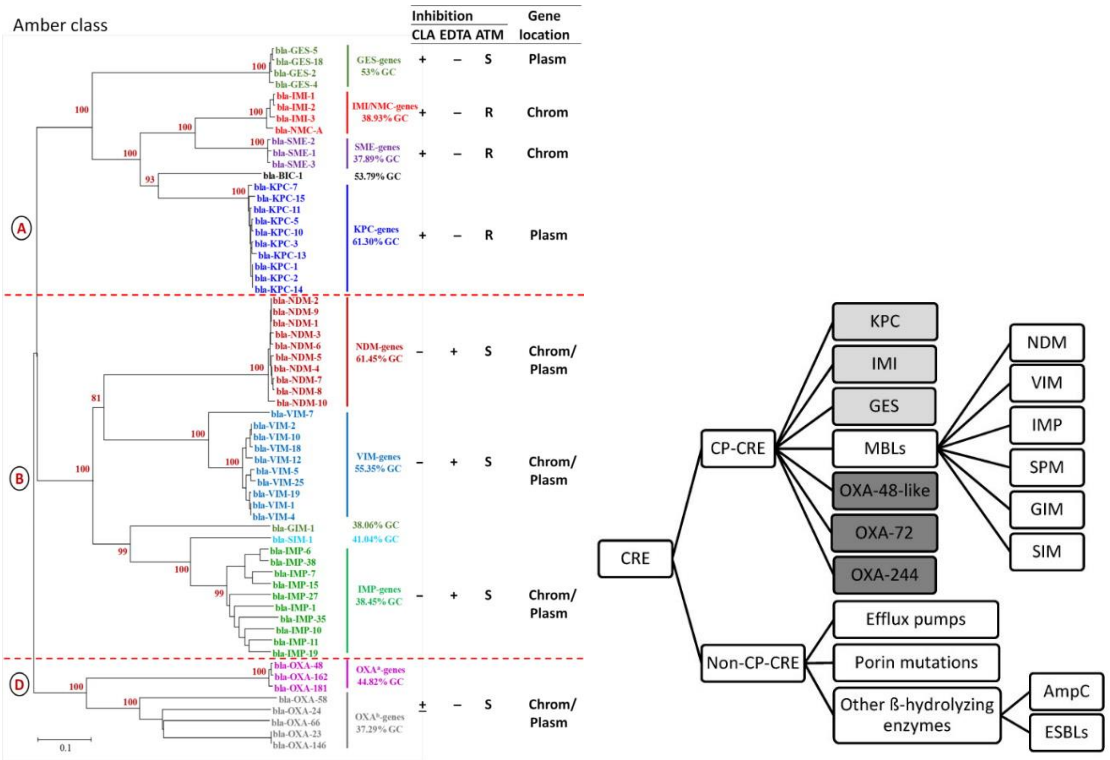

Figure 5: (A) Phylogenetic tree of the carbapenemase genes in Gram-negative bacilli: *Enterobacteriaceae, Pseudomonas* and *Acinetobacter* that divided into three classes: A, B and D. Figure obtained from [104]. (B) Classification of the different mechanisms of drug resistance in CRE which divided into two main subgroups: carbapenemase-producing CRE (CP-CRE) and non-carbapenemase-producing CRE (non-CP-CRE). Figure obtained from [105].

Epidemiologically, carbapenem-resistance has emerged worldwide in the last three decades. Since the first identification of *bla*$_{KPC}$ from *Klebsiella pneumoniae* in 1996 [106], other carbapenemase-genes have been reported at accelerating rates worldwide [107]. For example, *bla*$_{IMP-1}$ was first reported in Japan in 1991 in *Serratia marcessencs* [108] and is now endemic to Japan. Outbreaks and isolated instances of infections caused by Gram negative bacteria carrying *bla*$_{IMP}$ as well as *bla*$_{VIM}$ have now been made in the USA, Europe and Asia [109,110]. The New Delhi Metallo-β-lactamases (*bla*$_{NDM}$), first discovered in Sweden in 2008 in a patient returning from the India subcontinent [111], is now widespread across the globe. *bla*$_{OXA}$ was first described in *A. baumannii* in 1985 in Scotland. One variant of this gene, *bla*$_{OXA-48}$, has been identified predominantly in *K. pneumoniae* across Europe [112]. Figure 6 depicts the global distribution of major carbapenemase encoding genes.

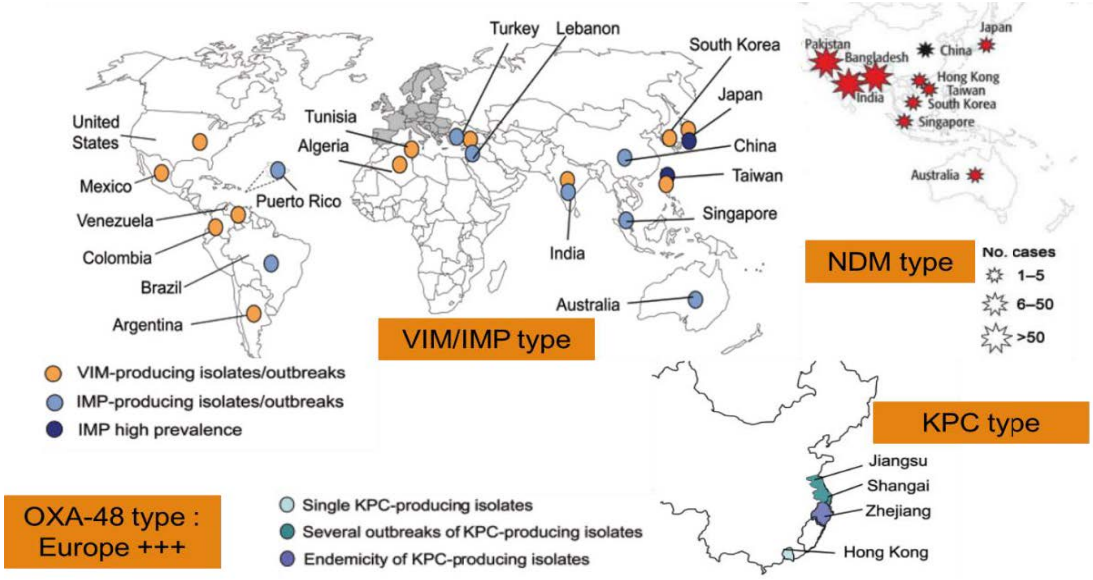

Figure 6: Distribution of carbapenemases in clinical Isolates worldwide. Although carbapenemases are prevalent, specific types, such as *bla*$_{KPC}$ *bla*$_{IMP}$ *bla*$_{VIM}$ and *bla*$_{NDM}$, exhibit a geographical distribution. Figure obtained from [112].

One of the biggest gaps in knowledge lies in identifying the phylogenetic relatedness between carbapenem-resistant GNB from the natural environment and humans, as well as animals, and understanding the driving forces behind the transfer of mobile horizontally acquired antibiotic resistance genes among different species.

2.3.2 Mobile genetic elements: vectors of AMR and pathogen evolution

Mobile genetic elements (MGEs) are genetic structures that can move between different organisms or locations within a genome, facilitating the transfer of resistance genes and contributing to the dissemination of AMR [113]. They play a significant role in the spread of antimicrobial resistance (AMR) and the evolution of pathogens.

MGEs are important vectors for AMR, carrying and transferring resistance genes between bacteria [98]. They can take on different structures and can be categorized into two main groups: i) intercellular MGEs, which are transmissible from one isolate to another, including plasmids, integrative conjugative elements (ICEs), genomic islands (GIs), and phages, and ii) intracellular MGEs, which cannot transfer themselves but can do so after integration into members of the first group. Intracellular MGEs comprise IS, transposons, and integrons [52, 53]. These elements have the capacity to harbor multiple resistance genes, often conferring resistance to diverse classes of antimicrobial agents.

When bacteria acquire MGEs containing resistance genes, they gain the ability to survive exposure to antibiotics, leading to treatment failures and the emergence of multidrug-resistant strains. Carbapenemase genes, along with other ARGs, are predominantly associated with MGEs, CRR, biocides, metals and pharmaceutical substances.

A significant characteristic of MGEs is their capacity for horizontal transfer, enabling the movement of resistance genes between bacteria, even across species. This horizontal gene transfer plays a crucial role in the often rapid dissemination of AMR by facilitating the transfer of resistance genes among diverse bacterial populations [114]. MGEs can transfer between bacteria occupying the same ecological niche or during interactions between environmental and clinical settings. For instance, MGEs can transfer resistance genes from environmental bacteria to human pathogens, leading to a critical impact on human health [115].

MGEs not only facilitate the dissemination of AMR but also contribute to pathogen evolution [116]. They can mediate the acquisition of virulence factors, enhancing the pathogenicity of bacteria. MGEs can carry genes encoding toxins, adhesion factors, or immune evasion mechanisms, allowing pathogens to better colonize and cause infections in hosts. The coexistence of resistance and virulent genes within MGEs can lead to the emergence of multidrug-resistant and highly virulent strains, posing significant challenges in public health [117]. Understanding the role of MGEs in AMR and pathogen evolution is crucial for addressing the global threat of AMR and developing effective strategies for surveillance and control [118].

2.3.2.1 Plasmids

Plasmids are extrachromosomal DNA found in bacteria that play a significant role in genetic exchange [119]. They can carry a variety of genes, including those involved in antimicrobial resistance, virulence factors, metabolic capabilities, and other adaptive functions. Plasmids are important components of the accessory genome – genes not part of an organisms conserved core genome - that playing a significant role in host and niche adaptation, disease etiology and enabling microorganisms to survive and thrive in diverse environments. An important characteristic of plasmids is their capacity to replicate independently from the bacterial chromosome. This allows plasmids to persist in bacterial populations even when selective pressures change. Plasmid maintenance systems, including multimer resolution systems, partitioning systems, post-segregation systems and restriction-modification systems, ensure the stable inheritance of plasmids during cell division and cell growth [120].

Plasmids vary in size, ranging from a few thousand base pairs to hundreds of kilobases [98]. They can exist as circular or linear DNA molecules. Circular plasmids are more common and tend to be more stable due to their ability to form closed loops. Plasmids can also be classified based on their copy number, with some existing as low-copy-number plasmids (1-5 copies per genome) and others as high-copy-number plasmids (10-100 copies per genome) [121].

Resistance genes located on plasmids provide bacteria with the capability to resist antibiotics [122]. These genes may encode enzymes that deactivate or modify drugs, efflux pumps that expel antibiotics from bacterial cells, or modified target sites that hinder antibiotic binding. This transferable resistance is a significant concern in the context of antimicrobial resistance, as plasmids can spread resistance genes among bacterial populations, leading to the emergence of multidrug-resistant strains [123].

Since the 1970s, plasmids have been categorized based on incompatibility [124], which denotes the incapacity of plasmids sharing similar replication and partition systems to coexist within the same host (F, P, I, X, etc.). For instance, *Enterobacteriaceae* has 27 Inc groups, *Pseudomonas* has 14 Inc groups, and *Staphylococcus* has approximately 18 Inc groups [124–126]. Some Inc groups in *Pseudomonas* are identical to those found in *Enterobacteriaceae*, such as IncP-1 (equivalent to IncP), IncP-3 (equivalent to IncA/C), IncP-4 (equivalent to IncQ), and IncP-6 (equivalent to IncG/U).

Notably, more than 75% of plasmids in *Enterobacteriales* belong to specific Inc groups, including IncF, IncA/C (equivalent to IncP-3), IncL/M, IncI, IncHI2/S, and IncN [127]. IncF plasmids are frequently encountered in clinical enterobacterial strains and play a significant role in the dissemination of antimicrobial resistance and virulence genes. These plasmids are responsible for the spread of carbapenemase, expanded-spectrum β-lactamase, and plasmid-mediated quinolone resistance genes [55]. IncM plasmids pose an emerging threat due they can carry multiple ARGs and are found in clinical, animal, and environmental isolates [128,129].

2.3.2.2 Insertion sequences

Insertion sequences (IS) are short genetic elements typically ranging in length from approximately 700 to 2500 base pairs that are commonly found in the genomes of bacteria. They are mobile and can move within the genome, leading to their name "insertion" sequences. IS elements play a significant role in the evolution and genetic diversity of bacteria [130]. IS elements are typically composed of a transposase gene,

which encodes the enzyme responsible for the movement of the element, flanked by inverted repeat sequences [131]. The transposase enzyme recognizes the inverted repeat sequences and catalyzes the excision and reinsertion of the IS element at different locations within the genome. This process is known as transposition. IS elements different from other transposases in that rarely carry genetic cargo other than the transposase.

IS elements differ from other transposons in that they rarely carry genetic cargo other than the transposase, which are determined by specific amino acids. The most common motifs include DDE (Asp, Asp, and Glu) and HUH (two His residues separated by a large hydrophobic amino acid) [98,132]. The classification of IS elements also depends on whether transposition follows a conservative, cut-and-paste mechanism, where the IS is simply excised from the donor genome and inserted into the recipient genome, or a replicative mechanism [133].

ISfinder [134] is a comprehensive tool for identifying IS elements and currently assigns names to IS based on the species in which they were first identified, followed by a number (e.g., IS*Aba1* for *A. baumannii*). However, IS elements were originally designated with numbers, such as IS6 , IS*26* and IS*257,* IS elements play an important role in the dissemination of resistance determinants in GNB [53,57].

IS elements facilitate genetic rearrangements including inversions and duplications, within the bacterial genome [136]. Their mechanism of action also facilitates deletion of adjacent DNA, often in a random manner. By inserting into different genomic locations, IS elements can disrupt genes or regulatory sequences, leading to the alteration of gene expression and potentially the acquisition or loss of phenotypic traits [137]. Moreover, IS elements can act as vehicles for the dissemination of other genetic elements, including antibiotic resistance genes or virulence factors. When an IS element inserts near these genes, it can promote their mobilisation and transfer to other bacteria through mechanisms such as transposition (via their encoded transposase) or recombination [98]. Horizontal gene transfer of genetic material, when mediated by IS elements, is a significant contributor to the spread of antimicrobial resistance among bacterial populations and a recognized means by which novel genes can be captured and introduced in clinically relevant bacterial lineage. The presence of IS elements in bacterial genomes enhances their ability to adapt and their capacity to evolve. IS elements can also contribute to the rapid evolution of bacterial populations in response

to selective pressures, such as exposure to antibiotics or changes in the environment [138].

2.3.2.3 Transposons

Transposons are genetic elements that can move from one location in a genome to another through a cut-and-paste mechanism. Their movement plays a significant role in driving genetic changes and has been instrumental in genome evolution. Additionally, transposons can be utilized to introduce foreign DNA into a genome when necessary [131]. Transposons are composed of three main components: the transposase gene, terminal inverted repeats (TIRs), and the intervening DNA sequence. The transposase enzyme catalyzes the excision and integration of the transposon, recognizing the TIRs as recognition sites [139]. The transposon sequence between the TIRs can carry various genes, including antibiotic and metal resistance genes (Partridge et al. 2018) and virulence genes [92]. IS elements are the simplest type of transposons. They consist of a gene encoding a transposase enzyme, which catalyses the transposition process, and short inverted repeats at the ends, which serve as recognition sites for the transposase. However, IS elements can insert themselves into other transposons, creating composite transposons that carry additional genes between the IS elements. These genes may confer antibiotic resistance or other traits to the host organism. IS elements can also cause mutations or rearrangements in the genome by inserting into or near genes, promoters, or regulatory regions [140]. Indeed, transposons are responsible for a wide range of mutations and genetic polymorphisms, thereby making a significant contribution to the genetic diversity of genomes [141]. So, they play a crucial role in the evolution and adaptation of bacteria. They also facilitate horizontal gene transfer, allowing genes carried by the transposon to be disseminated between different bacterial species or strains.

2.3.2.4 Integrative and conjugative elements

Integrative and conjugative elements (ICEs) are MGEs that are capable of integrating into the chromosome of the host bacterium and mediating their own transfer between bacterial cells through conjugation [142]. They range in size from approximately 18 kb to more than 500 kb and combine the characteristics of both plasmids and bacteriophages, allowing for the horizontal transfer of genetic material and contributing to the spread of diverse traits, including antibiotic resistance genes [98].

ICEs are typically composed of three functional modules: the integration/excision module, the conjugation module, and the accessory module [143]. The integration/excision module contains genes responsible for the site-specific integration of the ICE into the host chromosome and its subsequent excision. The conjugation module consists of genes involved in the production of the conjugative machinery required for DNA transfer between bacterial cells. The accessory module carries a variety of genes that can confer different phenotypic traits to the host bacterium, such as antibiotic resistance genes, virulence factors, metabolic enzymes or other traits that promote niche adaptation. ICEs are maintained by integrating and replicating with a replicon of the host genome, and they can propagate through conjugative transfer to recipient isolates after excision from the replicon as a circular covalently closed molecule [142]. These elements typically carry cargo genes and are considered modular mobile genetic elements. They integrate into the host genome via site-specific recombination and are vertically propagated during chromosomal replication and bacterial cell division [144]. Furthermore, by integrating into the host chromosome, ICEs have the ability to confer new phenotypes to bacterial isolates [145]. Their abilities of integration into the bacterial chromosome and mediate their own transfer contribute to the spread of diverse traits, including antibiotic resistance genes, among bacterial populations.

2.3.2.5 Integrons

Integrons are genetic elements that play a significant role in the acquisition and dissemination of antibiotic resistance genes among bacteria [146]. They are unable to move independently in their own right but they have the ability to acquire gene leading to the MDR phenotype [147]. Integrons are divided into class 1, 2 and 3. The class 1 integron are frequently associated with transposons on plasmids and are widely disseminated in GNB circulating in environmental clinical and veterinary settings.

Integrons possess a site-specific recombination system capable of integrating, expressing, and exchanging specific DNA elements in the form of gene cassettes [148]. They can capture, rearrange, and express gene cassettes through site-specific recombination. They consist of an integrase gene (*intI*), a promoter (Pc), a recombination site (*attI*) and a target attachment site (*attC)* [149]. The integrase enzyme encoded by the *intI* gene catalyzes the site-specific recombination events that allow the incorporation of gene cassettes into the integron structure [150]. In the clinical environment, IS26-associated integrons play a crucial role due to their tendency to integrate alongside other IS26 [151]. According to evolutionary reconstructions, a chromosomal class 1 integron

carried the biocide resistance gene *qacE* [152]. This integron mobilized and contained genes conferring resistance to biocides and mercury, which were extensively used before the antibiotic era, granting a significant selective advantage to the bacterial host. Subsequently, the sulphonamide resistance gene *sul1* was inserted, leading to the deletion of the terminus of *qacE* and the formation of *qacEΔ1-sul1*, which now resides in the 3′ conserved segment of clinical class 1 integron variants [153].

Despite class 1 integrons' ability to recognize and integrate a wide array of gene cassettes, there appears to be a prevalence of antimicrobial resistance genes among the diverse cassette functions associated with class 1 integrons [154].

2.3.2.6 Genomic islands

Genomic islands (GIs) are fragments of DNA that are inserted into the chromosome through HGT [155], meaning that it was acquired from another source and not transposed from one part of the chromosome to another [156]. GIs are typically large, discrete regions of the genome that differ in G+C composition from the rest of the bacterial genome. They play a crucial role in the evolution and adaptation of bacteria by facilitating the dissemination of ARGs, virulence genes, and the formation of catabolic pathways [157]. They typically have atypical G+C content and are often inserted at tRNA loci [158]. Many GIs are flanked by repeat structures and carry fragments of other mobile and accessory genetic elements [155].

GIs can be classified into different types based on their origin and characteristics. Pathogenicity islands (PAIs) are GIs that carry genes associated with virulence and are frequently found in pathogenic bacteria [159]. One example of a putative genomic island is IntIPac, which has been reported to promote antimicrobial resistance in *Acidithiobacillus ferrooxidans* [160]. Genomic islands have also been found to carry ESBL $bla_{\text{OXA-10}}$, a gene commonly found in *A. baumannii* [161] , as well as the $bla_{\text{GES-5}}$ gene cassette carried by the intI3-containing Tn*7221* structure observed in clinical *P. aeruginosa* strains [162–164]. GIs also play a key role in virulence. *Yersinia* high-pathogenicity island (HPI) contains genes responsible for synthesizing the siderophore yersiniabactin and is correlated with blood stream infections in mouse models [166].

The presence of GIs in bacterial genomes contributes to the evolution and adaptation of bacteria. GIs can provide selective advantages to bacteria by allowing them to colonise new niches, evade the host immune system, or acquire resistance to antimicrobial agents [165]. They play a crucial role in the emergence and spread of

multidrug-resistant pathogens, as they can carry multiple resistance genes and facilitate their dissemination among bacterial populations [155].

2.3.2.7 Phages

Phages, also referred to as bacteriophages, are viruses that exclusively infect and replicate within bacterial cells [167]. They have a unique ability to specifically target and infect bacteria, making them a powerful tool in understanding bacterial biology and their potential applications in various fields.

Phages exhibit a wide range of sizes, morphologies, and genomic organizations [168] . Being mobile across bacterial isolates, they are recognised as significant drivers of microbial composition and function, capable of exerting substantial impacts on microbial densities [169,170]. Phages, specifically particular phage virions, consist of three major components: a capsid that encloses the genome, a tail which functions as a conduit during infection to facilitate the transfer of the genome into the host cell, and a specialized adhesive system located at the tail's end that enables recognition of the host cell and penetration of its wall [88,89]. A prophage is a bacteriophage that has integrated its DNA into the bacterial genome. Prophages can exist in two different states: lytic and lysogenic [171]. During the lytic cycle, the phage undergoes replication and causes lysis of the host cell. In the lysogenic cycle, the phage DNA becomes part of the host genome and is transmitted to succeeding generations. Environmental stressors, such as starvation or exposure to toxic chemicals, can trigger the excision of the prophage, leading it to enter the lytic cycle[171].

Phages have been acknowledged for their role in promoting microbial diversity through a phenomenon known as "kill the winner" dynamics [172,173]. Although phages are unable to infect and replicate within human cells, they play a vital role in the human microbiome and serve as crucial mediators of genetic exchange between pathogenic and non-pathogenic bacteria [174,175].

2.4 One Health AMR genomic surveillance

The WHO has identified AMR as a major threat to public health and advocates for a One Health approach to tackle this global problem [176]. One Health recognizes the interconnectedness between human health, animal health, and the environment. AMR can spread through various means, including direct contact between humans and animals, exposure to antibiotic residues from human waste, or indirectly through

contaminated food or water. These routes create a well-connected transmission cycle, linking the microbiomes [43] (Figure 7).

Despite the knowledge that water bodies serve as important reservoirs of carbapenem-resistant bacteria, environmental surveillance of AMR currently lags behind in comparison to the other major sectors. This represents a significant gap in our understanding of AMR transmission dynamics.

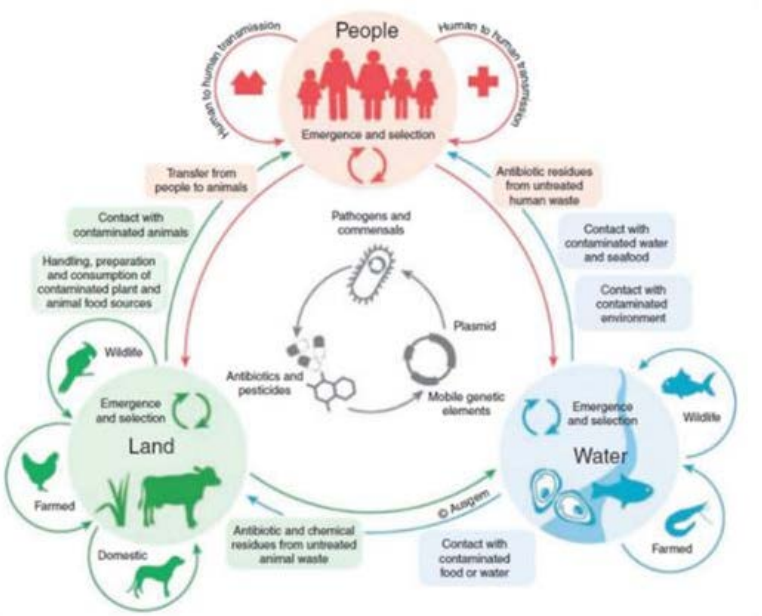

Figure 7: Spread of AMR bacteria, and the mobile genetic elements that they carry, between environment, animal and human. AMR is transmitted via diverse mechanisms, such as direct interactions between humans and animals, contact with antibiotic residues from human waste, or indirect transmission via contaminated food or water. These pathways contribute to a closely interconnected transmission cycle, connecting the microbiomes. Figure obtained from [43].

Diagnosis of bacterial infections is crucial for patient management, although it can be challenging due to similarities with other causes. The gold standard examination in clinical laboratories for these infections is culture [177]. However, microbiology culture faces various problems, including result delays, identification errors, and difficulties in culturing certain isolates [178–180]. Uncommon bacteria may be underdiagnosed as they often grow poorly in routine clinical microbiology culture, and even when cultured successfully, biochemical identification tests can provide erroneous or unidentified results. Misidentification can also occur with species-level identification using MALDI-TOF MS, highlighting the need for whole-genome sequencing (WGS) for accurate

diagnosis and to inform the development of improved diagnostic assays [178]. There have been concerted efforts to improve culture methods for a greater diversity of bacteria. Various effective targeted and open-ended approaches have been developed and applied successfully. Instances include the focused identification of particular bacteria in mixed plate cultures through colony hybridization, cultivation in simulated natural environments or in collaboration with 'helper' strains, and the adaptation of media preparation techniques or the creation of customized media. This may involve supplementing media with potential growth-stimulatory factors like siderophores [181].

While traditional molecular diagnosis methods like PCRs can screen for multiple genes in a single assay, they cannot match the scalability and comprehensive screening capabilities of WGS.

WGS involves analyzing the entire genomic DNA sequence of an isolate, providing the most comprehensive characterization of the genome [182]. It has uncovered the prevalent occurrence of multidrug resistance on a broad scale [183]. WGS enables correlation of STs, serotypes, and phylogroups with detailed relationships through phylogenetic analysis and gene data, allowing for characterization of AMR, virulence, and the presence of MGEs. Phylogenetic analysis using WGS data provides valuable insights into disease outbreak epidemiology [184–186]. Notably, advancements in software and tools have improved reproducibility and repeatability in bioinformatic analyses [187]. Public WGS databases have become essential resources for genomic epidemiological investigations. WGS analysis offers significant advantages over traditional molecular approaches in epidemiological investigations and AMR surveillance [188].

The increasing availability of WGS methods has led to a steady expansion in its use, including for tracking outbreaks of resistant organisms, identifying the significance of MGEs, characterizing bacterial pathogens, and contributing to our understanding of resistance evolution [189].

One Health AMR genomic surveillance provides a comprehensive approach that integrates genomic data from multiple sectors, including human health, animal health, and environmental health [176], to monitor and understand the dynamics of antimicrobial resistance (AMR) across different populations and ecosystems. As such, WGS is critical to understanding the AMR and pathogen evolution through a One Health lens.

Chapter 3. Materials and Methods

3.1 Summary

As the basis of this project, samples were collected from diverse waterbodies by colleagues at the University of South Australia. To monitor the environment, we utilised the qPCR LightCycler® 480 Instrument II (Roche Life Science) to assess the presence and abundance of bacteria in each sample. After serial dilutions, samples were plated onto commercial agar plates containing carbapenems (Oxoid Brilliance™ CRE Agar plates (Thermo Fisher Scientific Australia, Adelaide, SA)). Isolates that grew on agar supplemented with a carbapenem were identified using MALDI-TOF MS/MS (Bruker Daltonics), then DNA was extracted (QIAamp DNA mini kit; Qiagen, Hilden, Germany) and prepared for whole genome sequencing. Representative isolates from each clade can be selected for antibiotic minimal inhibitory concentration (MIC) testing when needed (Figure 8).

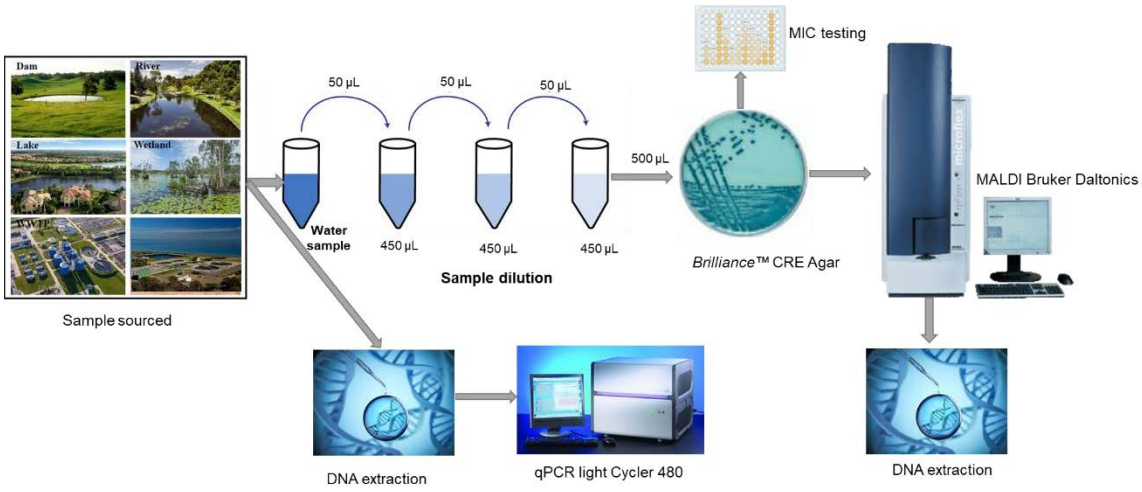

Figure 8: Methodology for Sample Collection and Data Acquisition from Collection Sites: The process involves dilution before plating, identification using MALDI, DNA extraction, and subsequent analysis through PCR and WGS.

Whole genome sequencing was performed in collaboration with the Quadram Institute in the UK. Sequencing was performed using an Illumina short read platform [190], with the resulting reads put through quality control (QC) using FastQC. Whole genome sequence assemblies were generated using the assembly pipeline Shovill (https://github.com/tseemann/shovill), which utilises the SPAdes genome assembler tool. Additional reference assemblies were downloaded from public database GenBank (https://www.ncbi.nlm.nih) (Figure 9).

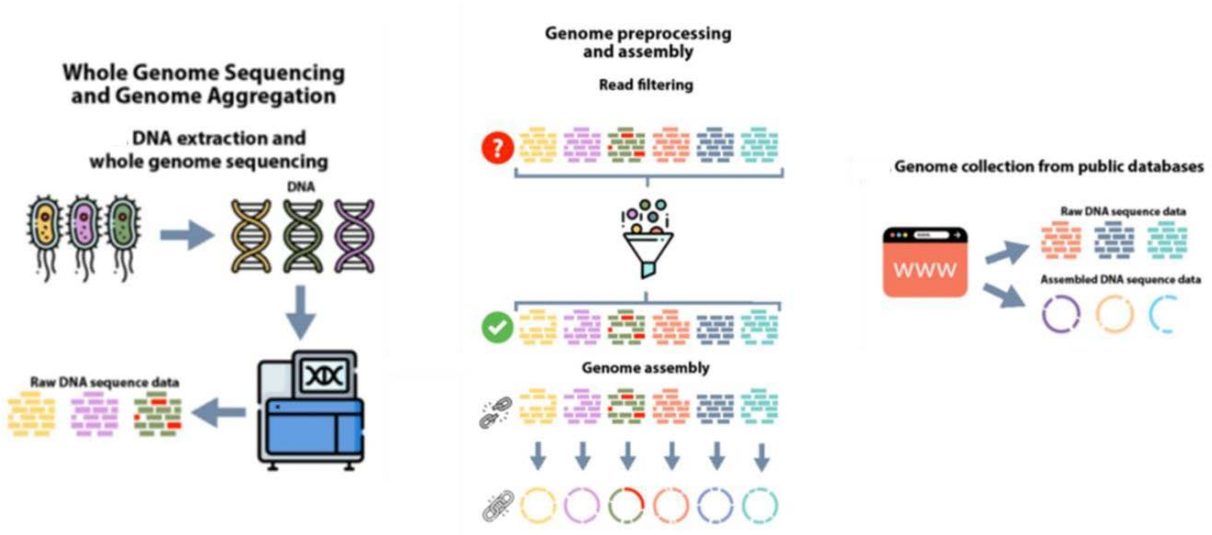

Figure 9: Whole-genome sequencing analysis using an Illumina short read platform with the resulting reads put through QC using FastQC. Genome sequence assemblies were generated by using the assembly pipeline Shovill (github.com/tseemann/shovill). Reference assemblies were downloaded from the public database GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Once genomes were assembled, QC was performed through our in-house quality control pipeline [191] (script available at https://github.com/maxlcummins/), which removes genomes of low quality (e.g. high contig numbers) and identifies potential contamination (e.g. multiple species calls). Each genome was then passed through numerous pipelines and software, to interrogate the data for information such as the presence of important genes [192–201], typing the chromosome and mobile genetic elements [134,202,203], and generating phylogenetic trees [204–206] to visualise the relatedness of sequence data [207–209], be it the whole genome or specific genes (Figure 10).

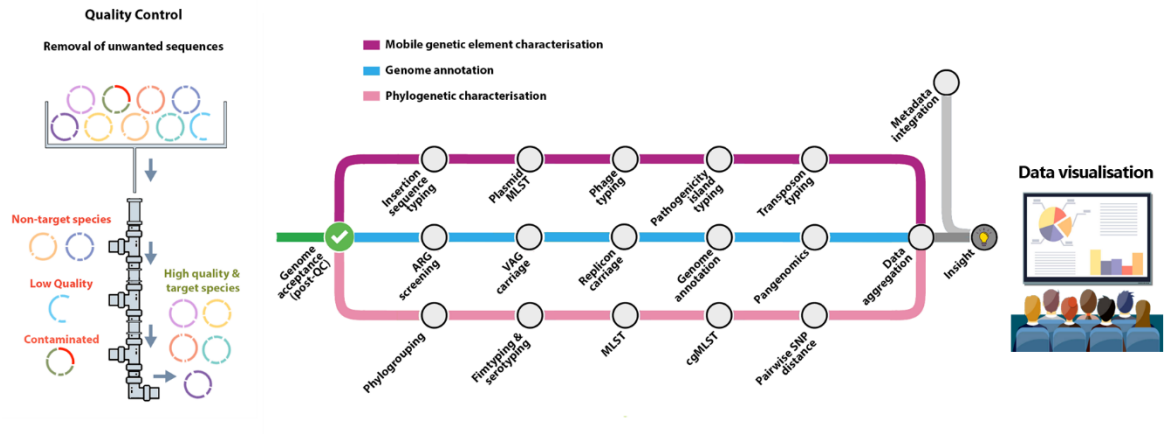

Figure 10: Overview of bioinformatics methodology. Once genomes were assembled, QC was performed through the quality control pipeline[191], which removes low quality and contaminated genomes to ensure outputs are good quality. Each genome was then passed through numerous pipelines and software such as phylogenetic characterisation pipeline[204–206], genome annotation [210,211] and mobile genetic element characterisation pipelines [134,202,203] with various data visualisation tools [207–209] to produce figures.

The workflow and tools chosen in this study are established in detail follows below.

3.2 Sample collection and bacterial isolation

The study focused on collecting various water samples, including influent wastewater from South Australia (SA), raw wastewater from two wastewater treatment plants in the South Coast of New South Wales (NSW), and landfill leachate from one NSW site. Over a one-year period (2018-2019), a total of 56 water samples from SA and 8 water samples from NSW were collected monthly in triplicate, resulting in the isolation of 665 bacterial strains from SA and 859 bacteria from NSW. The samples were processed immediately after collection, and bacterial isolates were cultured and identified using diverse techniques, such as Oxoid Brilliance™ CRE Agar plates and Plate Counting Agar (PCA) from Thermo Fisher Scientific. Single bacterial isolates, which were less than 24 hours old, were identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) [212] and preserved in glycerol stocks at -80°C for further analysis.

Additionally, during the breeding seasons of 2018 and 2019, silver gull chicks from NSW were sampled to investigate bacterial presence, and cloacal swabs were collected for culturing on MacConkey agar. The identification and storage of these isolates were conducted using similar methods to those used for wastewater samples.

3.3 DNA extraction and Whole-genome sequencing

DNA from a small piece of a colony (isolates) with MALDI-TOF MS scores of 2.000-3.000 (the log (score) value ranged from 0 to 3, a log (score) value ≥1.7 is indicative of a close relationship (i.e., at the genus level) and a log (score) value ≥2.0 is the set threshold for a match at the species level) were extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Nucleic acid quality was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA concentrations for all samples were measured by fluorometric quantitation using a Qubit instrument and High Sensitivity dsDNA HS Assay kit (Thermo Fisher Scientific), with purified DNA extracts stored at −20°C until sequencing.

WGS was performed on the Illumina NextSeq 500 [213] platform using a modified Nextera low input tagmentation approach (Foster-Nyarko et al., 2020). Genomic DNA was normalized to 0.5 ng µl−1 with 10 mM Tris-HCl before the library preparation. The pooled library was run at a final concentration of 1.8 pM on a mid-output flow cell following Illumina recommended denaturation and loading parameters. The raw data was demultiplexed using Basespace (https://basespace.illumina.com).

3.4 Bioinformatical methods

Computational analyses were conducted at UTS using the Interactive High-Performance Computing (HIPC) facility, which utilizes a virtual machine-based system. The code and commands utilized in this study are compatible with UNIX-based operating systems (Linux/Mac).

3.4.1 Genome quality control, assembly, and annotation

We used the Shovil (https://github.com/tseemann/shovill) program in conjunction with the SPAdes [214] genome assembler to assemble reads into contigs from sequenced DNA fragments. This approach efficiently merged individual reads, resulting in the creation of contigs. The robustness and accuracy of the SPAdes assembler ensured a more comprehensive representation of the target genomes. By leveraging Shovil and SPAdes, we successfully generated reliable genomic data, providing a strong foundation for our subsequent analyses.

After assembling the genomes, it required assembled genome lengths to be within the ranges observed for members of the genus identified in a snapshot of NCBI's RefSeq database as well as assemble to fewer scaffolds, defined for each species. Genomes were also analysed using FASTQC [191] (reads) and CheckM [215]

(assemblies) to determine their level of completeness and contamination. FASTQC examined various quality metrics, while CheckM estimated completeness and contamination by evaluating marker genes. Each genome had to meet three main criteria in FASTQC: genome size, an N50 value, and sequencing depth. For CheckM, genomes exhibiting a 10% or greater contamination score were excluded as were those with an under 90% completion score. Strains were also typed using mlst (http://github.com/tseemann/mlst) using scheme autodetection and where possible, only strains which encoded the appropriate MLST alleles [216] were included for further analysis.

Genomes were annotated using Prokka v1.14.6 [211] and managed using SnapGene v4.1.9 (snapgene.com). The RAST annotation pipeline [210] was also utilized on representatives to cross check annotations. In cases of ambiguity, additional BLAST searches were performed, and genes were annotated based on consensus.

3.4.2 Phylogenetic analysis

Phylogenetic analyses were performed using several different pipelines to achieve analyses at multiple resolutions, each selected depending on the range of genus or species included in each tree. The process for generating this analysis from short-read data involves two main steps after creating draft genome assemblies. Firstly, generating multisequence alignments, and secondly, constructing phylogenetic trees using these multisequence alignments. Additionally, pangenome analyses were used to identify all groups of orthologous genes within a population and the accessory genes.

The pipelines used in phylogenetic analysis in this study are as follows:

- Phylosift [206]: a technique that leverages a reference database comprising protein and RNA sequences to assess new genetic sequences for homologs and establish their phylogenetic relationship with entries in the database. The method focuses on 37 "elite" marker gene families, which are consistently present and typically exist in single copy across various organisms. This tool conducts phylogenetic analysis by placing short sequences onto reference phylogenies. This approach offers several conceptual advantages over taxonomic analysis, as it involves interpreting sequence data based on the placement within evolutionary relationships rather than relying on hierarchical classification information.

- Parsnp [217] (github.com/marbl/parsnp): A rapid core-genome multi-aligner, combined with Gingr, a dynamic visual platform, collectively offers interactive core-genome alignments and phylogenetic trees. Parsnp employs MUMs to recruit similar genomes and serve as anchors for the multiple alignment process. It takes a directory of MultiFASTA files as input for alignment and produces a core-genome alignment, variant calls, and an SNP tree as output. The generated outputs can be visually explored using Gingr.

- Mashtree [205]: a tool utilizes two key algorithms present in existing software packages. It employs the min-hash algorithm from Mash to create genome sketches, adjusting parameters such as sketch size for enhanced discriminatory power. Using Mash, Mashtree calculates distances between genomes and records them in a pairwise distance matrix. Subsequently, Mashtree applies the neighbor-joining algorithm from QuickTree to generate a dendrogram based on the Mash distance matrix, utilizing default options.

3.4.2.1 Multi-locus sequence typing

Multi-locus sequence typing (MLST) is a valuable classification system used for bacteria. It offers various schemes tailored to specific organisms. To ensure efficient and accurate MLST assignments, we utilize the mlst software (github.com/tseemann/mlst), which features automatic scheme detection and default settings for consistent and comparable analyses. This rigorous methodology contributes significantly to our understanding of bacterial evolution, transmission patterns, and epidemiological investigations.

3.4.2.2 Core genome-based alignment

Core genome phylogeny offers an alternative approach to SNP-based reference phylogenies. This method involves generating reference-independent alignments and utilizing an automated genome annotation pipeline Prokka [211]. Subsequently, we employed Roary [218] to produce the core genome alignment (via Mafft), which comprises the set of genes present in all members of the included isolates. The identity/similarity limits used to define putative orthologs in Roary for each of the genera. An all-against-all comparison is performed with BLASTP on the reduced sequences using default percentage sequence identity (95%).

3.4.2.3 Phylogenetic tree generation and visualization

SNP-based core genome phylogenetic analyses serve as useful tools for reconstructing evolutionary relationships among closely related sequences, such as strains within the same sequence type or clonal complex. To generate such alignments, we employed Snippy (http://github.com/tseemann/snippy), a reliable tool that incorporates recombination filtering software Gubbins [219]. Gubbins identifies regions with unusually high levels of SNPs, which may indicate potential recombination events. It then compares these regions to background levels of SNP accumulation and removes them if necessary. These SNP-based core genome phylogenies, implemented with Snippy and complemented by recombination filtering using Gubbins, was deployed through the automated pipeline snakemake [220], and provide valuable insights into the evolutionary relationships among closely related strains. Caution must be exercised when selecting a reference genome to serve as the basis of these alignments, as it can introduce potential biases in the analysis.

Additional phylogenetic trees were generated for specific cases. This included using PhyloSift [206], designed for analysing diverse isolates or multiple species. SNP-based phylogenetic trees were also generated using either the harvest suite (parsnp and gingr) [221], or Snplord (http://github.com/maxlcummins/ pipelord/tree/master/snplord), an automated snakemake pipeline [220] that incorporates SNP-sites for analysis. In specific cases, mashtree version 1.2.0 [205] was used with 100 bootstrap replicates. Custom R scripts were employed, utilizing various R packages such as R packages ggtree v1.16.6 [222], dplyr v0.8.3 [223], magrittr v1.5 [224], ggplot2 v3.2.1 [225], readr v1.3.1 [226], reshape2 v1.4.3 [227], tidytree v0.2.6 [228], and ggimage v0.2.8 http://cran.nexr.com/web/packages/ ggimage/vignettes/ggimage.html for visualising, and annotating the trees.

Alignments were converted into approximately maximum-likelihood trees using FastTree2 [229], which were then visualized using iTOL software v4 [208].

3.4.2.4 Pan genome analysis

Pangenome analysis was performed using Roary v3.11.2 [218]. The Roary pipeline enables the assessment of genetic diversity within a population by identifying core and accessory genes. Four categories are used: core (genes present in all genomes), soft core (genes present in >95% genomes), shell (genes present in >15% but <95% genomes), and cloud (genes present in <15% genomes). It efficiently clusters

genes based on sequence similarity and calculates the pangenome size, providing valuable insights into gene content variation among strains.

To improve the visualization and interpretation of the pangenome results, Phandango v1.3.0 [230] was utilized. This tool facilitates the identification of gene presence/absence patterns, genomic differences, and exploration of evolutionary relationships and gene dynamics in the studied microbial population.

3.4.3 Genotypic identification of novel species

The process of genomic identification of new species involved utilizing SpeciesFinder 2.0 0 [231] and Kraken2 [232] to first identify the genus of the isolate. Subsequently, we performed phylogenetic and SNP distance analyses to visualize the relationships among isolates with known and unknown species classification. To then classify any isolates with a significant phylogenetic distance from known species, we employed three principal bioinformatic tools.

For the pairwise genome comparisons, we used ANIb and ANIm algorithms from the JSpecies web server [197] with a 95% cut-off value for species delimitation [196]. Additionally, we employed the GGDC tool [233] to predict DNA-DNA hybridization results with a 70% cut-off value. Furthermore, we aligned complete 16S rRNA and rpoB sequences using Clustal Omega, with similarity cut-off values of 99.5% [195] and 97.7% [234], respectively.

These comprehensive analyses facilitated the identification of novel species and the determination of their relatedness to known species.

3.4.4 Gene screening

Using WGS data, we identified the presence or absence of specific genes of interest, such as ARGs, VAGs, plasmid replicons, and ISs. The NCBI (National Centre for Biotechnology Information) website and BLAST (Basic Local Alignment Search Tool) tool were commonly employed for this analysis, offering both online portal and command line options, enabling large-scale gene screening against extensive databases. Another approach utilised The Centre of Genomic Epidemiology (genomicepidemiology.org/), which hosts databases like Species Finder 2.0 [231], ResFinder [235], VirulenceFinder [236], PlasmidFinder [237], pMLST [202], and Serotype Finder (https://cge.food.dtu.dk/services/SerotypeFinder/), accessible through online screening

tools. These tools were used in conjunction with the following databases: VFDB [198], CARD [192], NCBI AMR FinderPlus [238] in HIPC platform.

In silico species identification was performed using Kraken2 [232]. Abritmar v1.0.11 (https://github.com/MDU-PHL/abritamr) and Abricate (https://github.com/tseemann/abricate) were employed to determine certain carriage ARGs, VAGs, and AMR-associated point mutations. A minimum nucleotide identity and coverage requirement of 90% were set for the detected genes.

3.4.5 Mobile Genetic Elements detection

This thesis explored three primary categories of MGEs: plasmids, pathogenicity islands (GIs), and integrative and conjugative elements (ICEs).

3.4.5.1 Plasmid analysis

While completed plasmids are seldom resolved using short reads, it is still possible to identify plasmid Inc groups especially for some of the most common plasmid incompatibility (aka plasmid family) types [239]. This data proves useful in the preliminary identification of specific lineages of plasmids, such as IncF, IncM, IncP, and HI2 plasmids. Mapping of reference plasmids was accomplished by employing Abricate with data containing the plasmid of interest, generating a simplified BLAST output, and performing comparisons of plasmid sequences against reference plasmids using BRIG [194] software.

Another technique for plasmid analysis involves determining the plasmid multilocus sequence types (pMLST), which was executed using pMLST (pubmlst.org/) [240]. The output was processed using custom scripts available at http://github.com/maxlcummins/pipelord/blob/main/scripts/combine_IncF_RST.py (for IncF plasmids) and http://github.com/ maxlcummins/pipelord/blob/main/scripts/combine_pMLST.py (for other plasmid types), as part of the aforementioned snakemake pipeline called Pipelord.

3.4.5.2 Genomic islands analysis

The analysis of putative genomic islands (GIs) was carried out using Islandviewer 4 [203], a tool for identifying genomic islands in bacterial genomes. To validate the presence of these putative GIs and gain further insights into their characteristics, a BLASTn search was conducted to compare these regions with the existing data

deposited NCBI database. This step helped confirm whether similar genomic islands had been previously reported in other studies or organisms.

To visualize and assess the genetic context of the identified GIs, the Easyfig [207] software was employed. Easyfig facilitates the comparative analysis of genomic regions and allows us to explore the arrangement and organization of genes within the putative GIs. This visualization was instrumental in understanding the potential functions and evolutionary relationships of these genomic islands.

3.4.5.3 Integrative and Conjugative Elements analysis

Islandviewer 4 [203], a bioinformatic tool renowned for its precision in identifying genomic islands, including (ICEs), was employed for the ICEs analysis. This involved comparing the ICEs with reference genomes in its comprehensive database to ensure accuracy. To verify the identified ICEs, a BLASTn search was performed, comparing their sequences with the vast genetic data available in the NCBI database. This allowed for determining their uniqueness and whether similar ICEs had been documented in previous studies or organisms. Furthermore, cross-referencing with the NCBI database provided valuable insights into the functions, host range, and potential roles of these ICEs across different bacterial species.

For a comprehensive understanding of the ICEs' genetic context and arrangement, we utilized BRIG [194] software, enabling a comparative analysis of ICEs against reference strains. The visualization process facilitated the examination of structural variations and gene content within the ICEs, offering significant insights into their evolutionary relationships and potential functions as mobile genetic elements.

Chapter 4. Genomic analysis of *Elizabethkingia* species from aquatic environments: evidence for potential clinical transmission

4.1 Declaration

This chapter represents a manuscript accepted for publication.

It responds to aim 2, 3 and 4.

Authors

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Author contribution statement

SH: writing the original draft, formal analysis, investigation, writing – review and editing. V.M.J: Writing – original draft, formal analysis, investigation, supervision, writing – review and editing, D.J.B: Investigation and resources. I.G.C: Investigation and resources. B.D: Investigation, conceptualization, validation, formal analysis, data curation, writing – review and editing. S.A: Investigation, formal analysis, data curation. E.D: Conceptualization, funding acquisition, supervision, writing – review and editing. D.B: Resources and methodology. M.J.B: Resources and methodology. P.N.A.H: Resources, supervision, writing – review and editing. E.R.W: Investigation, methodology, validation, writing – review and editing draft versions. S.P.D: Resources, supervision, funding acquisition, project administration, writingg- reviewing and editing draft versions.

Ethical approval on new species naming

The novel bacterial species described in this work, *Elizabethkingia umeracha*, was officially approved by the Peramangk, Kaurna, and Ngarrindjeri people. They are recognized as the traditional owners and custodians of the waters and lands where the environmental samples used in this research were collected. The name "Umeracha" was advised by these indigenous communities, and it holds the meaning "fine waterhole" in the Peramangk language. Additionally, the water samples used in our study were approved by the relevant authority for data publication.

Authors signatures

Sopheak Hem: Production Note: Signature removed prior to publication.

Veronica M Jarocki: Production Note: Signature removed prior to publication.

Dave J Baker: Production Note: Signature removed prior to publication.

Ian G Charles: Production Note: Signature removed prior to publication.

Barbara Drigo: Production Note: Signature removed prior to publication.

Sarah Aucote: Production Note: Signature removed prior to publication.

Erica Donner: Production Note: Signature removed prior to publication.

Delaney Burnard: Production Note: Signature removed prior to publication.

Michelle J Bauer: Production Note: Signature removed prior to publication.

Patrick N A Harris: Production Note: Signature removed prior to publication.

Ethan R Wyrsch: Production Note: Signature removed prior to publication.

Steven P Djordjevic: Production Note: Signature removed prior to publication.

4.2 Abstract

Elizabethkingia species are ubiquitous in aquatic environments, colonize water systems in healthcare settings and are emerging opportunistic pathogens with reports surfacing in 25 countries across six continents. *Elizabethkingia* infections are challenging to treat, and case fatality rates are high. Chromosomal *bla*$_{B}$, *bla*$_{GOB}$ and *bla*$_{CME}$ genes encoding carbapenemases and cephalosporinases are unique to *Elizabethkingia spp*. and reports of concomitant resistance to aminoglycosides, fluoroquinolones and sulfamethoxazole-trimethoprim are known. Here, we characterized whole-genome sequences of 94 *Elizabethkingia* isolates carrying multiple wide-spectrum metallo-β-lactamase (*bla*$_{B}$ *and bla*$_{GOB}$) and extended-spectrum serine-β-lactamase (*bla*$_{CME}$) genes from Australian aquatic environments and performed comparative phylogenomic analyses against national clinical and international strains. qPCR was performed to quantify the levels of *Elizabethkingia* species in the source environments. Antibiotic MIC testing revealed significant resistance to carbapenems and cephalosporins but susceptibility to fluoroquinolones, tetracyclines and trimethoprim-sulfamethoxazole. Phylogenetics show that three environmental *E. anophelis* isolates are closely related to *E. anophelis* from Australian clinical isolates (~36 SNPs), and a new species, *E. umeracha* sp. novel, was discovered. Genomic signatures provide insight into potentially shared origins and a capacity to transfer mobile genetic elements with both national and international isolates.

4.3 Background

The environment is a known reservoir for both opportunistic pathogens and antimicrobial resistant (AMR) bacteria [241]. It is important to investigate environmental microbial populations as prominent extended-spectrum β-lactamases (ESBLs), quinolone resistance genes and carbapenemases originated from marine and soil bacterium and have subsequently entered clinical isolates through horizontal gene transfer (HGT) [242,243] , as well as identifying potential infection transmission pathway [241].

Elizabethkingia species are aerobic, Gram-negative bacilli members of *Weeksellaceae* commonly found in the environment, particularly in soil and freshwater bodies, as well as insects and amphibians [244–247]. There are currently six identified *Elizabethkingia* species, all of which have undergone various taxonomic and nomenclature makeovers [53]. *Elizabethkingia meningoseptica,* isolated in 1959, has been known as *Flavobacterium meningosepticum* and *Chryseobacterium*

meningosepticum [248,249]. *Elizabethkingia miricola,* isolated from a Russian space station in 2003 [250], has been known as *Chryseobacterium miricola* and *Elizabethkingia* genomospecies 2 [251,252]. *Elizabethkingia anophelis,* isolated in 2011, has been described as *Elizabethkingia endophytica* and *Elizabethkingia* genomospecies 1 [66,251–253]. In addition, three new species were redefined in 2017 – *Elizabethkingia bruuniana, Elizabethkingia ursingii* and *Elizabethkingia occulta* - the former previously been referred to as *Elizabethkingia* genomospecies 3 and the latter two were both grouped as *Elizabethkingia* genomospecies 4 [251,252].

Interest in *Elizabethkingia* spp. is rising as they constitute difficult to treat emerging pathogens within hospitals and healthcare settings [254–256]. *Elizabethkingia* infections occur most often in newborns and immunocompromised patients, and the most common presentation is septicaemia [257]. However, reports of meningitis (caused by *E. meningoseptica* and *E. anophelis*), pneumonia, urinary tract infection, skin and soft tissue infections are also common [258,259]. Case fatality rates for *Elizabethkingia* spp. are high at ~25.2% in all species [260] and higher in cases of septicaemia and meningitis at 54% for *E. meningoseptica* [261] and 28.4 % for *E. anophelis* infections [262]. *Elizabethkingia* pathogenesis is largely unknown, though several virulence factor homologs have been reported, including capsule proteins, adhesins, iron uptake proteins and proteins contributing to biofilm formation [263–266].

Treatment of *Elizabethkingia* infections is complicated because most species are intrinsically resistant to clinically important antibiotics, including carbapenems and other β-lactams and aminoglycosides [53,264,265]. Resistance to fluoroquinolones and sulfamethoxazole-trimethoprim have also been observed [70]. Consistently, *Elizabethkingia* species genomes sequenced to date harbour multiple chromosomal antimicrobial resistance genes (ARGs), including genes bla_{B} and bla_{GOB}, associated with resistance to carbapenems, and extended-spectrum β-lactamase gene bla_{CME}, conferring resistance to all cephalosporins [68]. For fluoroquinolones resistance, mutations within conserved regions of DNA gyrase subunit A (GyrA) have been observed, including Ser83Ile and Ser83Arg [267]. ARGs have also been identified in *Elizabethkingia* integrative and conjugative elements (ICEs) [145], mobile genetic elements (MGEs) capable of integrating into a host genome and propagated during chromosomal replication and cell division [268]. Only two plasmids have been described and sequenced from two *Elizabethkingia* species: *E. anophelis* strain F3201 [145] and *E. miricola* strain EM_CHUV [269].

Cases of *Elizabethkingia* infections have been increasing over the past few decades, with reports surfacing in 25 countries across six continents [72,256,270–275]. *Elizabethkingia* mode of transmission remains unclear, but exposure to contaminated environments, especially waterbodies, medical devices, hemodialysis and mechanical ventilation equipment, hospital fomites, water faucets and healthcare worker hands, have all been implicated [68,70]. In addition, infections caused by *E. anophelis* have been linked to transmission events associated with mosquitoes in the Central African Republic [276]; however, this hypothesis is contentious due to a report of vertical transmission from mothers to infant of *E. anophelis* [272].

Elizabethkingia species are multidrug-resistant emerging pathogens with high case-fatality rates. To date, most studies on *Elizabethkingia* have characterized clinical isolates [277]. However, given that waterbodies are reservoirs and implicated in *Elizabethkingia* transmission pathways, this study provides a genomic analysis of whole-genome sequences derived from 94 *Elizabethkingia* isolates originating from aquatic environments in South Australia, as well as characterizing and comparing their genomic and antimicrobial resistance profiles to other publicly available *Elizabethkingia* environmental and clinical strains.

4.4 Methods

4.4.1 Sample collection and Isolation of carbapenem-resistant *Elizabethkingia* spp.

Water samples (~10 L) were collected in triplicate monthly from July 2018 to July 2019 in South Australia. The four locations represented two sources: (i) stagnant water (site A) and (ii) inland wetlands recharged by seasonal rainfall/runoff inflows (site B and C) or by a river (site D). Site A is a small rural reservoir created by damming a natural rainwater catchment area. It is fenced and not accessible to or impacted by livestock but is regularly visited by birds and particularly by ducks. Site B (wetland) is a recreation reserve covering an area of 19.4 hectares. Site C (wetland) covers 172 hectares and has a maximum capacity of 1200 megaliters.

The distance between the two wetland sites was ~10 km. Site D is a river 2,508 km in length, and the area sampled was flowing water near a wetland that covers a total of 42 hectares. Site D is used for recreational purpose only. At all sites, surface water samples were collected by dipping three sterile 10 L collection tanks below the surface. All samples were stored on ice directly after collection and processed within 2-3 hours.

Samples were processed on the day of collection. First, water samples were serially diluted and 500 µl from 2-3 consecutive 10-fold serial dilutions were plated in triplicate on Oxoid *Brilliance*™ CRE Agar plates (Thermo Fisher Scientific Australia, Adelaide, SA). Cultures were incubated at 25°C, 37°C and 44°C for 24 h. Next, using pre-sterilized toothpicks, single colonies growing on CRE Agar were plated on Plate Counting Agar (PCA; Thermo Fisher Scientific). PCA cultures were incubated at 37°C for 18-24 h, or until sufficient bacterial growth had occurred. A total of 667 bacteria were isolated and identified with Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonics) and preserved in glycerol stocks (40% v/v) at -80 °C.

4.4.2 MALDI-TOF MS species identification and DNA extraction

Fresh bacterial single isolates (<24 h old) were resuspended in 1 ml 70% ethanol, vortexed for 1 min, and centrifuged at 13,000 rpm for 2 min. The supernatant was removed, and the pellet re-dissolved with 5 µl of 70% formic acid (Baker; 90% stock) and 5 µl acetonitrile (CAN, LC-MS Grade, Merck). After 2 min centrifugation at 13,000 rpm, 1 µl of supernatant was spotted onto the target plate and left to dry. The sample was overlaid with α-cyano-4-hydroxycinnamic acid (HCCA) (1 µl) matrix (10 mg/ml^{-1}) and allowed to crystallize at room temperature. One µl Bacterial test standard (Bruker Daltonics) in 50% (v/v) ACN containing 2.5% (v/v) trifluoroacetic acid (LC-MS Grade; Thermo Fisher Scientific) was spotted, left to dry and overlaid with HCCA for calibration. MALDI-TOF MS analysis was acquired on an autoflexTM speed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) operated in linear positive mode under MALDI Biotyper 3.0 Real-time Classification (version 3.1, Bruker Daltonics) and FlexControl (version 3.4, Bruker Daltonics) software.

Spectra were acquired in the mass range of 2000 to 20 000 Da with variable laser power, and a total of 1200 sum spectra were collected in 40 shot steps. The sample spectra were identified against an MSP database library (5989 MSP entries). Identification scores of 2.300–3.000 indicate highly probable species identification, scores of 2.000–2.299 indicate secure genus identification and probable species identification, scores of 1.700–1.999 indicate probable genus identification, and a score of ≤1.699 indicates that the identification is not reliable.

Water samples were concentrated by vacuum filtration through a 0.2 µm nitrocellulose filter (Millipore) then stored at −80°C until DNA extraction. Total genomic

DNA from each 0.2 µm filter was extracted using the DNeasy PowerWater kit (Qiagen) according to the manufacturer's instructions. DNA from MALDI-TOF MS identified colonies with scores 2.000-3.00 were extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Nucleic acid quality (*i.e.*, 260/280 ratio) was measured with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA concentrations for all samples were measured by fluorometric quantitation using a Qubit instrument and High Sensitivity dsDNA HS Assay kit (Thermo Fisher Scientific), and purified DNA extracts were stored at −20°C until used.

4.4.3 Real-Time multiplex PCR assay

This PCR is for detection of *Elizabethkingia* spp. and differentiation between *E. anophelis and E. meningoseptica* . *E. anophelis* strains preserved in glycerol stocks (40% v/v) at -80 °C were grown on PCA for 24 h at 37°C. Total DNA from all *Elizabethkingia* reference strains, including the positive controls *E. anophelis* DSM 23781T and the negative control *E. meningoseptica* ATCC 13253T, were extracted using QIAamp DNA Mini kit (Qiagen) according to the manufacturer's protocols. The DNA concentration was determined using a Qubit 2.0 fluorometer (Life Technologies). Standards were prepared by serial diluting the DNAs and by calculating *E. anophelis* and *E. meningoseptica* copy number with the following equation: *Elizabethkingia* copy number = (concentration of template in ng × *NL*) / ($n \times 10^9 \times 660$) where *NL* is the Avogadro constant (6.02×10^{23}), *n* is the genome length of the standard in base pairs or nucleotides and *660* is the average molecular weight of double-stranded DNA. All qPCR analyses were carried out in duplicate on a LightCycler® 480 Instrument II (Roche Life Science) with positive, negative, and non-template controls included. Individual real-time qPCR assays were used to quantify *E. anophelis*, *E. meningoseptica* and *Elizabethkingia* spp. genome copies using a multiplex probe assay with the primers and probes described in Table 1.

Table 1: Gene targets, primers and probes used in this study

Gene target	**Target organism**	**Primer/Probe ID**	**Fluorophore Quencher**	**Final reaction conc (µM)**	**Product size (bp)**	**Primer sequence (5′-3′)**	**Reference**
secY	*Elizabethkingia* spp.	SECYF1_4		0.01		GTTTTTACGTTCACGCTCATCTTGGT	[278]
		SECY R2		0.07	146	AGTAAGCCTAAAAGCCCAGAAG	
		SECYP2_5	FAM/BHQ1	0.05		TTGCAAGTATACAGAACCAAGGAGGAAGCAAG	
pheT	*E. meningoseptica*	TIGR472_F7		0.1		TTTAAACTGGATGTGGAAGATGCTGAT	[278]
		TIGR472_R1_2		0.05	90	CCACTCTGGGGACTCTTCTACCTGT	
		TIGR472_P3	Quasar 670/BHQ3	0.05		GCGTTATCTGGGAGCTGTAATTGAAGG	
lepA	*E. anophelis*	TIGR1393F22		0.07		CATGTGAAGGGGCGCTACTTATTGT	[278]
		T1393R3WT		0.1	142	TCAGGGTTTGCAGAAGGAAGGTC	
		TIGR1393P1	CalRed 610/BHQ1	0.02		ACCTGGCTTTGGAAAATGACCTTACC	

Amplification was done in 25 µl reaction volumes consisting of 10 µl of the LightCycler® 480 SYBR Green I Master (Roche Life Science), 5 µl of DNAse/RNAse free water (Roche Life Science), 5 µl of the primer-probe mixture, and 5 µl of template DNA within the concentration range of 40 to 50 ng/µl. The cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 64°C for 30 s [278]. Fluorescence data were acquired at the end of the annealing step of each cycle. All mixes were made using a Biomek Automated Liquid Handler (Beckman Coulter) to avoid pipetting errors.

The efficiency of the different real-time PCRs ranged from 97–100%. Secondary structures were not encountered in any of the runs. The threshold of each single run was placed above any baseline activity and within the exponential increase phase. The cycle thresholds (C_T) were determined by a mathematical analysis of the resulting curve using the software manufactured by Roche Life Science. The C_T values of the non-template controls were always 40 or above, indicating no amplification. Dissociation curves were determined for qPCR products to confirm product integrity and the absence of PCR inhibitors. Among the different qPCR coefficients, attention was given to the R coefficient, which was used to analyze the standard curves obtained by linear regression analysis. Most of the samples, and all standards, were assessed with a minimum of two runs to confirm the reproducibility of the quantification.

Real-Time PCR datasets were analyzed using analysis of variance (ANOVA). To evaluate the absolute abundance of gene copy numbers in water samples, F-tests were used to compare variance. Normality was tested with a Shapiro-Wilks test and by inspection of residuals, and variance homogeneity by Levene's test. When data failed to satisfy one of these tests, an appropriate transformation was applied (log or square-root transformation). Tukey's honestly significant difference (HSD) method and the modified version for unequal sample size (Unequal N HSD) were used for post hoc comparisons with a 0.05 grouping baseline. Graphs were drawn using GraphPad Prism version 9 (GraphPad Software Inc.).

4.4.4 Whole-genome sequencing

Whole-genome sequencing (WGS) was performed as described previously (Foster-Nyarko et al., 2020). Briefly, WGS was performed on the Illumina NextSeq 500 platform using a modified Nextera low input tagmentation approach. Genomic DNA was normalized to 0.5 ng µl−1 with 10 mM Tris-HCl before the library preparation. The pooled

library was run at a final concentration of 1.8 pM on a mid-output flow cell following Illumina recommended denaturation and loading parameters. Data was uploaded to Basespace (www. basespace.illumina.com), where the raw data was converted to FASTQ files for each sample.

4.4.5 Phylogenetic analysis

Maximum-likelihood phylogenetic trees were constructed using PhyloSift [206], and single nucleotide polymorphism (SNP)-based phylogenetic trees were made using Snplord (github.com/maxlcummins/ pipelord/tree/master/snplord), an automated snakemake [220] pipeline that utilizes snippy (github.com/tseemann/snippy), Gubbins [219] and SNP-sites (github.com/sanger-pathogens/snp-sites). All trees were resolved using FastTree2 [229] and visualized using the Interactive Tree Of Life (iTOL) software v4 [208]. In addition to the 94 *Elizabethkingia* draft genomes presented in this study, 54 *Elizabethkingia* genomes sourced from Genbank [279] were included in the phylogenetic analyses.

The *Elizabethkingia* pangenome was calculated using Roary v3.11.2 [218] and visualized using Phandango v1.3.0 [230]. A pangenome wide gene association study on novel *Elizabethkingia* spp. isolates was performed using Scoary [280]. A pairwise genome distance matrix was generated using Mash [281] and used to create a classical (metric) multidimensional scaling (MDS) plot using R Studio v4.0.2 and the gglot2 v3.3.0 package. MDS plots for virulence-associated genes and ARGs were also created in R, using gene presence/absence matrices (1 = present; 0 = absent).

4.4.6 Genotyping

In silico species identification was performed using SpeciesFinder 2.0 [231] and Kraken2 [232]. To determine novel *Elizabethkingia* species, pairwise genome comparisons were performed using both the average nucleotide identity BLASTn (ANIb) and ANI MUMer (ANIm) algorithms available on the JSpecies web server [197] using a 95% cut-off value for species delimitation [196]. Predicted DNA-DNA hybridization (DDH) results were ascertained using the Genome-to-Genome Distance Calculator (GGDC) tool [233] with a 70% cut-off value for species delimitation using the recommended Formula 2. Complete 16S rRNA and *rpoB* sequences were aligned using Clustal Omega and 99.5% [195] and 97.7% [234] similarity cut-off values were used, respectively.

Virulence-associated genes, ARGs and plasmid replicons were screened for using Abricate (github.com/ tseemann/abricate) in conjunction with the following

databases: VFDB [198], CARD [192], NCBI AMR FinderPlus [238] and PlasmidFinder [282]. Virulence factors were also screened using the VFDB Set A of experimentally determined virulence factors and BLASTp with > 40% identity and E^{-10} cut-off values.

4.4.7 Genome annotation

Draft genomes were annotated using Prokka v1.14.6 [211] and managed using SnapGene v4.1.9 (snapgene.com). The RAST annotation pipeline [210] was also utilized on eight genomes representative of each clade to cross check annotations. Putative genomic islands (GIs) and ICEs were identified by Islandviewer 4 [203] using the following reference genomes: *E. anophelis* strain CSID_3015183681 (CP015068.2), *E. anophelis* strain F3543 (CP014340.1), *E. miricola* strain EM798-26 (CP023746.1) and *E. genomospecies 4* strain G4123 (CP016377.1). BLASTn was utilized to determine whether putative GIs, ICE and AMR regions identified in this study had been previously deposited into NCBI. Aliview software v3.0 (GPLv3) [283] was used to view the sequence alignment of AMR genes.

4.4.8 Minimum Inhibitory Concentration testing

Representative isolates from each *Elizabethkingia* clade and isolates harbouring unique combinations of bla_{B} *and* bla_{GOB} genes were selected for Minimum Inhibitory Concentration (MIC) testing (n=10) against 38 clinically relevant antimicrobials as described previously [72]. Antibiotic testing plates were hand prepared, inoculated and incubated in accordance with AS ISO 20776.1-2017. Quality Control of antibiotic and testing isolates was in accordance to Clinical and Laboratory Standards Institute (CLSI) M100 ED31:2021; plate reading in accordance to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reading guide v 3.0 2021. Both the guidelines of the EUCAST pharmacokinetic-pharmacodynamic (PK-PD) “non-species” breakpoints [284] and the non-*Enterobacteriaceae* breakpoints of the CLSI [285] were used in the AMR phenotypic analysis.

4.5 Results

In this study, 94 *Elizabethkingia* isolates were collected from aquatic environments in South Australia from 2018-2019. Strains sourced from wetlands (site B & C) constituted the majority [n=70 (B=50, C=20); 75%], followed by dam (site A, n=22; 23%) and then river (site D, n=2; 2%) samples. Associated metadata on all isolates used, including 54 sourced from outside this collection used in phylogenetic and gene screening analyses, is available in Appendix 1.

Genome assembly using draft genomes were assembled using shovill v1.0.4. Genome size ranged from 4039979 bp to 4660922 bp, with an average size of 4459168 bp. The number of contigs per genome ranged from 25 to 160, with a mean of 55. Read depth ranged from 23.26 to 80.63, with a mean of 38.79. Full assembly statistics can be viewed in Appendix 2.

4.5.1 Absolute quantification of *Elizabethkingia* species

Quantitative data targeting a generic *Elizabethkingia* spp. gene marker and *E. anophelis* and *E. meningoseptica* markers were used to estimate the absolute abundance of each in samples from the four aquatic sites. The absolute abundance of *Elizabethkingia* spp. in the dam sample was on average 7.6 x 10^3 gene copies/mL, representing 1.36 x 10^{-6} % of the total bacterial community (16S rRNA qPCR-based). In the wetland samples, *Elizabethkingia* spp. ranged from 3.5 x 10^4 genes/mL to 4.6 x 10^4 genes/mL, representing 6.25 x 10^{-6} % to 8.21 x 10^{-6} % of the total bacterial community (Figure 11). In each case, the absolute abundance of *E. anophelis* was a factor of ten lower than the total *Elizabethkingia* spp. absolute abundance, indicating that it is not the dominant species within the aquatic environments. *E. meningoseptica* were detected and gene copies/mL were ranging from 23 (site A; dam) to 50 copies/mL (site B, C and D; wetlands) on average.

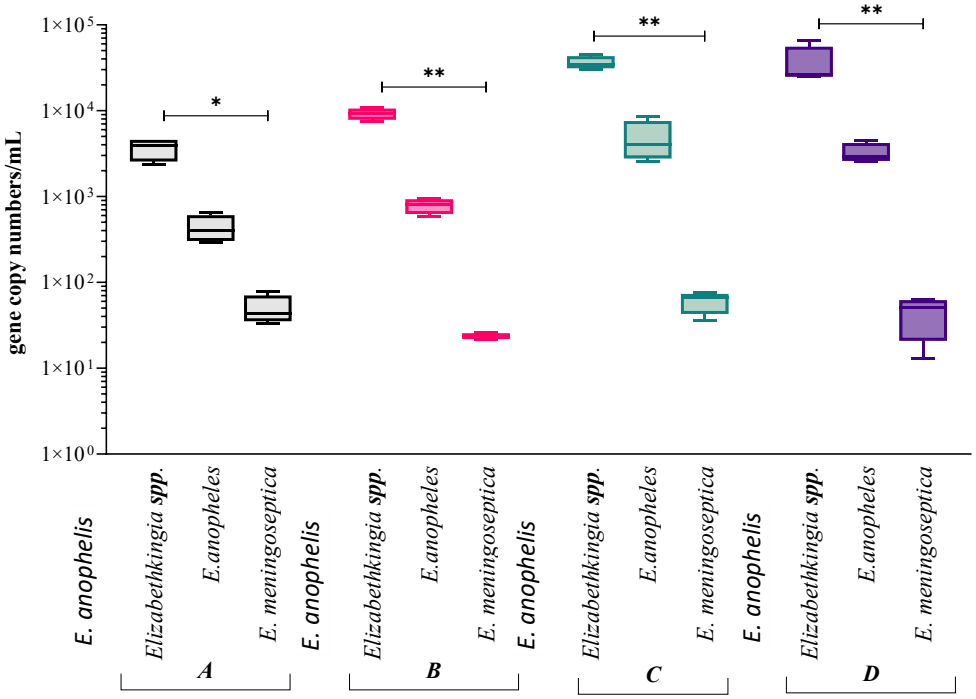

Figure 11. *Elizabethkingia* spp., *E. anophelis* and *E. meningoseptica* average absolute abundance determined by qPCR analysis of total DNA extracted from filtered waters (site A, B, C and D). Data are expressed as log10 genes copies per mL, samples (n = 32).

4.5.2 Identification of *Elizabethkingia* species

The speciation of *Elizabethkingia* isolates varied considerably between the typing techniques implemented (Table 2). Of the 94 isolates sourced from Australian aquatic environments, the most prominent species identified by MALDI-TOF MS was *E. miricola* (n=54; 57%), by Kraken2 was *E. anophelis* (n=93; 99%), and according to SpeciesFinder 2.0 *E.* genomospecies 4 was most prevalent (n=77; 82%). Our phylogenetic characterization (detailed below) classified 71 isolates as *E. miricola* (76%), 16 isolates as *E. anophelis* (17%) and seven isolates as a potentially novel species (7%).

Table 2: Aquatic environmental *Elizabethkingia* species (n=94) identified by MALDI-TOF MS, Kraken2, Species Finder, and phylogenetic analysis.

Species Identification	MALDI-TOF MS	Kraken2	SpeciesFinder 2.0	Phylogenetic characterization
E. anophelis	0	93 (99%)	16 (17%)	16 (17%)
E. meningoseptica	11 (12%)	0	0	0
E. miricola	54 (57%)	0	1 (1%)	71 (76%)
*E. ursingii**	0	1 (1%)	77 (82%)	0
Elizabethkingia spp.	16 (17%)	0	0	7 (7%)
Non-Reliable Identification	13 (14%)	0	0	0

*Also known as *E.* genomospecies 4

4.5.3 Phylogenetic analysis

A phylogenetic tree comprised of 148 *Elizabethkingia* isolates was constructed using Phylosift (Figure 12) with 94 isolates from the Australian aquatic environments (this collection), 27 isolates from Australian clinical samples and Australian hospital environments, and 27 global strains available from Genbank. Where metadata was available, *Elizabethkingia* isolates were derived from the environment (n=102), humans (n=42), Anopheles gambiae (n=2), and one isolate each from Zea mays (corn) and a frog. The distribution of species in the phylogenetic tree were *E. anophelis* (n=52), *E. meningoseptica* (n=5), *E. miricola* (n=78), *E. bruuniana* (n=3), *E. ursingii* (n=2), *E. occulta* (n=1) and a novel clade of *Elizabethkingia* sp. (n=7). The seven species of

Elizabethkingia were clearly separated from each other, with *E. meningoseptica* appearing the most distant from the other species.

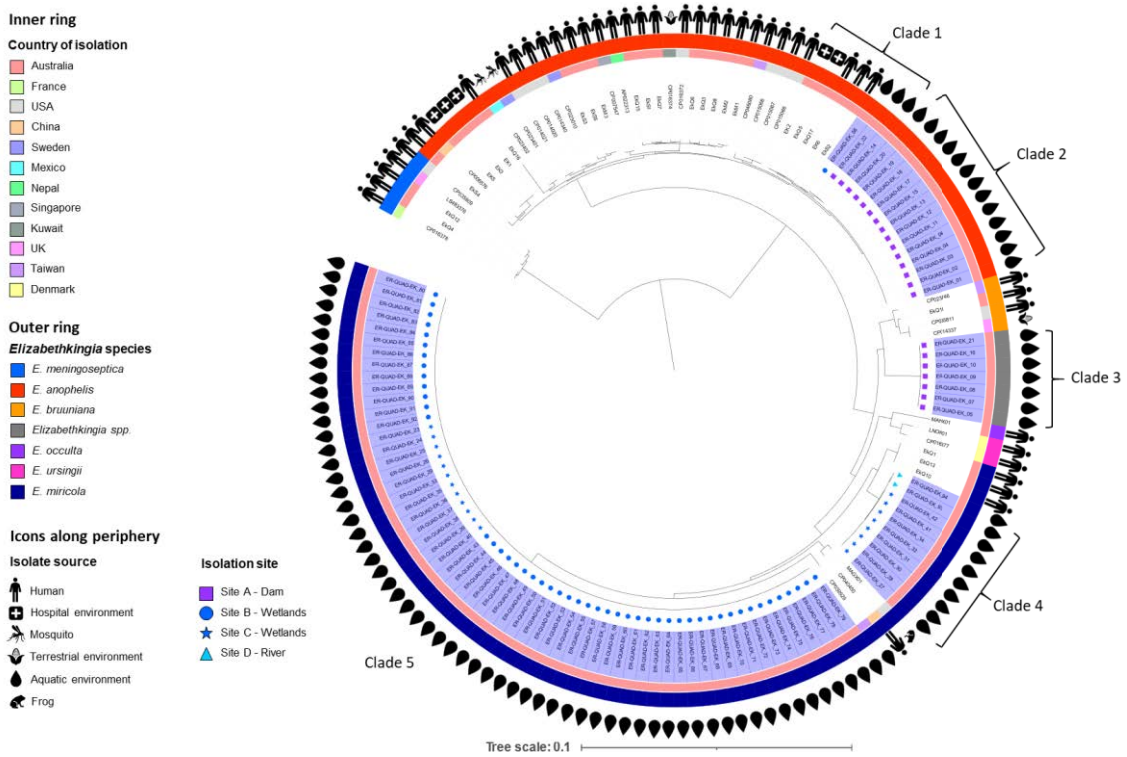

Figure 12: *Eizabethkingia* phylogeny. Mid-point rooted phylogenic tree and geographic data of *Elizabethkingia* species using Phylosift. Samples from this collection are coloured in purple.

The *Elizabethkingia* isolates from our aquatic environment study formed five clades that branched alongside international and clinical isolates.

Clade 1 contained three *E. anophelis* from the aquatic environmental study (ER-QUAD-EK_54, QUAD-EK_14 and QUAD-EK_22) that were closely related to Australian clinical isolates (EkS2, EkQ5 and EkQ17) with an average of 36 SNV (Single nucleotide variants) across 87% of the core genome (EkS2 as reference) and hospital environment isolates EK2 and EK6 with an average of 42 SNV. The two *E. anophelis* isolates from dam samples, QUAD-EK_14 and QUAD-EK_22, were separated by 2 SNV, while ER-QUAD-EK_54 from the Australian wetland samples differed by an average of 33 SNV from dam isolates.

Clade 2 consists of 13 clonal *E. anophelis* from our study that differ by an average of 2 SNV between each other and 807 SNV from our *E. anophelis* isolates situated in Clade 1 (pairwise SNP matrices for *E. anophelis* isolates provided in Appendix 3).

Clade 3 appears as a novel clade, represented by seven isolates (ER-QUAD-EK_21, QUAD-EK_08, QUAD-EK_09, QUAD-EK_10, QUAD-EK_16, QUAD-EK_07, and QUAD-EK_05) isolated from an Australian dam. Isolates within this apparently novel clade of the *Elizabethkingia* were most closely related to *E. bruuniana* but appeared genetically distinct in a progressive Mauve analysis (Appendix 11) and differed by an average of 124,216 SNPs to *E. bruuniana* isolate EkQ11.

Clade 4 constitutes ten *E. miricola* isolates with an average of 66 SNV between each other (range 0 – 197 SNPs) across 83% of the core genome (EkQ1 as reference). These isolates branch alongside three *E. miricola* isolates from Australian clinical samples (EkQ10, EkQ13 and EkQ1) however, the SNV between these two branches is ~ 21,539.

Clade 5 represents a group of 61 clonal *E. miricola* isolates (average 7 SNV) from Australian wetlands, with the closest relative strain CP03929, from a water sample from Taiwan, at ~21,629 SNPs difference. Pairwise SNP matrices for *E. miricola* isolates provided in Appendix 3.

4.5.4 Identification of proposed new species *Elizabethkingia umeracha* sp. nov.

The seven isolates in clade 3, with an average 124,216 SNV to *E. bruuniana* isolate EkQ11, were investigated to determine whether they constituted a closely related, yet distinct species to *E. bruuniana*. For this purpose, 16S rRNA and rpoB sequence identities as well as ANIb, ANIm and GGDC values (the latter mimicking DDH values) were calculated (averages presented in Table 3; full analysis in Appendix 4). Except for a single ER-QUAD-EK_05 16S rRNA sequence identity result (99.7%), all other values placed these seven isolates as representing a novel *Elizabethkingia* species.

We therefore propose that these seven isolates constitute a provisional novel species and propose the name *Elizabethkingia umeracha*; Umeracha meaning “fine waterhole” in Peramangk language. We respectfully acknowledge the Peramangk people as the traditional owners and custodians of the waters and lands of the Adelaide Hills where these isolates originated.

Table 3. *E. umeracha* sp. nov. is a separate species from *E. bruuniana*, as evidenced by ANIb, ANIm, GGDC, and 16S rRNA and rpoB sequence identity.

	Average values for seven *E. umeracha* sp. nov. isolates				
	ANIb (>95% cutoff)	ANIm (>95% cutoff)	Predicted DDH (>70% cutoff)	16S rRNA (>99.5% cutoff)	rpoB (>97.7% cutoff)
E. bruuniana str. ATCC 33958 (CP035811)	76.70 ± 0.73 SD	78.18 ± 0.57 SD	49.23 ± 0.04 SD	99.28 ± 0.17 SD	97.59 ± 0 SD
E. bruuniana str. G0146 (CP014337)	76.41 ± 0.71 SD	77.90 ± 0.65 SD	45.18 ± 0.07 SD	99.28 ± 0.17 SD	97.59 ± 0 SD
E. bruuniana str. EkQ11 (SRS5502615)	76.57 ± 0.70 SD	78.27 ± 0.65 SD	49.17 ± 0.43 SD	99.41 ± 0.18 SD	97.62 ± 0 SD

4.5.5 Pangenome analysis

A pangenome analysis of all available *Elizabethkingia* genomes (n=148) demonstrated high genetic diversity (Figure 13). The *Elizabethkingia* spp. pangenome consisted of 28,240 genes, with a core genome of only 76 genes and an accessory genome of 28,164 genes (443 soft-core, 6057 shell and 21,664 cloud genes).

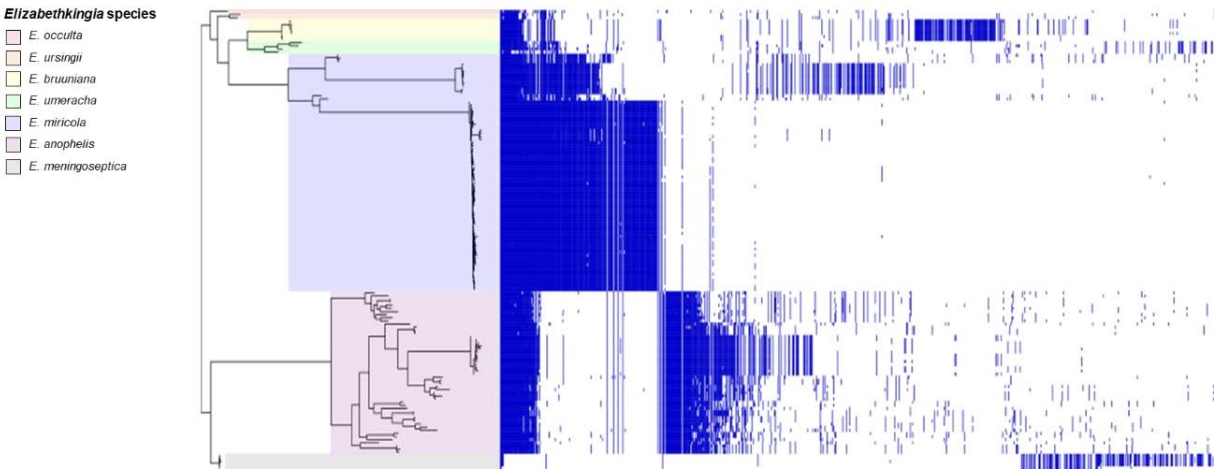

Figure 13: *Elizabethkingia* pangenome. Pangenome analysis of 148 *Elizabethkingia* species from the Australian environment and clinical isolates alongside international strains.

A pairwise genome distance MDS plot of *Elizabethkingia* genomes (Figure 14) demonstrated tight clustering of all *E. anophelis* isolates, while *E. meningoseptica* isolates were the most distinct, both regarding other species and also between the *E. meningoseptica* isolates. The remaining *Elizabethkingia* species formed a more diffuse cluster with no clear distinction between human and environmental isolates and with *E. umeracha* sp. nov. isolates situated at the peripheries (Figure 13, pink triangles).

The gene presence/absence matrix generated by Roary (Appendix 5) was fed into Scoary to calculate any differentiating genes present in *E. umeracha* sp. nov. isolates. A total of 1886 genes were only identified in these seven isolates (100% specificity, 100% sensitivity; Appendix 6). More than half of these genes (n=1110; 58.8%) encode hypothetical proteins however of the remaining genes, 537 were fed into STRING which identified several functional enrichments, the highest scoring being tryptophan biosynthesis (1.04 strength) and molybdenum cofactor biosynthesis (1.04 strength) (full analysis available in Appendix 7).

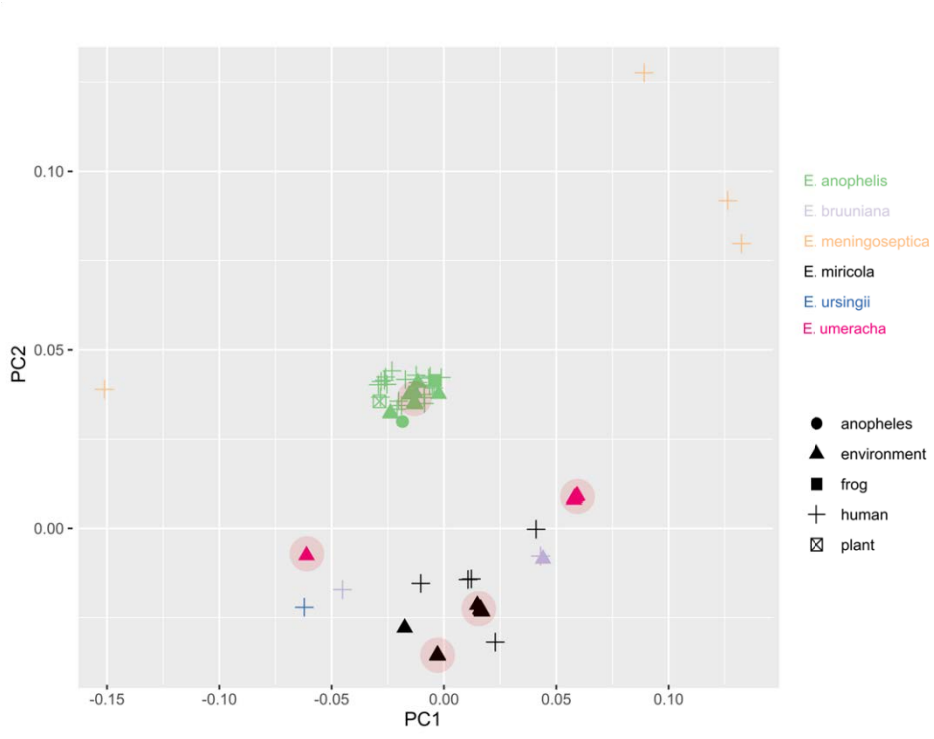

Figure 14: *Elizabethkingia* pairwise genome distances. MDS illustrating pairwise genome distances calculated using Mash. Colored by species, shapes represent isolate source. Red areas are isolates from this collection.

4.5.6 Virulent gene identification

Using the VFDB, a total of 107 putative virulence factors were identified in this collection with 62 (56%) of these being shared across the three identified species (Figure 15A). However, several virulence factors were species specific. In *E. miricola*, unique virulence factors included homologs of adhesin/invasin Cj1136 (found in all *E. miricola* isolates, n=71; 100%, notably absent in *E. miricola* isolates outside this collection) [286], capsule protein Cps41 (n=71; 100%) [287], adenylate cyclase CyaB (n=8; 11%) [288], ABC-transporter HlyB (n=8; 11%) [289], toxins RtxB (n=11; 15%), RtxE (n=10; 14%) [290] and SmcL (n=71, 100%) [291], immune evasion protein GtrB (n=8; 11%) [292], intracellular growth protein PrsA2 (n=1) [293] and iron uptake protein YbtP (n=71; 100%) [294]. The virulence factors only identified in *E. anophelis* isolates were homologs of capsule proteins WbaP (n=1)[295] Cj1440c (n=10; 63%, only found in *E. anophelis* from this collection) [296], FTT_0790 (n=1), FTT_0797 (n=16; 100%), and FTT_0798 (n=3; 19%) [297], lipopolysaccharide proteins BplB, BplG [298], and KfoC [299] (all n=16; 100%), immune evasion protein OmpA [300] (n=16; 100%), and stress protein MucD [301] (n=1). Only one unique virulence factor homolog was identified in *E. umeracha* sp. nov. – capsular protein NeuB [302] - however it was only identified in two isolates (29%).

An MDS analysis on putative virulence factors identified in this collection as well as genomes sourced outside of this collection (Figure 15B; virulence factor BLAST results and heatmaps in Appendix 8) demonstrated that our *E. anophelis* isolates clustered with a subset of known pathogenic *E. anophelis* isolates while our *E. miricola* isolates formed a cluster of their own. *E. umeracha* sp. nov. isolates formed two separate clusters, one standalone, the other amongst pathogenic *E. bruuniana* and *E. miricola* isolates and an *E. usingii* isolate.

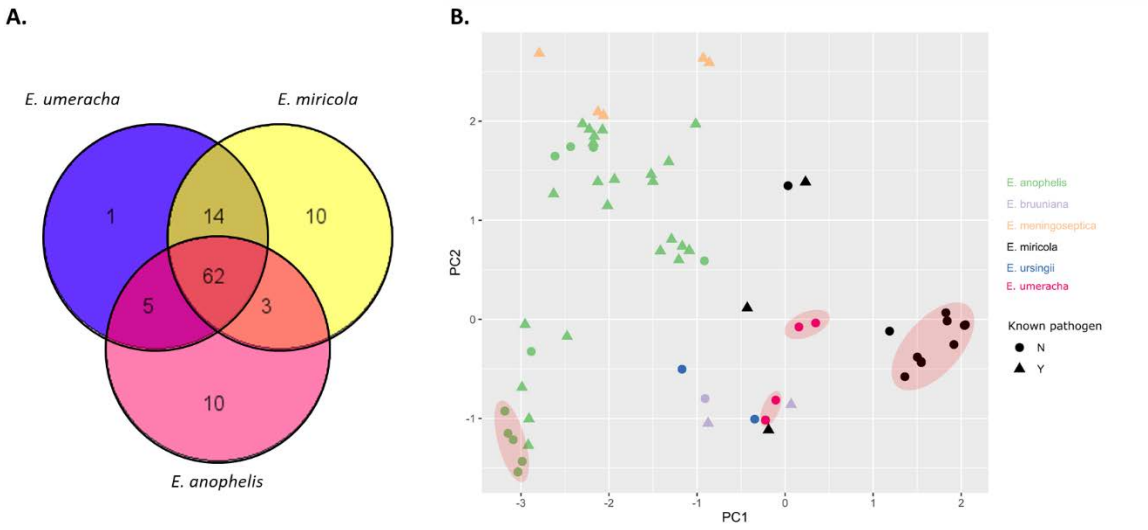

Figure 15: Virulence factors of *Elizabethkingia* species. A) Venn diagram of distribution of putative virulence factors across the three *Elizabethkingia* species identified in this collection. B) MDS analysis of putative virulence factors identified in 148 *Elizabethkingia* isolates. Colored by species. Triangles = known pathogen; circles = ability to cause disease unknown. Red areas = isolates from this collection.

4.5.7. Beta-lactamase resistance genes and Other ARGs

All 94 *Elizabethkingia* isolates from this Australian aquatic collection carried *bla*$_{B}$ (subclass B1) and *bla*$_{GOB}$ (subclass B3) genes encoding resistance to carbapenems and a *bla*$_{CME}$ gene encoding resistance to cephalosporins.

MUSCLE alignments with all available reference sequences of *bla*$_{B}$ and *bla*$_{GOB}$ were generated to compare species/allele distributions (Figure 16).

All *Elizabethkingia* species in our analysis carried *bla*$_{GOB}$, with an interesting distribution of several distinct signature deletions of 2-4 amino acids within the different alleles (Appendix 12). However, none of these deletions are expected to alter gene reading frame given they appear in multiples of three nucleotides. For the *bla*$_{B}$ gene distribution (Figure 15; right side tree), we saw four to five primary clades in the tree structure which were generally grouped by species.

Regarding metallo-β-lactamase allele combinations, one *E. anophelis* isolate (ER-QUAD-EK_56) carried a novel *bla*$_{GOB}$ variant and the remaining 15 *E. anophelis* isolates from this study carried *bla*$_{GOB-20}$. Of these 15 isolates, 13 carried *bla*$_{B-11}$. Interestingly, the remaining 3 *E. anophelis* isolates (ER-QUAD-EK-14, -22, and -56), carried a *bla*$_{B-1-like}$ gene also shared by three Australian clinical isolates (EkS2, EkQ5, EkQ17) and two Australian hospital environment isolates (EK2 and EK6).

Together these eight isolates formed the closely related (~36 SNPs to clinical isolates; ~42 SNPs to hospital environment isolates) clade 1 depicted in Figure 12.

The ten *E. miricola* isolates of clade 4 uniquely carried $bla_{\text{GOB-25-like}}$ genes and all carried $bla_{\text{B6-like}}$ genes. These $bla_{\text{B6-like}}$ genes were also identified in three Australian clinical *E. miricola* isolates (EkQ1, EkQ10 and EkQ13). The remaining 61 *E. miricola* isolates from this study uniquely carried $bla_{\text{GOB19-like}}$ and $bla_{\text{B26-like}}$ genes. Notably, *E. umaracha* sp. nov. isolates carried novel alleles of both metallo-β-lactamase genes.

Chromosomal extended-spectrum β-lactamase bla_{CME} has two types known: $bla_{\text{CME-1}}$ and $bla_{\text{CME-2.}}$ $bla_{\text{CME-1}}$ appeared to be the closest allele related to the Australian aquatic environmental *E. anophelis* (ER-QUARD-EK_14, 22, 56) from wetland and dam samples. $bla_{\text{CME-2}}$ was present at very high levels of variation from the aquatic environment isolates. Interestingly, the two distinct *E. miricola* clades appear to possess each a novel bla_{CME} allele, and a third novel allele appears in the *E. umeracha* sp. nov. (Appendix 13).

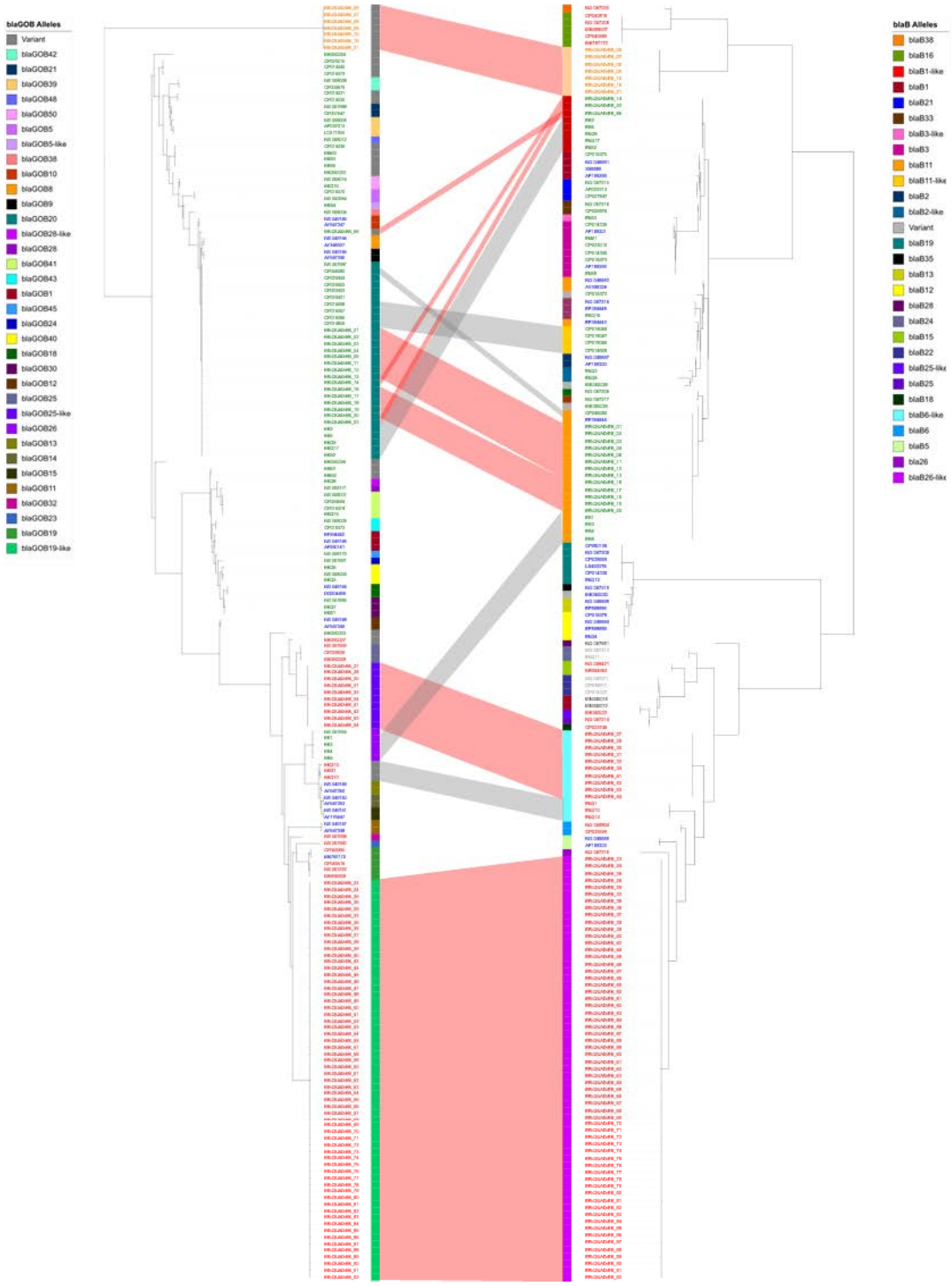

Figure 16: *Elizabethkiniga bla_{GOB} and bla_{B}* alleles. Phylogenetic trees of all *Elizabethkingia bla_{B} and bla_{GOB} alleles.* Left side is the tree of bla_{GOB} alleles and right side is the tree of *blaB alleles.* Labels are colored in red for *E. miricola,* green for *E. anophelis*, *blue for E. meningoceptica* and orange for *E. umeracha* sp. nov. Connecting space between the trees links sequences from the same isolate. Available allele numbers are presented as colored strips.

No other ARGs were detected in any of the Australian aquatic environment isolates. We also searched for the known mutations in gyrA (Ser83Ile or Ser83Arg) that encode resistance to ciprofloxacin and levofloxacin (fluoroquinolone), however none were detected.

4.5.8 AMR phenotypic analysis: MIC testing

Ten representative isolates of *E. anophelis, E. miricola* and *E. umeracha* sp. nov., harboring unique combinations of bla_{B}, bla_{GOB} and bla_{CME} genes from each *Elizabethkingia* clade were tested for MIC against 38 clinically relevant antimicrobials (Table 4). To date, *Elizabethkingia* species lack their own defined breakpoint, so they have been interpreted by using EUCAST non-species and NCSI non-*Enterobacteriaceae* PK-PD breakpoints (Appendix 9). All isolates tested showed remarkable resistance to carbapenems, cephalosporins, penicillins including carboxypenicillin and monobactam. Regarding different $bla_{GOB,}$ bla_{B} and bla_{CME} combinations, some differences in resistance profiles were noted (Table 4), including piperacillin/tazobactam resistance in only $bla_{B\text{-}26\text{-}like}$/$bla_{GOB\text{-}19\text{-}like}$/$bla_{CME\text{-}variant}$ *E. miricola* isolates, and cefepime resistance in two *E. anophelis* isolates (one $bla_{B\text{-}1\text{-}like}$/$bla_{GOB\text{-}20}$/$bla_{CME\text{-}1}$ and one $bla_{B\text{-}1\text{-}like}$/$bla_{GOB\text{-}variant}$/$bla_{CME\text{-}1}$).

Isolates were also resistant to antibiotic classes other than carbapenem and ESBLs, including aminoglycosides and glycylcycline (Table 4). One isolate, *E. anophelis* ER-QUAD-EK_14 was highly resistant to chloramphenicol and trimethoprim/ sulfamethoxazole. Azithromycin and rifampicin have no corresponding breakpoint in EUCAST or CLSI, however, tested isolates had a very low MIC (up to the lowest range tested), suggesting a potential sensitive profile. Vancomycin and teicoplanin also lack a breakpoint but their MICs were very high, indicating non-susceptibility. Likewise for the glycopeptides and colistin, which showed higher MIC than the top of concentration tested, suggesting potential resistance of *Elizabethkingia* against these antibiotics.

Table 4: MIC data of aquatic environmental *Elizabethkingia* isolates from South Australia against clinically relevant antimicrobials. Cells colors: red = resistant, yellow = intermediate, green = sensitive, No colour: Antimicrobial, Range tested (µg/mL) and MIC 90 (µg/mL). *bla*$_{B}$, *bla*$_{GOB}$ and *bla*$_{CME}$ alleles shown under isolate names; ^{V} = variant ^{L} = like.

			E. anophelis			***E. umeracha* sp. nov.**				***E. miricola***		
Antimicrobial	**Range tested (µg/mL)**	**MIC 90 (µg/mL)**	**ER-QUAD-EK_14** B-1^{L} GOB-20 CME-1	**ER-QUAD-EK_56** B-1^{L} GOB^{V} CME-1	**ER-QUAD-EK_18** B-11 GOB20 CME-1	**ER-QUAD-EK_08** B^{V} GOB^{V} CME^{V}	**ER-QUAD-EK_09** B^{V} GOB^{V} CME^{V}	**ER-QUAD-EK_10** B^{V} GOB^{V} CME^{V}	**ER-QUAD-EK_21** B^{V} GOB^{V} CME^{V}	**ER-QUAD-EK_94** B-6^{L} GOB-25^{L} CME^{V}	**ER-QUAD-EK_64** B-26^{L} GOB-19^{L} CME^{V}	**ER-QUAD-EK_92** B-26^{L} GOB19 ^{L}CME^{V}
Amoxicillin	2-32	> 32	>32	>32	32	>32	>32	>32	>32	>32	>32	>32
Ampicillin	2-32	> 32	>32	>32	32	>32	>32	>32	>32	>32	>32	>32
Amoxicillin/clavulanic acid	4-128	> 128	8	8	16	8	16	16	8	8	16	16
Piperacillin/tazobactam	1-64	> 64	<1	<1	<1	<1	<1	2	<1	4	8	8
Ampicillin/sulbactam	8-128	> 128	32	16	16	32	32	64	32	32	32	32
Temocillin	2-32	> 32	> 32	16	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32
Cephalexin	4-64	N/A	> 64	> 64	64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
Cefazolin	0.25-32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	> 32	32	> 32	> 32

Cefuroxime	1-16	> 16	>16	>16	16	>16	>16	>16	>16	>16	> 16	>16
Cefoxitin	8-256	N/A	< 8	< 8	16	32	32	32	32	16	32	32
Cefotaxime	0.03-8	> 8	> 8	>8	8	> 8	8	8	4	2	4	4
Ceftazidime	0.12-16	> 16	16	> 16	> 16	> 16	> 16	> 16	> 16	4	> 16	> 16
Ceftriaxone	0.03-4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	2	> 4	> 4
Cefiderocol	0.03-32	N/A	8	4	8	4	4	4	1	1	4	8
Cefepime	0.06-16	> 16	>16	16	8	8	4	4	4	1	4	4
Ceftaroline	0.5-16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16
Ceftolozane/tazobactam	0.5-16	16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16
Meropenem	0.015-32	> 16	16	16	32	32	32	32	32	32	32	32
Tebipenem	0.03-8	N/A	8	4	8	8	8	8	4	> 8	8	8
Etrapenem	0.015-4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
Aztreonam	0.5-16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16	> 16
Amikacin	1-64	64	32	4	32	32	32	32	32	16	16	16
Gentamicin	0.25-16	>16	8	2	> 16	16	8	8	8	8	4	2
Tobramycin	0.015-64	>16	>64	32	>64	> 64	16	64	64	64	64	64
Azithromycin	4-64	N/A	8	< 4	8	< 4	< 4	< 4	< 4	< 4	< 4	< 4
Ciprofloxacin	0.015-4	2	1	0.12	0.25	0.25	0.25	0.12	0.06	0.5	0.25	0.25
Levofloxacin	0.06-8	1	0.25	0.125	0.25	0.125	0.125	0.125	< 0.06	0.25	0.25	0.125

Trimethoprim	0.5-16	N/A	8	2	1	< 0.5	2	< 0.5	< 0.5	<0.5	2	2
Trimethoprim/sulfamethoxazole	0.12/2.38-32/608	2.38-152	8/152	1.0/19.0	0.5/9.5	> 0.12/2.38	0.5/9.5	0.25/4.75	<0.12/2.38	<0.12/2.38	0.5/9.5	0.5/9.5
Vancomycin	0.12-32	N/A	8	8	8	4	>32	4	4	8	8	8
Teicoplanin	2-64	N/A	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64
Minocycline	0.25-16	1	0.5	< 0.125	0.25	0.5	0.5	0.5	< 0.125	0.5	0.25	0.5
Doxycycline	0.015-64	> 64	2	0.5	0.5	0.5	0.5	1	0.25	0.5	1	1
Tigecycline	0.12-8	2	4	2	8	4	4	4	8	4	2	4
Rifampicin	0.12-32	N/A	< 0.125	< 0.125	0.25	< 0.125	2	< 0.125	< 0.125	< 0.125	0.25	< 0.125
Colistin	0.25-8	N/A	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
Polymixin B	0.25-8	N/A	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8	> 8
Chloramphenicol	2-128	>128	128	16	32	8	8	8	8	16	8	8

4.5.9 Mobile genetic elements characterization: ICEs, Plasmid and Phage

Integrative conjugative elements (ICEs) were identified in 67 / 94 (71.3%) of the aquatic environments *Elizabethkingia* spp. by comparing to ICEs publicly available data in Genbank using three types of ICEs [145]: ICEEaI from strain CSID3015183678, ICEEaII from strain NUHP1 and ICEEaIII from strain R26. The alignments demonstrated imperfect matches to the reference sequences, however all matches were closest to the type III ICE from *E. anophelis* strain R26.

Two plasmids have been described from *Elizabethkingia* species so far (Accessions CP016375.1 and CM003640.1), however neither were detected in this study. In our analysis, both plasmid sequences were aligned to the aquatic and clinical *Elizabethkingia* isolates explored here. Thirteen (81%) of the environmental *E. anophelis* in clade 2 showed low-quality matches to plasmid CP016375.1 (average 8% coverage at 90% identity) (Appendix 14). Alignments to the second reference plasmid from *E. miricola* strain EM_CHUV (CM003640.1) revealed little to no homology.

We performed a phage analysis on 94 *Elizabethkingia* genomes via Phaster, with hits detected for each isolate (data not shown). While most hits were recorded as 'incomplete' due to scaffolding of the WGS, fourteen isolates were detected with complete phages (Appendix 10). From these fourteen isolates, coming from multiple species, we selected four ranges of hit length to group the phages: 48.7 kb, 21.4-27.1 kb, 10.4 -18.8 kb, and 4.9-9.7 kb. Alignments were generated to identify similar phages amongst the different size ranges, with only the largest (48.7 kb) appearing conserved in multiple isolates. Initially, this phage was detected in two *E. miricola*, one from a wetland and another from a river, with alignments demonstrating different chromosomal locations. By aligning the large phage against the Australian *Elizabethkingia* sequences, we identified 17 environment and clinical *Elizabethkingia* isolates to carry it: two *E. anophelis* (one from wetland and another from human bronchial alveolar lavage), nine *E. miricola* (seven from wetlands and two from a river), one *E. bruuniana* (human blood) and five *E. umeracha* sp. nov. (dam). While short-read assembly data prevents detailed comparisons of these phages across the sequences, the *Elizabthekingia* species do share considerable mobile DNA.

4.6 Discussion

Elizabethkingia spp. are emerging pathogens and the only known organisms with multiple chromosomal metallo-β-lactamase genes, offering inherent resistance to carbapenems [303]. *Elizabethkingia* species are considered environmental bacteria, with

water bodies serving as environmental reservoirs. Contaminated water is implicated in *Elizabethkingia* spp. transmission pathways [304], yet with the exception of insects [305] , frogs [247,306], reptiles [307] and spacecraft [250], most studies on *Elizabethkingia* genus have focused on clinical isolates and isolates taken from hospital environments, leaving *Elizabethkingia* species dwelling in aquatic environments unexplored. Here we characterized WGS of 94 *Elizabethkingia* derived from dam, river, and wetland samples from South Australia, thereby providing the first study of *Elizabethkingia* from diverse aquatic environments. Furthermore, we provide comparative genomics analyses of these environmental isolates with clinical *Elizabethkingia* isolates originating from Australia and worldwide.

Correctly identifying *Elizabethkingia* species is paramount as not only is the literature on *Elizabethkingia* spp. convoluted due to various nomenclature changes, but standard commercial microbial identification systems, such as biochemical tests and mass spectrometry (MS) using standard databases, cannot currently differentiate *E. anophelis*, *E. bruuniana*, *E. ursingii* or *E. occulta* and these are often misidentified as either *E. meningoseptica* or *E. miricola* [72,273,308,309]. Consistently, our initial MALDI-TOF MS results misidentified 11 isolates as *E. meningoseptica*. Interestingly, all speciation methods we used, including MALDI-TOF MS, Kraken2 and SpeciesFinder, gave conflicting results. The issues regarding MS and Kraken2 misidentifications likely arose from using standard databases [53] while SpeciesFinder uses 16S rRNA gene sequences, which are known to be limited for taxonomic purposes [231] with studies demonstrating less than 30% accuracy for aerobic bacteria to the species level [310]. These data suggest that *in lieu* of WGS, future *Elizabethkingia* spp. studies should be cautious in using 16S rRNA for speciation and ensure any utilized MS databases include all *Elizabethkingia* species.

The fact that *E. anophelis* was misidentified here and elsewhere [308,309,311,312] as *E. meningosepticum* by conventional clinical methods has led to speculation that *E. anophelis* is not only underrepresented but may actually be the primary species to cause disease in humans. This hypothesis is strengthened by recent reports of life-threatening *E. anophelis* infections in Asia, Australia, and the USA [53,72]. The 16 *E. anophelis* isolates identified here in dam and wetland samples formed two clades which differed by approximately 807 SNPs. The single wetland isolate and two dam isolates were found to differ by only ~36 SNPs to three *E. anophelis* isolates originating from sepsis patients in Queensland, Australia, and ~42 SNPs to two *E. anophelis* isolates derived from sinks located in a Queensland hospital [72]. Screening

for putative virulence factors did not identify any specific to the three environmental isolates and the three sepsis isolates, however several putative virulence factors were found to be unique to *E. anophelis* in general, including homologs of lipopolysaccharide biosynthesis proteins and serum resistance protein OmpA.

The majority of Australian aquatic environment isolates from this study were *E. miricola*. *E. miricola* is known to cause sepsis, oral and urinary tract infections [53,254,313] though reports of infection are less frequent than that for *E. meningoseptica* and *E. anophelis*. Environmental *E. miricola* from this study formed two distinct clades. While a clade of ten isolates were phylogenetically positioned next to three Australian clinical *E. miricola* isolates taken from sputum samples [72], the average SNP difference between these environmental and clinical isolates was 21,539. Nevertheless, environmental *E. miricola* shared putative virulence factors with clinical strains including homologs of capsule protein, Cps4I [314], haemolytic toxin SmcL [291], and iron acquisition protein YbtP [294]. The remaining 61 *E. miricola* isolates formed a clonal clade with an average difference of 7 SNPs. Despite the highly clonal nature of these isolates, they originated from two wetland sites approximately 10 km apart. Identifying potential transmission pathways, such as through wildlife, will be critical for future epidemiology.

The remaining seven *Elizabethkingia* isolates originated from dam samples and were phylogenetically placed proximal to *E. bruuniana* strains, though SNP analyses demonstrated the two branches to differ by ~124,216 SNPs. This considerable difference prompted an investigation as to whether these seven isolates represent a novel *Elizabethkingia* species. WGS-based ANI and *in silico* DDH analyses are known to be robust speciation methods that have proven more accurate than even the gold-standard conventional DDH for bacterial species delineation [53,233,315–317]. Here, both analyses clearly indicated that these isolates were a distinct species. The *rpoB* gene is also used in speciation and possesses a higher resolution for delineation than the 16S rRNA gene [234]. Here, all seven isolates fell under the 97.7% similarity cut-off to be classified as *E. bruuniana*. Furthermore, we identified 1886 genes unique to these isolates and where functional assignment was possible, these genes were mainly involved in cellular and metabolic processes. Together these data indicate a closely related but distinct species to *E. bruuniana* and we propose the name *E. umeracha* sp. nov. Though these isolates were shown here to share 62 putative virulence factors with *E. anophelis* and *E. miricola* isolates, future studies are required to determine whether this new species is pathogenic.

Metallo-β-lactamases (MBLs) are a worldwide concern as they confer resistance against carbapenems and almost all β-lactams [318], making pathogens carrying MBLs very difficult to treat. *Elizabethkingia* are currently the only known organisms to carry two chromosomal MBLs (bla_{B} and bla_{GOB}) and additionally carry a chromosomal bla_{CME} gene conferring resistance to cephalosporins [68]. Here we found that the three species of *Elizabethkingia* residing in aquatic environments all carried multiple known alleles, as well as novel variants of bla_{B}, bla_{GOB} and bla_{CME} genes. Regardless of allelic combinations, MIC testing demonstrated all were highly resistant to carbapenems, penicillins and monobactams. All tested isolates were also resistant to cephalosporins, though one *E. miricola* isolate carrying a bla_{CME} variant was susceptible to cefepime. Intravenous vancomycin has been cited as the favourable treatment option for *Elizabethkingia* infections [319]. While most tested isolates had MICs of vancomycin at 4 or 8 μg/mL, one *E. umeracha* sp. nov. isolate had >32 μg/mL, suggesting that a conventional dose of vancomycin would not be effective [318]. In addition to carbapenems and β-lactams, several isolates were found resistant to aminoglycosides and one *E. anophelis* isolate was highly resistant to trimethoprim/sulfamethoxazole and chloramphenicol. However, no additional AMR genes were identified, suggesting the presence of novel AMR mechanisms.

Environmental bacteria often harbour important AMR genes that are later captured and disseminated by more common human pathogens. For example, the ESBL gene, $bla_{CTX\text{-}M}$, now endemic among *Enterobacteriaceae*, likely originated from *Kluyvera ascorbate*, a soil bacteria [320]. The acquisition and spread of resistance, as well as virulence genes, is facilitated by MGEs. ICEs are MGEs that integrate into a host chromosome and can bestow new phenotypes [145]. In the 2015-2016 *E. anophelis* outbreak in the USA, which led to 66 confirmed cases of sepsis and 19 deaths, an ICE was identified in all of the outbreak clones. This ICE interrupted the *mutY* gene which led to a hypermutator phenotype [274]. ICEs were identified in 71% of 94 environmental *Elizabethkingia* isolates, however these bore no similarity to the ICE associated with the USA outbreak and only ~50% similarity to the type III ICE found in *E. anophelis* strain R26 [305], the first *E. anophelis* strain isolated. As such we have extended knowledge on the types of ICE that are found in *Elizabethkingia* spp..

In conclusion, we presented the first WGS analysis of *Elizabethkingia* species found in aquatic environments and discovered that they carry diverse bla_{B} bla_{GOB} and bla_{CME} genes and are highly resistant to carbapenems, cephalosporins, monobactams and other beta-lactams. Some isolates were also resistant to additional antibiotic classes

suggesting the presence of yet undiscovered AMR mechanisms. We uncovered environmental *E. anophelis* isolates that were very closely related to sepsis-causing clinical strains, thus identifying water bodies as an important reservoir for pathogenic *Elizabethkingia* spp. and highlighting the potential for cross-habitat movement. Finally, we discovered a proposed novel species, *E. umeracha* sp. nov., representatives of which appear resistant to vancomycin and carry novel metallo β-lactamase and extended-spectrum serine β-lactamase gene alleles.

Chapter 5. Genomic analysis of Carbapenem-resistant *Comamonas* in water matrices: implications for public health and wastewater treatments

5.1 Declaration

This chapter represents a manuscript accepted for publication.

It responds to aim 2 and 3.

Authors

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Author contributions

SH: writing the original draft, formal analysis, investigation, writing – review and editing. ERW: Investigation, Methodology, Validation, Conceptualisation, Supervision, Writing – review & editing; BD: Investigation, Conceptualization, Validation, Formal analysis, Data Curation, Writing – Review & Editing; ED: Conceptualisation, Funding acquisition, Supervision, Writing – Review & Editing; IGC: Investigation, Resources; DJB: Resources, Methodology; VMJ: Writing – original draft, Formal analysis, Investigation, Supervision, Conceptualization, Writing – review & editing the final draft; SPD: Conceptualisation, Resources, Supervision, Funding acquisition, Project administration, Writing – review & editing the final draft.

Ethical approval

The water samples used in our study were approved by the relevant authority for data publication.

Authors signatures

Sopheak Hem: Production Note: Signature removed prior to publication.

Ethan R. WyrscH: Production Note: Signature removed prior to publication.

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Steven P. Djordjevic: Production Note: Signature removed prior to publication.

5.2 Abstract

Comamonas spp. are Gram-negative bacteria that catabolise a wide range of organic and inorganic substrates. *Comamonas* are abundant in aquatic and soil environments, including wastewater, and can cause opportunistic infections in humans. Because of their importance in wastewater bioaugmentation and bioremediation, the identification of *Comamonas* species harbouring genes encoding carbapenemases and other clinically important antibiotic resistance genes warrant further investigation. Here, we present an analysis of 39 whole-genome sequences comprising three *Comamonas* species from aquatic environments in South Australia that were recovered on media supplemented with carbapenems. The analysis includes a detailed description of 33 *C. denitrificans* isolates, some of which carried chromosomally acquired bla_{GES-5}, bla_{OXA} and aminoglycoside resistance (*aadA*) genes located on putative genomic islands (GIs). All bla_{GES-5} and bla_{OXA}-containing GIs appear to be unique to this Australian collection of *C. denitrificans.* Notably, most ORFs within the GIs, including all AMR genes, had adjacent *attC* sites indicating that these ORFs are mobile gene cassettes. One *C. denitrificans* isolate carried an IncP-1 plasmid with genes involved in xenobiotic degradation and response to oxidative stress. Our assessment of the sequences highlights the very distant nature of *C. denitrificans* to the other *Comamonas* species and its apparent disposition to acquire antimicrobial resistance genes chromosomally.

5.3 Background

Comamonas spp. are Gram-negative bacilli that reside within the family *Comamonadaceae* and the phylum Proteobacteria [321]. *Comamonas* spp. are commonly found in a variety of environmental samples and are among the common bacteria in soil [322–324], wetlands [325–327] and wastewater [54,328]. The ability of *Comamonas* spp. to catabolise a wide range of organic and inorganic substrates, including amino acids, aromatic compounds, carboxylic acids, heavy metals, and steroids, have been extensively documented [329–331]. Furthermore, several studies have documented the roles *Comamonas* spp. play in wastewater bioaugmentation and bioremediation [332–334]. *Comamonas denitrificans* was first identified because of its significant ability to grow in anoxic conditions and denitrify sludge [335,336], a by-product during sewage treatment rich in antimicrobial residues, metals, pharmaceuticals, and drug-resistant microbial communities [337,338]. Unlike other species in the *Comamonas* genus, *C. denitrificans* can reduce nitrate to nitrogen gas, both in aerobic and anaerobic conditions [336] and has a notable capacity of biofilm formation [339].

While *Comamonas* spp. regularly feature in environmental microbial communities, several species have been described as aggressive opportunistic pathogens capable of surviving in hospital environments [340]. Reports of *Comamonas* spp. causing disease in humans have been increasing in multiple countries across six continents [79,341–346]. *Comamonas testosteroni* is the species most frequently associated with human disease and is known to cause invasive infections such as cellulitis [347], peritonitis [348], endocarditis [76], meningitis [349], endophthalmitis [350], pneumonia [351], and appendicitis associated with bacteraemia [344]. However, other *Comamonas* species, such as *Comamonas terrigena* and *Comamonas kerstersii*, can also cause pathologies, including eye and intra-abdominal infections [352,353]. Due to the omnipresence of *Comamonas* spp. in the environment and its apparent capacity to cause opportunistic infection, it is important to understand if members of the genus display resistance to antibiotics. Antimicrobial resistance (AMR) poses a global public health threat, and the increase in resistance to "last-resort drugs", such as carbapenems, is alarming [354–356]. The environment is an important reservoir of resistant bacteria, antimicrobial-resistant genes (ARGs) and antimicrobial residues, and there is mounting evidence suggesting the dispersal of environmental and clinical ARGs between wildlife, agriculture, and humans [357–359]. Carbapenem-resistant bacteria have been isolated from both natural water bodies [360,361], wastewater [362–364], wildlife and synanthropic species that frequent wastewater [359,365]. Airline waste [366] and wastewater in particular has been flagged as a hotspot for AMR evolution given the high abundance of bacteria combined with sublethal antibiotic concentrations derived from anthropogenic sources, including agriculture, industry, hospitals and households [338,364].

To date, only one carbapenem resistance gene has been identified in any *Comamonas* spp. (bla_{IMP-8} in *Comamonas thiooxydans*) [55], and in general, data regarding ARG carriage in *Comamonas* is limited. Published case reports suggest that *C. testosteroni* is susceptible to common antibiotics such as aminoglycosides, fluoroquinolones, ceftazidime, carbapenems and piperacillin-tazobactam [321,367]. However, in a severe meningitis case caused by *C. testosteroni*, the pathogen was resistant to 3rd generation cephalosporin but sensitive to carbapenem [349]. *C. denitrificans* has been reported to survive high doses of amoxicillin [368], and a genomic analysis of a C. aquatica isolate from the Daguan River, China, identified genes encoding resistance to several antibiotics, including cephalosporins, penicillins, and bacitracin but the genes that could account for these resistances were not reported [369].

Given the growing number of studies identifying carbapenem-resistant bacteria in water matrices and the abundance of *Comamonas* in the environment, this study sought to shed light on the potential for *Comamonas* species to acquire resistance to critically important antibiotics in Australia. Here we provide a whole-genome sequence (WGS) and phylogenic analysis of 39 carbapenem-resistant *Comamonas* spp. isolates, including 33 diverse *C. denitrificans* isolates, the majority of which carried acquired chromosomal carbapenemase bla_{GES-5} and β-lactamase bla_{OXA} genes.

5.4 Methods

5.4.1 Sample collection and bacterial isolation

Within a period of one year, from July 2018 to July 2019, water samples (~10 L) were collected in triplicate, monthly, from three sources: influent wastewater, a lake and a wetland in South Australia.

Influent wastewater was collected from four municipal wastewater treatment plants (hereafter referred as WWTPs A, B, C and D). WWTP A serves ≈150,000 inhabitants and treats approximately 33 ML/day of primarily domestic sewage. WWTP B serves ≈198,000 inhabitants and treats around 55 ML/day, primarily from households and commercial establishments with minor industrial inputs. WWTP C serves ≈700,000 inhabitants and treats around 175 ML/day with a large industrial/commercial component, as well as residential, and hospital sources. WWTP D is a rural wastewater treatment plant which serves 5,000 inhabitants and treats around 1.2 ML/day primarily from households and seasonal meat processing facilities. Raw wastewater to all WWTPs is classified as low-to-medium organic strength [370]. All samples were stored on ice directly after collection and processed within 2-3 hrs.

The lake is a shallow artificial lake fed by recycled water, which is composed of a mix of treated wastewater and cleansed stormwater. The lake is sporadically fringed by aquatic reads and vegetation and attracts silver gulls (*Larus novaehollandiae*), pigeons (*Columba livia*), Eurasian coots (*Fulica atra*) and indigenous and migratory birds.

The inland wetland convers 42 hectares and is recharged by seasonal rainfall/runoff inflows. The annual mean rainfall is 438 mm (11.4 mm February; 59.6 mm July) and mean annual temperature ranging between 21.6°C (28.2°C January) to 11.5°C (7.1°C July) (bom.gov.au). The wetland is habitat to over 160 species of indigenous and migratory birds, including purple swamp hem, stilt and herons, as well as *Larus novaehollandiae, Fulica atra, Columba livia* and *Haliaeetus leucogaster*. The water of

this inland wetland is harvested and pumped underground into natural sandy limestone aquifers.

5.4.2 Isolation of Carbapenem-resistant *Comamonas* species and MALDI-TOF MS species identification

All sample were plated, in triplicate, on Oxoid Brilliance™ CRE Agar plates (Thermo Fisher Scientific Australia, Adelaide, SA) after 10-fold serial dilutions, using 500 μL from 2-3 consecutive dilutions. All cultures were incubated at 25°C, 37°C and 44°C for 24 hrs. Single colonies growing on CRE Agar were picked up and steaked on a Plate Counting Agar (PCA; Thermo Fisher Scientific). Then, PCA cultures were incubated at 37°C for 18-24 hrs to have sufficient bacterial growth. A total of 40 *Comamonas* isolates were sampled, with their identity resolved initially by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). MALDI-TOF MS was performed using Bruker Daltonics, operated in linear positive mode under MALDI Biotyper 3.0 Real-time Classification (version 3.1, Bruker Daltonics) [371]. All isolates were preserved in glycerol (40% v/v) at -80 °C.

5.4.3 DNA extraction and WGS

DNA from MALDI-TOF MS identified colonies with scores 2.000-3.000 were extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Nucleic acid quality was measured with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA concentrations for all samples were measured by fluorometric quantitation using a Qubit instrument and High Sensitivity dsDNA HS Assay kit (Thermo Fisher Scientific), and purified DNA extracts were stored at −80°C until sequencing.

WGS was performed on an Illumina Nextseq500 instrument according to the manufacturer's instructions. Data was uploaded to Basespace (www. basespace.illumina.com) where the raw data was converted to 8 FASTQ files for each sample. Genome assembly was performed using Shovill v1.0.4 (https://github.com/tseemann/shovill) using the SPAdes option. A single genome was cut due to high scaffolding, with the remaining 39 sequences passing basic quality control using assembly-stats (https://github.com/sanger-pathogens/assembly-stats).

5.4.4 Phylogenetic analysis

A phylogenetic analysis was performed on *Comamonas* isolates from multiple species, including: 39 draft genomes from aquatic environments in South Australia (this

study) alongside 49 *Comamonas* genomes (22 complete, 27 draft) sourced from Genbank (downloaded on 05 March 2021) [279] (https://ncbi.nlm.nih.gov/genbank/) including five clinical *Comamonas* isolates. Genbank sourced genomes are referred to by their accession numbers throughout the manuscript. A maximum-likelihood phylogenetic tree of all species were constructed using PhyloSift [206], and single nucleotide polymorphism (SNP)-based phylogenetic analyses were generated using Parsnp [217] (https://github.com/marbl/parsnp). All trees were resolved using FastTree2 [372] and visualised using Interactive Tree Of Life (iTOL) software v4 [208] (https://itol.embl.de/). The *C. denitrificans* pangenome was calculated using Roary v3.11.2 [218] (https://github.com/sanger-pathogens/Roary) and visualised using Phandango v1.3.0 [373] (https://jameshadfield.github.io/phandango/#/main). A pangenome-wide gene association study on *C. denitrificans* isolates was performed using Scoary [280] (https://github.com/AdmiralenOla/Scoary).

5.4.5 Genotyping

In addition to MALDI-TOF MS, *in silico* species identification was also performed using Kraken2 [232] (https://ccb.jhu.edu/software/kraken2/). To confirm the identification of *C. denitrificans* and *C. testosteroni*, pairwise genome comparisons were performed against *C. denitrificans* JAFNME01 and *C. testosteroni* NZ_CP067086.1 using both the average nucleotide identity BLASTn (ANIb) and ANI MUMer (ANIm) algorithms available on the JSpecies web server (https://jspecies.ribohost.com/jspeciesws) using a 95% cut-off value for species delimitation [197]. Predicted DNA-DNA hybridisation (DDH) results were ascertained using the Genome-to-Genome Distance Calculator (GGDC) tool [233] (https://ggdc.dsmz.de) with a 70% cut-off value for species delimitation using the recommended Formula 2. Virulence-associated genes, antimicrobial resistance (AMR) genes, metal resistance genes and plasmid replicons were screened for using ABRicate (https://github.com/tseemann/abricate) in conjunction with the following databases: VFDB [198] (https://mgc.ac.cn/VFs), CARD [192] (https://https://card.mcmaster.ca), MEGARes 2.0 [374] (https://megares.meglab.org) and PlasmidFinder [282] (https://cge.cbs.dtu.dk/services/ PlasmidFinder), respectively.

5.4.6 Genome annotation

All draft environmental genomes in this study were annotated using prokka (https://github.com/tseemann/prokka) and managed using SnapGene v4.1.9 (https://snapgene.com). The RAST annotation pipeline [210] (https://rast.nmpdr.org/rast.cgi) was also utilised on genomes representative of each

clade to cross-check annotations. Transposons were identified using TnCentral (https://tncentral.proteininformationresource.org). Putative genomic islands (GIs) were identified by Islandviewer 4 [203] (https://pathogenomics.sfu.ca/islandviewer/) using reference genome *Comamonas* sp. strain NLF 7-7 (accession NZ_CP042344.1), and confirmed in detail using progressive Mauve [375]. Mobile gene cassette-associated recombination (*attC*) sites were screened for using HattCI (https://github.com/maribuon/HattCI)[376]. Only *attC* sites with Vscores >7.5 were considered. GIs were visualised using EasyFig [377] (https://mjsull.github.io/Easyfig/). BLASTn was utilised to determine whether putative GIs and AMR regions identified in this study had been previously deposited into NCBI. The Aliview software v3.0 (GPLv3) [283] (https://github.com/AliView), utilising MUSCLE and FastTree2 was used to align gene alleles and perform maximum-likelihood phylogenetic analyses. Plasmid comparisons were performed using the BLAST Ring Image Generator (BRIG) [378] (http://brig.sourceforge.net/).

5.5 Results

From a total of 471 isolates cultured from CRE-agar plates inoculated with samples from aquatic environments in South Australia, including wastewater, wetland, and lake samples from 2018-2019, 39 isolates were *Comamonas* spp.. *Comamonas* constituted the third most prevalent genus isolated from wastewater and are the subject of this study. Most *Comamonas* spp. were sourced from influent wastewater (n=37; 94.8%) with single isolates from a lake (n=1; 2.6%) and a wetland (n=1; 2.6%). Associated metadata on all 39 isolates described here as well as 48 *Comamonas* spp. sourced from Genbank is available in Appendix 15.

Draft genomes were assembled using shovill v1.0.4. Consistent with an earlier whole-genome sequencing study [345], genome sizes ranged between 2782493 bp to 4055492 bp with an average size of 3101902 bp. The number of scaffolds per genome ranged from 29 to 321, with a mean of 128.9. Full assembly statistics can be viewed in Appendix 16. Draft genomes have been deposited in the NCBI database under the accession numbers SAMN25632339-SAMN25632377, and BioProject PRJNA803140.

5.5.1 Identification of *Comamonas* species

Speciation of *Comamonas* isolates remains a challenge and varies between the typing techniques used (Table 5). MALDI-TOF MS of the 39 isolates sourced from Australian aquatic environments predicted *Comamonas* sp. for 25 (62.5%) and one *C. aquatica*. Kraken2 was also unreliable with 11 (28%) non-*Comamonas* identifications.

Kraken2 predicted 28 isolates as *Comamonas* species (72%), but some were misidentified as *Pseudomonas aeruginosa* (n=1) and *Acidovorax* spp. (n=10). Using our phylogenetic approach, all 39 isolates were identified as *Comamonas* spp., specifically, 33 as *C. denitrificans* (84.6%), five as *C. testosteroni* (12.5%) and one *C. aquatica* (2.5%) isolate.

Table 5: *Comamonas* Speciation. Aquatic environmental *Comamonas* species (n=39) as identified by MALDI-TOF MS, Kraken2, and WGS phylogenetic analysis.

Species Identification	MALDI-TOF MS	Kraken2*	WGS Phylogenetic characterisation
C. aquatic	1 (2.6%)	12 (30.7%)	1(2.6%)
C. testosterone	0	6 (15.4%)	5 (12.8%)
C. denitrificans	0	0	33 (84.6%)
C. kerstersii	0	8 (20.5%)	0
Comamonas sp.	25 (64.1%)	2 (5.1%)	0
Non-Reliable Identification	13 (33.3%)	0	0

*Kraken2 typing also resulted in *Pseudomonas aeruginosa* (n=1; 2.6%), *Acidovorax carolinensis* (n=2; 5.1%), *Acidovorax* spp. (n=8; 20.5%)

5.5.2 Phylogenetic analysis

A phylogenetic tree comprising 88 *Comamonas* spp. genomes was constructed using Phylosift (Figure 17) with 39 isolates from the Australian aquatic environment in this collection and 49 global strains available from Genbank. The isolates were derived from diverse sources, primarily environmental samples (n=75), humans (n=5) and various animal waste (n=5). The count of species post-phylogeny was as follows: *C. denitrificans* (n=34), *C. testosteroni* (n=19), *C. thiooxydens* (n=12), *C. aquatica* (n=4), *C. kerstersii* (n=3), *C. terrigena* (n=2) and one isolate each of *C. badia*, *C. composti*, *C. granuli*, *C. jiangduensis*, *C. koreensis*, *C. odontotermitis*, *C. piscis*, *C. sediminis* and C. serinivorans. Four isolates could not be characterised to the species level.

Based on the distribution of sequence alignments by PhyloSift, *Comamonas* species were separated into three primary clusters, with a clear separation between species in all cases except *C. testosteroni* and *C. thiooxydans*, which were closely related. *C. denitrificans* was notably separated from other *Comamonas* species, mirroring prior analyses of RNA sequences [379].

Comamonas group 1 (Figure 17) comprised of five species: *C. serinivorans*, *C. sediminis*, *C. granuli*, *C. badia* and an undefined *Comamonas* sp. strain NLF 7-7 (Sus scrofa, Korea).

Group 2 was defined as *C. denitrificans* only, including the environmental/ wastewater isolates from this study plus the only available *C. denitrificans* genome sequence from GenBank (JAFNME01, from sediment, Denmark).

Group 3 constituted isolates across at least eleven species based on current nomenclature, with clade structure suggesting up to thirteen species may be present. Our five *C. testosteroni* isolates were most closely related to *C. testosteroni* NFYY023 from human faeces, which sits apart from the *C. testosteroni*/*C. thiooxydans* subclade, however *C. testosteroni* NFYY023 was ~90,000 SNPs from the closest *C. testosteroni* from this collection.

It is likely that our five isolates constitute a closely related yet distinct species to *C. testosteroni*. Using the NCBIs *C. testosteroni* representative strain (NZ_CP067086) as a reference, we performed ANIb, ANIm and in silico DDH analyses as determine whether the isolates belong to *C. testosteroni*. All five isolates had ANIb and ANIm values of 83% and 87%, respectively, which are below the 95% cut-off values for species delimitation, and the in silico DDH analysis returned a 0.04% probability of being the same species as NZ_CP067086 (Appendix 17). Definitive novel species characterisations will be the subject of future studies.

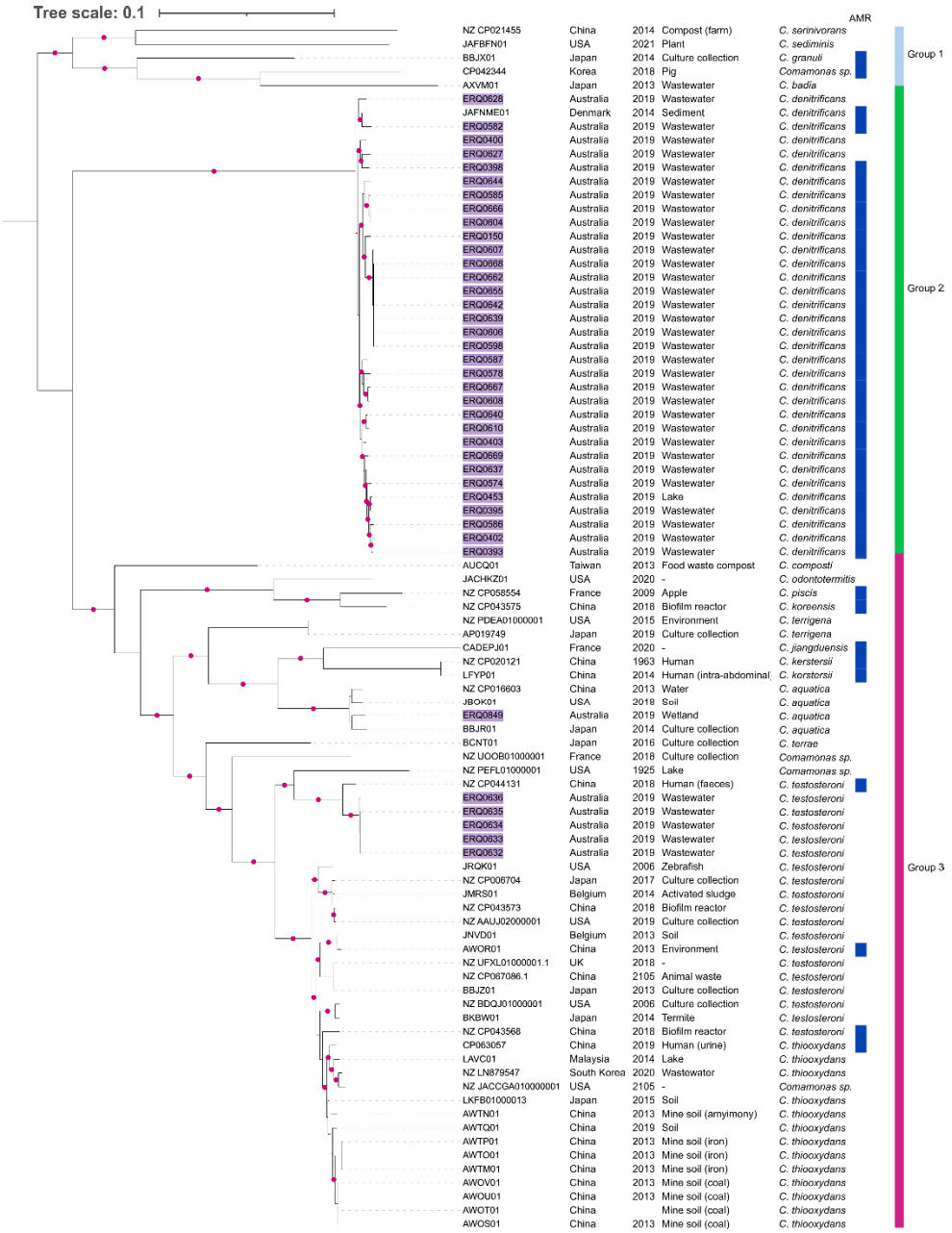

Figure 17: *Comamonas* phylogeny. Mid-point rooted Maximum-likelihood phylogeny and associated metadata of 88 *Comamonas* species using Phylosift. Isolates from this collection are coloured in purple. Bootstrap values > 0.9 are shown as pink dots. Blue strip designates isolates carrying ARGs.

To illustrate the more nuanced distribution of *C. denitrificans* in our study, particularly against the representative from Denmark, a Parsnp tree was constructed (Figure 17). Firstly, despite the majority coming from the same Australian wastewater source, we noted a diverse set of clades that generally mirror the PhyloSift distribution, where we acquired only one to three representatives of many potential subclades. The

exception was Group 2 (Figure 18), which sat as a closely related subclade (as low as 7 SNVs between 8 isolates). The external international reference sat most distant in the distribution, at approximately 6000 SNPs from both its closest neighbours in clades 1 and to clade 2, suggesting significantly diverged lineages of the species.

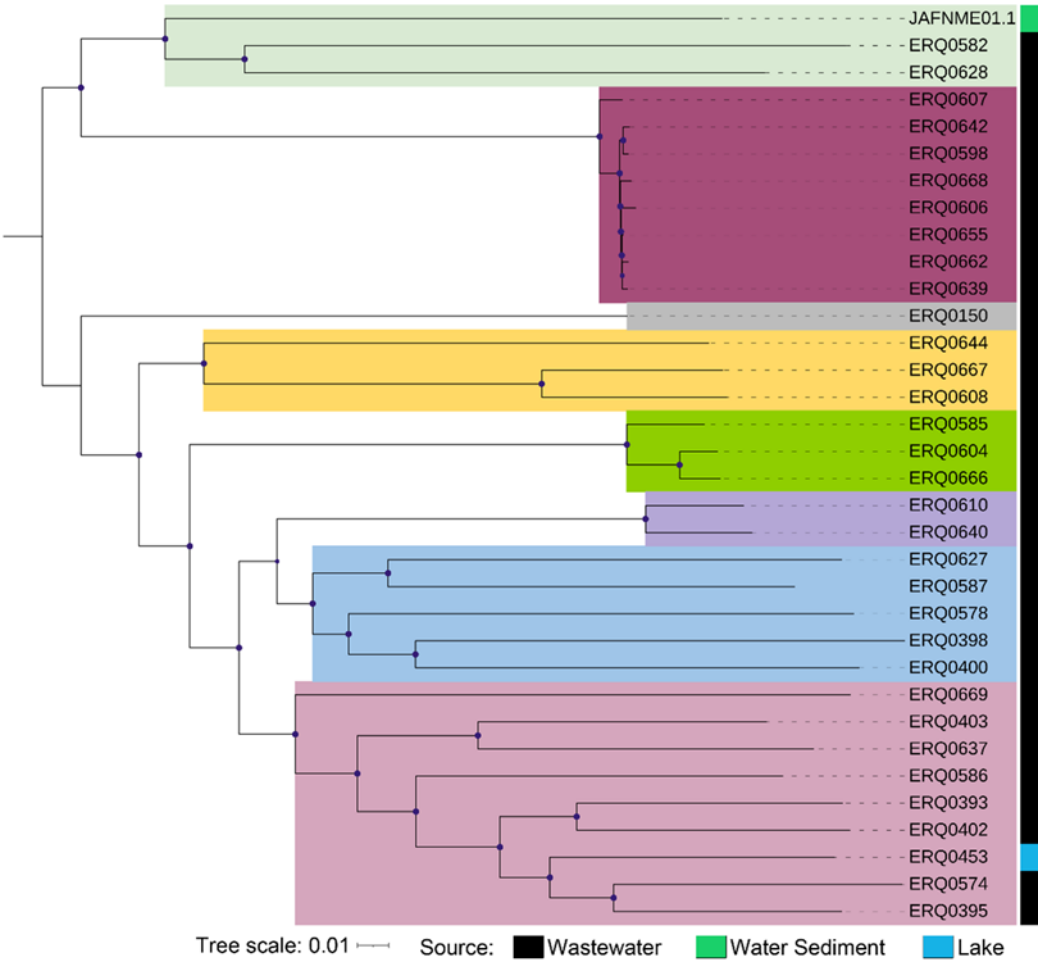

Figure 18: *C. dentrificans* phylogeny. Phylogenetic relationships of Australian *C. denitrificans.* The tree was generated from a SNP-based alignment via Parsnp, with *C. denitrificans* JAFNM01 used as a reference.

5.5.3 Pangenome analysis of *Comamonas denitrificans*

An analysis of the *C. denitrificans* pan-genome supported the sub-clade associations as determined by the core-genome phylogeny and the high genetic diversity of the species (Figure 19). The *C. denitrificans* pangenome consisted of 7,219 genes, with a core genome of only 946 genes and an accessory genome of 5173 genes (147 soft-core, 2,637 shell and 2,389 cloud genes). A range of multiple alignment methods, L-INS-i (accurate; for alignment of <~200 sequences), FFT-NS-2 (fast; for alignment of <~30,000 sequences) through MAFFT. The distribution of genes generally matched the

phylogenetic analysis presented in Figure 18, with notable divergence in pangenome data for clades 1 (light green), 7 (blue) and 8 (pink), which had some isolates with distinct genotypes distant from the closest evolutionary neighbours. This comparison aligns with the highly variant nature of the isolates presented here, suggesting that up to twenty-five distinct lineages of *C. denitrificans* are now available as WGS.

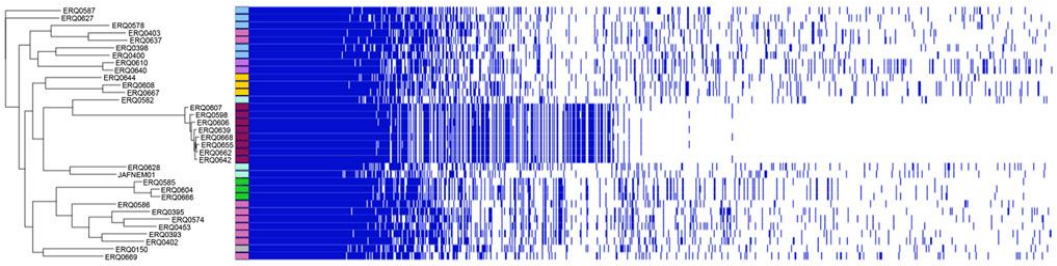

Figure 19: *C. denitrificans* pangenome. Pangenome analysis of 33 *C. denitrificans* from Australian aquatic environments and *C. denitrificans* JAFNEM01 sourced from Genbank. Colour groupings match clades designated in Figure 2. Phylogenetic clustering by accessory genomes.

5.5.4 Antimicrobial resistance genes identification

All 88 *Comamonas* spp. were screened for ARGs (Figure 20). In the Australian collection, 30 out of the 33 *C. denitrificans* isolates (29 from wastewater, one from a lake sample) carried at least one ARG or detergent resistance gene. Despite being selected for phenotypic carbapenem resistance, none of the other isolates from the Australian collection (n=6) carried any known resistance gene. Among the 33 Australian *C. denitrificans* isolates, 13 carried carbapenemase gene *bla*$_{\text{GES-5}}$, nine hosted β-lactamase gene *bla*$_{\text{OXA-10}}$, two carried *bla*$_{\text{OXA-101,}}$ and two carried *bla*$_{\text{OXA-5-like}}$ alleles. Therefore, we could not account for resistance to the carbapenem supplement in Oxoid Brilliance CRE Agar plates in 26/39 *Comamonas* spp isolates.

A total of 21 Australian *C. denitrificans* isolates carried at least one aminoglycoside resistance gene, *aadA5* being the most common (n=9) followed by *aadA13* (n=6) (Figure 19). *C. denitrificans* isolate ERQ0403 carried a combination of clinically important antimicrobial resistance genes (*bla*$_{\text{GES-5}}$, *bla*$_{\text{OXA-10}}$) and aminoglycoside resistance (*aadA13*). All but one Australian *C. denitrificans* harboured detergent resistance gene *qacL* (Figure 20).

A subset of *Comamonas* isolates sourced from Genbank (n=11; 22%) also carried ARGs (Figure 17 and Figure 20), five of which, sourced from either humans or biofilm

reactors, had multidrug resistance profiles (Figure 20). However, outside of the Australian *C. denitrificans* isolates, only one isolate, a *C. thiooxydans* sourced from a human sample, carried a carbapenem resistance gene (*bla*$_{IMP-8}$).

Metal resistance genes were also identified using the MEGAres 2.0 database. All *C. denitrificans* isolates originating from Australia carried genes conferring resistance to copper (*cop* genes), and 11 (33%) additionally carried mercury resistance genes (*mer* genes). The singular Australian *C. aquatica* isolate also carried copper resistance genes, as did two *C. aquatica* isolates sourced from Genbank. The five Australian *C. testosteroni* isolates did not carry metal resistance genes; however, eight *C. testosteroni* isolates sourced from Genbank all carried mercury resistance genes, with one isolate carrying an additional gene conferring resistance to chromium. Virulence-associated genes were also screened for using the VFDB; however, none were identified in any isolate using a >80% nucleotide identity and >80% gene coverage cut-off.

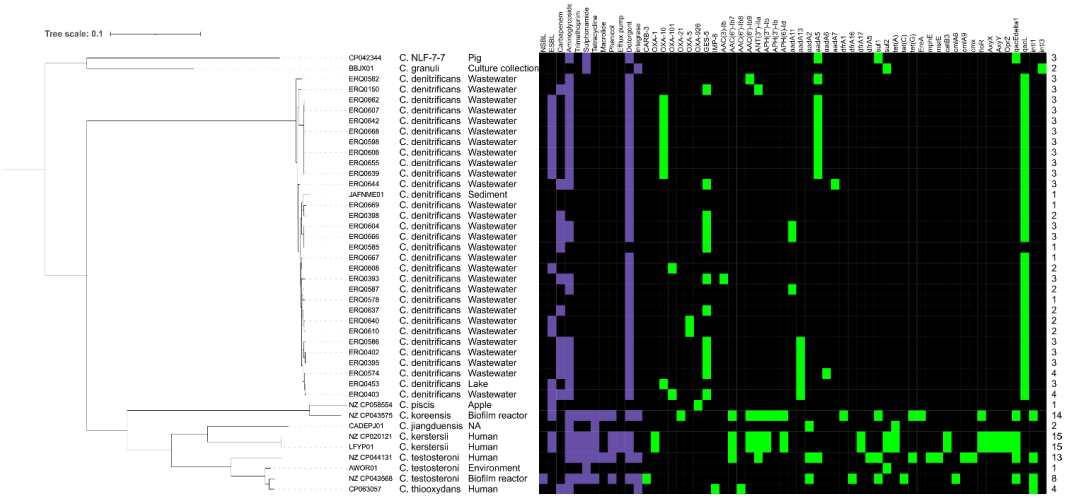

Figure 20: Antimicrobial resistance genes (ARGs) in *Comamonas* spp. Heatmap of ARGs identified in all available *Comamonas* spp. genomes. Specific ARGs, detergent resistance genes and integrases are shown in green. ARGs shown in green. Resistance conferred by gene carriage is shown in purple. NSBL = narrow spectrum beta-lactamase.

C. denitrificans chromosomally acquired *bla*$_{GES-5}$ and *bla*$_{OXA}$ alleles

Regarding genetic context, *bla*$_{GES-5}$ and *bla*$_{OXA}$ genes appeared on putative genomic islands (GIs). The clade of *C. denitrificans* encoding *bla*$_{GES-5}$ presented as a set of diverse lineages (Figure 19). Upon comparing the genetic context around *bla*$_{GES-5}$, it was determined that two chromosomal sites were hosting the gene, with related isolates ERQ0150, ERQ0395, ERQ0585 encoding the gene in the same chromosomal loci. One isolate, ERQ0644, had acquired the gene at a separate chromosomal site. Two reference

strains (JAFNME01 and ERQ0400) were used to resolve these chromosomal sites across contigs present in the isolates hosting AMR (Figure 21A, 5C). In addition to *bla*$_{GES-5}$, the GI present in ERQ0395 also harboured *aadA13* (aminoglycoside resistance), *qacL* (quaternary ammonium compound resistance) and several DUF1010 genes. Though the GIs represented in Figure 21A lacked any tRNA gene or known integrase, they were identified as GIs based on atypical codon usage by SIGI-HMM [380], and most ORFs within the GIs, including all AMR genes, had adjacent *attC* sites indicating that these ORFs are mobile gene cassettes. The *attC* sites associated with *bla*$_{GES-5}$ in ERQ0585 and ERQ0150 shared 98% sequence identity but only ~50% sequence identity with the ERQ0395 *bla*$_{GES-5}$ *attC* site. However, the *bla*$_{GES-5}$ gene and associated *attC* site in ERQ0395 was identical to that carried by an *intI3*-containing Tn*7221* (also called Tn*Pfu1*; Tn*402* family) in *Pseudomonas aeruginosa* pIPM3H3-GES5. Unlike the three aforementioned *bla*$_{GES-5}$-containing GIs, the GI carrying *bla*$_{GES-5}$ in ERQ0644, located at a different chromosomal insertion site (Figure 21C), contained a phage associated integrase *intIPac*, which was also partially present in ERQ0400 (Figure 21C).

A comparison of two representative GIs from EQR0640 and EQR0655, encoding *bla*$_{OXA-10}$ and *bla*$_{OX-5-like,}$ respectively, were also visualised (Figure 21B). Alignments demonstrated that GIs hosting these AMR genes were inserted at the exact same chromosomal site as three GIs containing *bla*$_{GES-5}$ (Figure 21A). The shared chromosomal sequence at each structure's termini included *lysR* (transcriptional regulator) and a *fic*-family gene, often found in mobile GIs [381] at one end and a partial *lysR* and an *mmsA* (CoA-acylating methylmalonate-semialdehyde dehydrogenase) at the other. Like the *bla*$_{GES-5}$ GIs, most ORFs, including *bla*$_{OXA-5-like}$ and two *qacL* genes in EQR0640 and *bla*$_{OXA-10}$ and *aadA5* in EQR0655, were associated with *attC* sites, though lacked any known integrase or tRNA gene. All *bla*$_{GES-5}$ and *bla*$_{OXA}$-containing GIs appear to be unique to this Australian collection of *C. denitrificans,* with no significant homology to GIs previously deposited in Genbank, including a well characterised *bla*$_{GES-5}$-containing GI residing in *P. aeruginosa* [162].

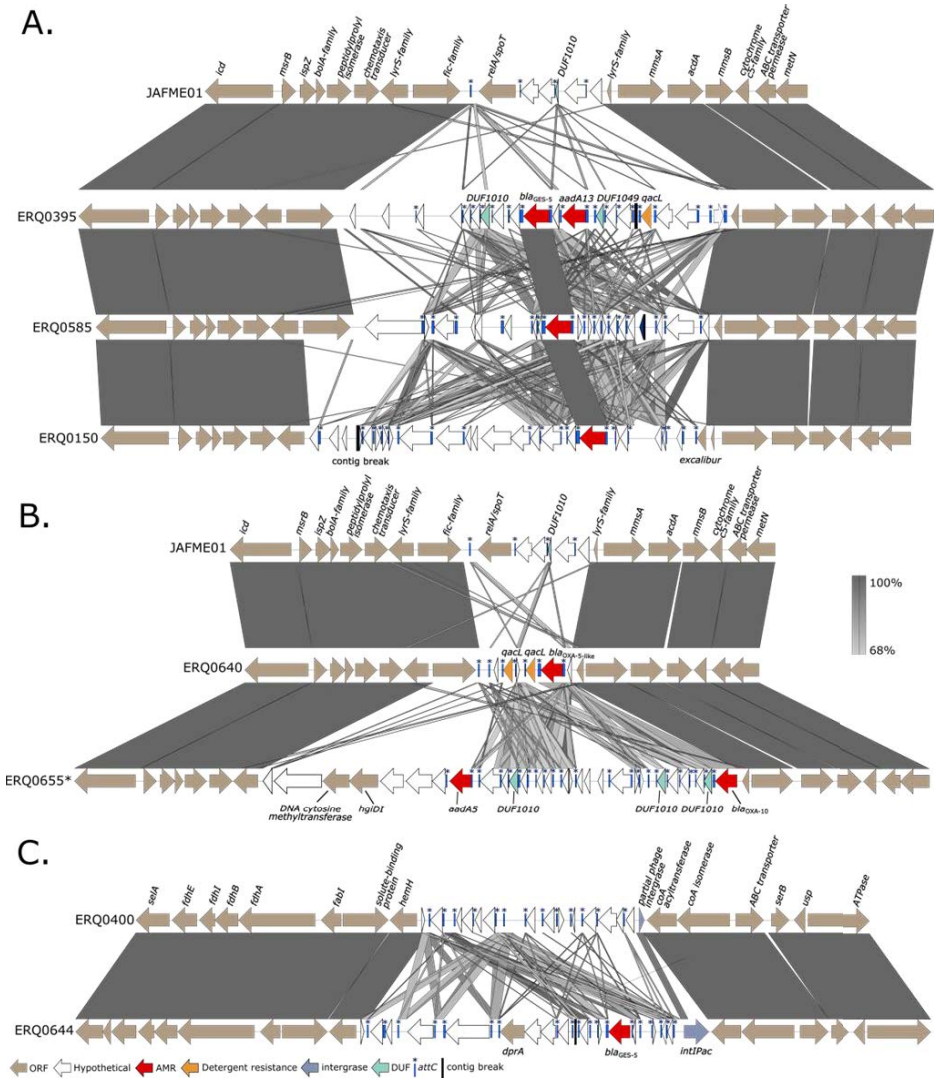

Figure 21: Genomic islands (GIs) carrying AMR determinants. A) Comparison of three GIs carrying *bla*$_{GES-5}$ at the chromosomal insertion site. B) Comparison of two GIs, one carrying *bla*$_{OXA-10}$, the other *bla*$_{OXA-5-like}$. GI in the same genetic context as A). C) Comparison of GI carrying *bla*$_{GES-5}$. The genetic context differs to A) and B) and is associated with intergrase *intIPac.*

5.5.5 *Comamonas denitrificans* IncP-1 plasmid

All 88 *Comamonas* genomes were screened for plasmid replicons, and three were identified: IncP-1 in *C. denitrificans* isolate ERQ0628 and a *C. jiangduensis* isolate (CADEPJ01), and rep21 in a *C. kerstersii* strain J29 (NZ_LFYP00000000.1). The IncP-1 replicon (*trfA*) in ERQ0628 was located on a 62,429 bp contig, beginning with a partial *traI* gene and ending with a partial *traE* gene. Another contig (3980 bp) contained both partial *traI* and *traE* genes present at the contig start and end. These two contigs were concatenated to produce a putative complete IncP-1 plasmid sequence. Future long-read sequencing is needed to investigate any potential additional insertions into the *tra*

region following *traI* or *traE*, though previously insertions have only been reported following *traM,* between *oriV* and *trfA* and between *trb* and *tra* regions [382]. Nevertheless, a BLAST search of the NCBI database was used to identify high sequence identity IncP-1 plasmids and the top 15 hits (ranging between 98.78 - 99.95% sequence identity and 40 – 79% sequence coverage) were used for content comparisons (Figure 21).

All compared IncP-1 plasmids possessed an IncP-type conjugation *tra* region and the IncP *kor-par-kle* regulon region. The remaining regions present in ERQ628 IncP-1 (Figure 22; left) were less common. An 11,709 bp inserted region containing a mercury resistance *mer* operon carried by Tn*5031* (Tn*402* family) was identified in two other similar plasmids, one carried by *Alicycliphilus denitrificans* pALIDE02 (CP002451) and the other by *Delftia acidovorans* pUO1 (AB06332) [383]. These two Tn*5031* sequences were near-identical, differing by 1 SNP. *A. denitrificans* pALIDE02 was the only other plasmid to possess a 7,964 bp inserted region containing genes involved in stress response and xenobiotic degradation, including two genes encoding glutathione S-transferases and a short-chain dehydrogenase/reductase (SDR). The ERQ628 IncP-1 plasmid also possesses a unique 12,843 bp inserted region flanked by two IS*1071* elements and contained genes involved in oxidative stress response, including *msrA, msrB* and *ydiU.* In *D. acidovorans* pUO1 (Figure 21; right) IS*1071* elements flank genes responsible for haloacetate degradation (*dehH1*, *dehH2*). Notably, the ERQ0628 IncP-1 plasmid only contained *trbA,* and the remaining *trb* region was missing (Figure 22; right).

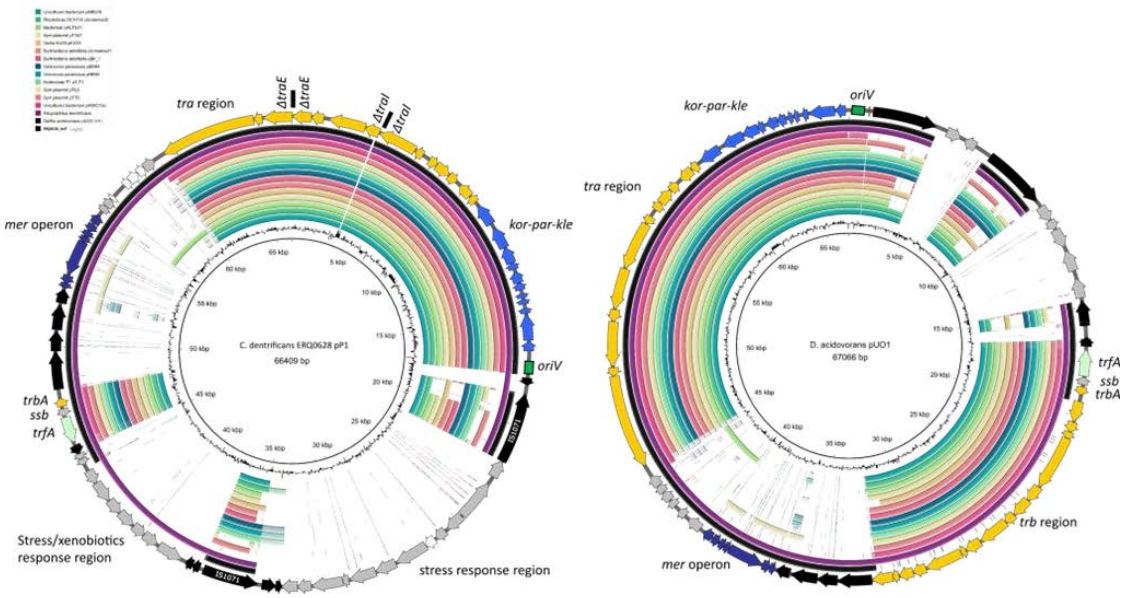

Figure 22: BRIG comparisons of IncP-1 plasmids. Left: Plasmid map of putative IncP-1 plasmid from ERQ0628 and BLAST alignments to other IncP-1 plasmids. Contig breaks noted in the *tra* operon. Right: Plasmid map of *D. acidovorans* pUO1 and BLAST alignments to other IncP-1 plasmids.

5.6 Discussion

Wastewater has been flagged as a hotspot for interaction between diverse bacteria, genetic exchange, and AMR evolution and dissemination across ecosystems [384]. However, despite the abundance of *Comamonas* species in wastewater, its ability to acquire AMR is poorly understood. Here we add 39 draft *Comamonas* genomes, sourced from South Australian municipal wastewater, a lake, and a wetland, to make up a total pool of 88 *Comamonas* WGS currently available. Our analyses show: i) carbapenem-resistant *Comamonas* was most prevalent in wastewater, ii) *C. denitrificans* isolates carried genomic islands encoding carbapenemase bla_{GES-5} or extended-spectrum beta-lactamase (ESBL) bla_{OXA} alleles iii) *Comamonas* carry genes conferring resistance to copper and mercury, iv) *C. denitrificans*, the most commonly isolated species, was genetically diverse, and v) a *C. denitrificans* isolate carried an IncP-1 plasmid with genes involved in xenobiotic degradation and response to oxidative stress.

5.6.1 Carbapenem-resistant *Comamonas* is present in aquatic environments: implications for public health and wastewater treatments

Carbapenem-resistant Gram-negative bacteria are an urgent threat to global health [385,386] and have been detected in both wastewater and surface water worldwide [363,387–389]. Here we isolated 39 carbapenem-resistant *Comamonas* isolates: 32 *C. denitrificans* and five *C. testosteroni* from wastewater and, one *C. denitrificans* from a wetland and one *C. aquatica* from a lake with public access.

To the best of our knowledge, this is the first report of carbapenem resistance in *C. denitrificans* and *C. aquatica*, though there has been at least one report of a carbapenem-resistant *C. testosteroni* infection in Turkey in 2015 [390]. Infections caused by carbapenem-resistant bacteria are associated with poor prognoses and increased morbidity and mortality rates [391]. *C. testosteroni* has been isolated as the infectious agent in various sites, including blood, peritoneal fluid, cerebrospinal fluid, urine, and different tissues [392]. *C. aquatica* reportedly has caused bacteremia and septic shock [393], and *C. denitrificans* has been detected in clinical samples [79].

Therefore, the emergence of carbapenem-resistance in these organisms warrants closer public health surveillance. To that end, it is important to note that

Comamonas spp. grow poorly on routine clinical media [79], and biochemical tests often provide erroneous *Pseudomonas* spp. identifications [321]. Similarly, as shown here and elsewhere, MALDI-TOF MS can misidentify *Comamonas* at the species level (K. Moriya et al., 2015). Furthermore, *C. denitrificans* and *C. nitrativorans* have highly similar 16S rRNA sequences and cannot be differentiated by phenotypic analysis [321]. Conversely, WGS represents a high-resolution method that can accurately identify *Comamonas* species.

The presence of carbapenem-resistant *Comamonas* in wastewater also has implications for wastewater treatments. Wastewater treatment plants receive large volumes of sewerage enriched in inorganic constitutes, such as metals and chemicals, and organic constituents, such as faeces and food waste [395,396].

Biological treatment of wastewater using microbes is effective and easy to implement at a low cost, particularly when using indigenous microbial communities [397]. A recent meta-analysis of 20 diverse metagenomic wastewater samples across 11 countries found that *Comamonas* spp. was dominant in industrial, biological, and municipal wastewater [398].

In wastewater, *Comamonas* spp. play important roles in the degradation of benzene, ethylbenzene, and toluene [399], dye[332], naphthalene-2-sulphonic acid [400], organics [333], pyridine [334], quinoline and phenol [401], in denitrification [402] and bioremediation of petrochemicals [403]. These capabilities, in addition to high biofilm-forming properties, make *Comamonas* good candidates for biofilm systems wastewater treatment [404,405].

However, a recent study demonstrated that spiking wastewater biofilm reactors with streptomycin and oxytetracycline caused the acquisition of class 1 integron integrases (*intI1*) and *aadA* genes (aminoglycoside resistance) in *Comamonas* strains [406]. Given this recent observation, it is perhaps unsurprising that genes conferring resistance to aminoglycosides were identified in 30 *C. denitrificans* isolates (91%) in our collection with *aadA5* the most prevalent (n=9), and while *intI1* was not identified in this collection, the potential for capture should not be overlooked. On that note, bacteria in wastewater exist as heterogeneous communities, and the presence of *Comamonas* is positively correlated with the presence of *Acinetobacter, Aeromonas* and *Pseudomonas,* suggesting these microbes form a symbiotic relationship [398]. Horizontal gene transfer occurs in proximal bacteria, and all three species are known producers of carbapenemases [363,407]. Therefore, a symbiotic relationship with these organisms

may not only involve the cooperative degradation of pollutants, but also the exchange of AMR determinants.

5.6.2 C. denitrificans carry genomic islands encoding either *bla*$_{GES-5}$ or *bla*$_{OXA}$ alleles

Genes encoding carbapenemases confer resistance to carbapenems, penicillins, cephalosporins, and monobactams, thus severely limiting treatment options [391]. While all 39 *Comamonas* isolates described here had a carbapenem-resistant phenotype, only 13 isolates, all *C. denitrificans,* carried a known carbapenemase gene – *bla*$_{GES-5}$ – suggesting novel AMR mechanisms for the remaining isolates.

The *bla*$_{GES-5}$ gene cassette has previously been detected in the genus of *Citrobacter, Enterobacter, Escherichia, Leclercia,* and *Lelliottia* found in wastewater [363,387,408], in *Citrobacter* and *Klebsiella* in surface water [361,409] and was commonly associated with class 1 and 3 integrons. The gene has also been detected in clinical *P. aeruginosa* strains carried by plasmids and genomic islands [162–164].

Genomic islands are fragments of DNA inserted into the chromosome via horizontal gene transfer and play an important role in the evolution and adaptation of bacteria through the dissemination of ARGs and virulence genes and the formation of catabolic pathways [157]. We were able to resolve four examples in which a *bla*$_{GES-5}$ gene cassette was located on predicted genomic islands, and in one instance the *bla*$_{GES-5}$ gene cassette was identical to that carried by *intI3*-containing Tn*7221* structure found on a *P. aeruginosa* plasmid.

However, genomic islands are generally characterised by atypical G+C content and codon usage, the presence of integrons, and are typically inserted at tRNA loci [158]. Of the four putative genomic islands, only one carried an integrase - *IntIPac* - previously reported promoting AMR in *Acidithiobacillus ferrooxidans* [160]. Apart from atypical G+C content and codon usage, the remaining three genomic islands did not contain any typical genomic island characteristics and appeared to consist of a collection of gene cassettes located in the same chromosomal loci.

Curiously, we also resolved two predicted genomic islands, one carrying ESBL *bla*$_{OXA-10}$, commonly found in *Acinetobacter baumannii* [161], and the other a novel *bla*$_{OXA-5-like}$ gene, both located at the same chromosomal loci as the three *bla*$_{GES-5}$ containing genomic islands. Though rare, gene cassettes have been found outside an integron context [410], however the mechanisms behind their insertion has not been determined.

5.6.3 Comamonas spp. carry heavy metal and biocide resistance genes

In addition to residual antibiotics, it has been postulated that the high AMR levels in water matrices are also due to the presence of metals and biocides, which can exert selective pressure for ARGs through co-selection [411].

Though we did not find any apparent correlation between ARG, heavy metal, and biocide carriage, we did identify genes conferring resistance to copper (*cop* genes) in all *C. denitrificans,* and the singular *C. aquatica* isolate in this collection. Eleven *C. denitrificans* isolates (33%) additionally carried mercury resistance genes (*mer* genes), and 29 *C. denitrificans* isolates (89%) carried detergent-resistance gene *qacL*.

Interestingly, a study on the association of metal tolerance with antibiotic susceptibility in *C. acidovorans* found mercury resistant phenotypes were resistant to nitrofuran, beta-lactams, aminoglycosides, glycopeptides and tetracycline, while copper resistant phenotypes were susceptible to all tested antibiotics [412]. However, ARG carriage was not determined.

5.6.4 IncP-1 plasmid present in *C. denitrificans*

Sensing stressful conditions, and adjusting cellular metabolism accordingly, is essential for bacteria to survive in variable environments such as wastewater [413]. IncP-1 plasmids are promiscuous self-transmissible plasmids with broad host ranges and an ability to swiftly acquire and transfer genes involved in the degradation of introduced xenobiotics [414].

IncP-1 plasmids have been previously identified in *Comamonas* spp. isolated from wastewater that were involved in the degradation of dyes [415]. However, only one IncP-1 replicon was identified in *Comamonas* from Australian aquatic environments; a *C. denitrificans* isolate from wastewater.

The IncP-1 replicon was situated on a putative 66,409 bp plasmid containing three inserted regions. One inserted region contained a functional *mer* operon, a well-characterised metal resistance system capable of degrading highly toxic mercury into volatile, non-toxic forms [416]. Another inserted region carried genes involved in oxidative stress response and was flanked by IS*1071* elements. IS*1071* are known to flank many catabolic genes in diverse Gram-negative and Gram-positive bacteria [417] and have been shown to transpose at high frequencies in *C. testosteroni* [418]. The last inserted region carried two glutathione S-transferases, known to degrade a wide range of toxic chemicals, including carcinogens, environmental pollutants, and oxidative stress

products [419], and a short-chain dehydrogenase/reductase, involved in the metabolism of aromatic hydrocarbons, including steroids and sugars [417]. Unlike most, if not all, IncP-1 plasmids, the majority of *trb* genes, which form the sex pili, were missing in the *C. denitrificans* IncP-1 plasmid, meaning that in all probability, it is non-conjugative.

Chapter 6. Genomic analysis of *Citrobacter* from Australian wastewater and silver gulls reveals novel sequence types carrying critically important antibiotic resistance genes

6.1 Declaration

This chapter represents a manuscript accepted for publication.

It responds to aim 2, 3 and 4.

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Author contributions

SH: writing the original draft, formal analysis, investigation, writing – review and editing. MLC: writing the original draft, formal analysis, investigation, writing – review and editing; ERW: Investigation, Methodology, Validation, Conceptualisation, Supervision, Writing – review & editing; BD: Investigation, Conceptualization, Validation, Formal analysis, Data Curation, Writing – Review & Editing; KM: Investigation, Resources; BJH: Resources, Methodology; MSS:

Resources, Methodology, JG: Investigation, Resources; DRB: Resources, Methodology ;CJ: Investigation, Resources; ATD: Investigation, Resources, JY: Investigation, Resources, FH: Investigation, Resources– review and editing; ED: Conceptualisation, Funding acquisition, Supervision, Writing – Review & Editing; VMJ: Writing – original draft, Formal analysis, Investigation, Supervision, Conceptualization, Writing – review & editing the final draft; SPD: Conceptualisation, Resources, Supervision, Funding acquisition, Project administration, Writing – review & editing the final draft.

Ethical approval

The water samples used in our study were approved by the relevant authority for data publication.

Authors signatures

Sopheak Hem: Production Note: Signature removed prior to publication.

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6.2 Abstract

Antimicrobial resistance (AMR) is a major public health concern, and environmental bacteria have been recognized as important reservoirs of antimicrobial resistance genes (ARGs). *Citrobacter*, a common environmental bacterium and opportunistic pathogen in humans and other animals, has been largely understudied in terms of its diversity and AMR potential. Whole-genome sequencing on 77 *Citrobacter* isolates obtained from Australian silver gull (*Chroicocephalus novaehollandiae*) and influent wastewater samples was performed here, revealing a diverse *Citrobacter* population, with seven different species and 33 sequence types, 17 of which were novel. From silver gulls we isolated a broader range of species with little to no mobilised ARG carriage. Wastewater isolates carried a heavy burden of ARGs (up to 21 ARGs, conferring resistance to nine classes of antibiotics), with several novel multidrug-resistant lineages identified, including *C. braakii* ST218 novel, which carried ARGs conferring resistance to eight to nine classes of antibiotics, and *C. freundii* ST23 novel, which carried two carbapenemase genes, $bla_{\text{IMP-4}}$ in class 1 integron structure, and $bla_{\text{KPC-2}}$.

Additionally, we identified an MDR *C. portucalensis* isolate carrying $bla_{\text{NDM-1}}$, $bla_{\text{SHV-12}}$, and *mcr-9*. We identified IncC, IncM2, and IncP6 plasmids as the likely vectors for many of the critically important mobilised ARGs. Phylogenetic analyses were performed to assess any epidemiological linkages between isolation sources, demonstrating low relatedness across sources beyond the ST level. However, these analyses did reveal some closer relationships between strains from disparate wastewater sources despite their collection some 13,000 kilometres apart. These findings support the need for future surveillance of *Citrobacter* populations in wastewater and wildlife populations to monitor for potential opportunistic human pathogens.

6.3 Background

Antimicrobial resistance is an urgent and global health threat, the management of which requires an understanding of the mechanisms of the evolution and dissemination of mobile. antimicrobial resistance genes and antimicrobial resistant bacteria. While most studies reporting on antimicrobial resistance (AMR) come from clinical settings, the environment is an important reservoir of AMR bacteria, with a 2017 United Nations (UN) report highlighting AMR as an “emerging issue of environmental concern” [420]. Nevertheless, the burden of AMR remains poorly understood in the environment, and current surveillance systems often neglect environmental sampling [421,422]. Environmental bacteria, the most prevalent but least studied bacteria, serve

as sources of ARGs that can become incorporated into human and animal opportunistic pathogens via mobile genetic elements (MGEs), such as plasmids[423,424].

Citrobacter spp. are ubiquitous in soil, food, water bodies and are part of the human and animal commensal intestinal microbiota [425]. However, some *Citrobacter* spp., such as *Citrobacter freundii*, *Citrobacter koseri and Citrobacter braakii,* are known opportunistic human pathogens responsible for a plethora of diseases. *C. freundii* infections are diverse in their manifestations and include diarrhoeal disease but also extraintestinal infections of the urinary and respiratory tracts, wounds, and blood [426]. *C. braakii* has been known to cause peritonitis and foodborne diseases [425,427–429], and *C. koseri* causes meningitis and brain abscess in neonates and immunocompromised individuals [430]. Invasive *Citrobacter* infections are often fatal with mortality rates estimated to be between 33% and 48% [431–435]. In infants, meningitis caused by *Citrobacter* has a mortality rate of approximately 30% and of those who survive, more than 80% sustain various degrees of cognitive deficits [436].

Pathogenic *Citrobacter* spp. present treatment challenges as they are generally resistant to cephalothin and cefazolin (1st generation cephalosporins), most penicillins, and β-lactamase inhibitor/β-lactam combinations due to their carriage of the chromosomal inducible β-lactamase AmpC [88,89] – encoded by bla_{CMY}. AmpC type β-lactamases may also be carried on plasmids and confer additional resistance to cefoxitin or cefotetan, and oxyimino-cephalosporins (Jacoby, 2009b; Tan et al., 2009). At least two plasmid-mediated AmpC β-lactamases thought to originate from *C. freundii* (bla_{LAT-1} and bla_{CFE-1}) [437] have now been captured by multiple plasmid lineages and disseminated into diverse species of *Enterobacteriaceae* [438]. Like extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases mediate resistance to 3rd generation cephalosporins and β-lactams but unlike ESBLs they are not inhibited by clavulanic acid (Fallah et al., 2020). Both AmpC β-lactamases and ESBLs pose a serious global health burden [439,440].

Citrobacter species have also been increasingly emerging as important carriers and disseminators of carbapenemases. Carbapenems are considered drugs of “last resort”, thus the global rise of carbapenem-resistant *Enterobacterales* (CRE) in recent decades has become an urgent and global health threat. Metallo-β-lactamases (MBLs), encoded by genes such as bla_{IMP} and $bla_{NDM,}$ confer resistance to carbapenems and hydrolyse almost all β-lactams, rendering treatment of pathogens carrying MBLs challenging. A study on 512 genomes of CRE isolates from 61 German hospitals across three years found that *Citrobacter* spp. were increasingly detected and that their carriage of carbapenemase genes (bla_{KPC}, bla_{OXA-48}, bla_{VIM} and bla_{NDM}) were comparable to *K.*

pneumoniae isolates from the same study. These genes were largely carried by IncN and IncL/M plasmids [90]. Additionally, several multidrug resistant (MDR) strains of a recently identified species, *Citrobacter portucalensis*, have been reported globally since it was first differentiated from *C. freundii* in 2017. One clinical isolate from China was reported carrying 29 antimicrobial resistance genes (ARGs) including bla_{CMY-35}, bla_{KPC-2} and bla_{NDM-1} [441].

Similarly, environmental populations of *Citrobacter* may pose an AMR threat. Indeed, CRE carrying AMR plasmids have already been isolated from environmental samples including natural waterbodies [442], wildlife and synanthropic species [443], and wastewater [444]. Wastewater in particular has been flagged as a hotspot for AMR evolution given the high bacterial load of these substrates, their contamination with sublethal antibiotic concentrations derived from anthropogenic sources [445] and their exposure to antimicrobials used in sanitation processes [446]. Wildlife that frequents wastewater holding lagoons, particularly wild bird species, may contribute to the dissemination of clinically important ARGs[447,448]. In Australia, silver gulls (*Chroicocephalus novaehollandiae*) have been reported harbouring CRE and ESBL-producing *Enterobacterales,* particularly *Escherichia coli* and *Salmonella* spp. [449–457].

The population structure of *Citrobacter* in silver gulls and in Australian wastewater is currently unknown. To begin addressing these knowledge gaps, we undertook whole genome sequencing (WGS) of 77 isolates of environmental *Citrobacter spp.* isolated from Australian wastewater (n=60) and silver gulls (n=17). Here, we report on their phylogenetic structure, carriage of virulence genes, AMR genes and MGEs. We also present a phylogenetic analysis of these genomes and explore their relatedness to a collection of 970 additional *Citrobacter* genomes sourced from public databases.

6.4 Methods

6.4.1 Bacterial isolation and DNA extraction

Bacterial isolates used in this study are detailed in Appendix 18. Of the 77 *Citrobacter* spp. isolates sequenced in this study, 60 were obtained from wastewater (55 from South Australia (SA) and 5 from New South Wales (NSW)), with the remainder of isolates being sourced from silver gulls (n=17) from NSW. Influent wastewater samples from SA were collected as previously described [458]. From NSW, raw wastewater (influent) samples were collected from two WWTP in the South Coast. Because indiscriminate disposal of antibiotics can lead to significant level of antibiotics in landfill leachate, landfill leachate samples were also collected from one NSW site as a

reference. Briefly, over a one-year period from July 2018 to July 2019, a total of 56 water samples from SA and 8 water samples from NSW were collected monthly in triplicate. This corresponded to the isolation of a total of 665 bacteria from SA and 859 bacteria from NSW, respectively. Samples were collected in sterile 10 L collection tanks and processed within 2-3 hrs of collection. 500µL from 2-3 consecutive 10-fold serial dilutions were plated in triplicates on Oxoid *Brilliance*™ CRE Agar plates [Carbapenem Resistant *Enterobacteriaceae,* Thermo Fisher Scientific]. Cultures were incubated at 25°C, 37°C and 44°C. Incubation times of under 24 hrs were used to avoid biases associated with the inactivation of carbapenem in the culture medium by CRE that produce extracellular carbapenem degrading β-lactamases. Using pre-sterilized toothpicks, single colonies growing on CRE Agar were plated on Plate Counting Agar [PCA; Thermo Fisher Scientific]. PCA cultures were incubated at 37°C for ~18-24 hrs.

Silver gull chicks (1-6 weeks post hatching) were sampled on Big Island, Five Islands Nature Reserve, in the Illawarra region of NSW during the breeding seasons of 2018 and 2019. A cloacal swab was collected using a rayon-tipped swab subsequently stored in amies [Copan, Italy]. Samples were stored on ice while in the field (24-72 hrs) prior to culture. Swabs were placed in 1 ml Luria-Bertani (LB) broth, vortexed for 30 s, then 10 µl was streaked onto MacConkey agar [Edwards, Australia) and incubated overnight at 37ºC. In 2018, individual colonies were selected from these initial cultures and recultured overnight at 37ºC on Brilliant Green agar (Edwards, Australia) before being stored in 1 ml of storage medium (Milli-Q water containing 1% casamino acids and 10% glycerol and autoclaved) at -80ºC. Isolates were cultured for DNA extraction by suspending 10 µl of the storage solution in LB and incubating at 37ºC overnight. In 2019, all growth from the initial MacConkey agar plate was collected and stored at -80ºC in 1 ml of storage medium. Subsequently, 10^{-9} dilutions of these stored samples were prepared in sterile PBS and 150 ml was used to grow bacterial lawns on MacConkey agar. Individual colonies from these lawns were then recultured on Brilliant Green agar [Edwards, Australia] from which they were recultured overnight at 37ºC in LB for identification and DNA extraction.

Bacteria isolated from cultures were identified by MALDI-TOF MS and preserved in glycerol stocks (40% v/v) at -80°C. MALDI-TOF MS was performed in linear positive mode under MALDI Biotyper 3.0 Real-time Classification [v3.1, Bruker Daltonics]. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit [Qiagen] for wastewater and using a Qubit and dsDNA HS Assay kit [Thermo Fisher Scientific] for silver gull isolates, according to manufacturer's instructions. The purity of isolated DNA

was determined using Nanodrop 1000 spectrophotometer [Thermo Fisher Scientific] while its concentration determined. Purified DNA extracts were stored at −20°C.

6.4.2 Whole-genome sequencing, read filtering and genomic assembly

Libraries were prepared for whole-genome sequencing using the Hackflex protocol [459], a modified version of the Illumina DNA Prep. Sequencing was performed on an Illumina Nextseq500 instrument according to the manufacturer's instructions [213] before raw data demultiplexed using Basespace (https://basespace.illumina.com). Reads were filtered using fastp [460] v0.20.1 (default settings) and genomes assembled using Shovill v1.0.4. (https://github.com/tseemann/shovill) (parameters: "--minlen 200") via a snakemake workflow available at https://github.com/maxlcummins/pipelord. All downstream genomic analysis steps utilised filtered reads or the resulting assemblies from Shovill. Sequences (in the form of unfiltered reads) have been deposited on GenBank under bioproject number PRJNA100606.

6.4.3 Quality control

Genome lengths within the ranges observed for members of the genus *Citrobacter* in a snapshot of NCBI's RefSeq database (accessed March 2022) (3,095,000 to 6,770,000 bp) were included for further analysis where they assembled to 1000 or fewer scaffolds and have an N50 of ≥ 15,000, as determined by analysis with assembly-stats v1.0.1 (https://github.com/sanger-pathogens/assembly-stats). Genomes were also analysed using CheckM [215] to determine their level of completeness and contamination. Genomes exhibiting a 10% or greater contamination score were excluded as were those with an under 90% completion score. Strains were also typed using mlst (https://github.com/tseemann/mlst) using scheme autodetection and only strains which contained *Citrobacter* MLST alleles [216] were included for further analysis. Data pertaining to these metrics for samples under investigation are available in Appendix 18.

6.4.4 Species identification

Similar to wet-lab methods, species classification of *Citrobacter* using genomic data is often inaccurate and this is reflected in the poor correlation between phylogeny of publicly available *Citrobacter* genomes and their associated species identities, especially for species other than *C. freundii*. To enable accurate species classification of our *Citrobacter* genomes we downloaded an 1,171 additional *Citrobacter* genomes from RefSeq, subjected them to the quality control (970 passed the quality control procedures described above) and performed a mash-based phylogenetic analysis using BacSort (https://github.com/rrwick/Bacsort). Species identities were then curated in accordance

with their location in the resulting phylogeny and BacSort was then used to generate species identities for genomes from the study collection and is available as Appendix 21.

6.4.5 Phylogenetic trees and generation of pairwise SNP distances

Due to the phylogenetic diversity of the cohort under investigation, all phylogenetic trees generated using mashtree utilised v1.2.0 [205] with 100 bootstrap replicates. Trees were visualised and annotated using custom R scripts available at https://github.com/maxlcummins/*Citrobacter* utilizing R packages ggtree v1.16.6 [222], dplyr v0.8.3 [223], magrittr v1.5 [224], ggplot2 v3.2.1 [225], readr v1.3.1 [226], reshape2 v1.4.3 [227], tidytree v0.2.6 [228], and ggimage v0.2.8 http://cran.nexr.com/web/packages/ggimage/vignettes/ggimage.html.

Pairwise SNP distances between strains were determined using Split Kmer Analysis v1.0 [200] using “ska fastq” (default settings) and “ska distance” (parameters: -s 100 -i 0.95). Genomes were clustered into closely related groups when their SNP distances differed by 100 or fewer SNPs and genomes shared 95% of split kmers.

6.4.6 Genotypic characterisation

Carriage of antibiotic resistance genes (ARGs) and AMR-associated point mutations was determined using Abritamr v1.0.11 (https://github.com/MDU-PHL/abritamr). Carriage of other genes associated with virulence, insertion sequences (IS) and plasmids was determined using abricate (https://github.com/tseemann/abricate) with nucleotide databases from virulence factor database [198], ISFinder [461] (is.biotoul.fr) and PlasmidFinder [462], respectively. Plasmid comparisons were performed using the BLAST Ring Image Generator (BRIG) [378] (http://brig.sourceforge.net/). All databases utilised are available at https://github.com/maxlcummins/Citrobacter/ dbs/abricate. Genes detected by abricate were required to have minimum nucleotide identity and coverage of 90%.

Determination of plasmid multilocus sequence types (pMLST) was performed using pMLST (pubmlst.org/) [240] with output processed using custom scripts available at https://github.com/maxlcummins/pipelord/blob/main/scripts/combine_ IncF_RST.py (IncF plasmids) and https://github.com/maxlcummins/pipelord/blob/main/scripts/combine_pMLST.py (other plasmid types) as part of the aforementioned snakemake pipeline Pipelord.

For our purposes we define: i) AMR as carrying one or more genes or SNPs associated with resistance to one or more classes of antibiotic; ii) MDR as carrying genes or SNPs associated with resistance to three or more classes of antibiotic; iii) critically

important antimicrobial (CIA) resistance as carriage of genes conferring resistance to the highest priority CIAs [463].

6.5 Results

6.5.1 Identification of *Citrobacter* spp.

Phylogenomic analysis generated using BacSort revealed Australian wastewater and silver gulls host diverse *Citrobacter* species (Table 6). Among the 77 sequenced isolates seven species were identified, two of which, *C. freundii* and *C. braakii*, were present in both gull and wastewater samples (Figure 23). We identified 33 *Citrobacter* sequence types (STs) among which 16/33 (48.5%) were previously known and the remaining 17/33 (51.5%) were novel (either comprising previously unreported alleles or allelic combinations). These novel STs were submitted to pubmlst.org and assigned STs (Appendix 19). A total of 12 STs were identified among the 17 gull sourced isolates, of which five were novel, whilst among the 60 wastewater isolates 21 STs were identified, of which 12 were novel. *C. braakii* from wastewater in SA were predominantly identified as ST110 (66.7%, n=14/21) while *C. freundii* ST150 was found to be the most common ST of this species (24.3%, n=9/32). Among wastewater isolates from NSW, the novel ST1105 was the most prevalent ST comprising 80% (n = 4/5) of such strains. Novel STs was found in all the seven species identified in our study collection.

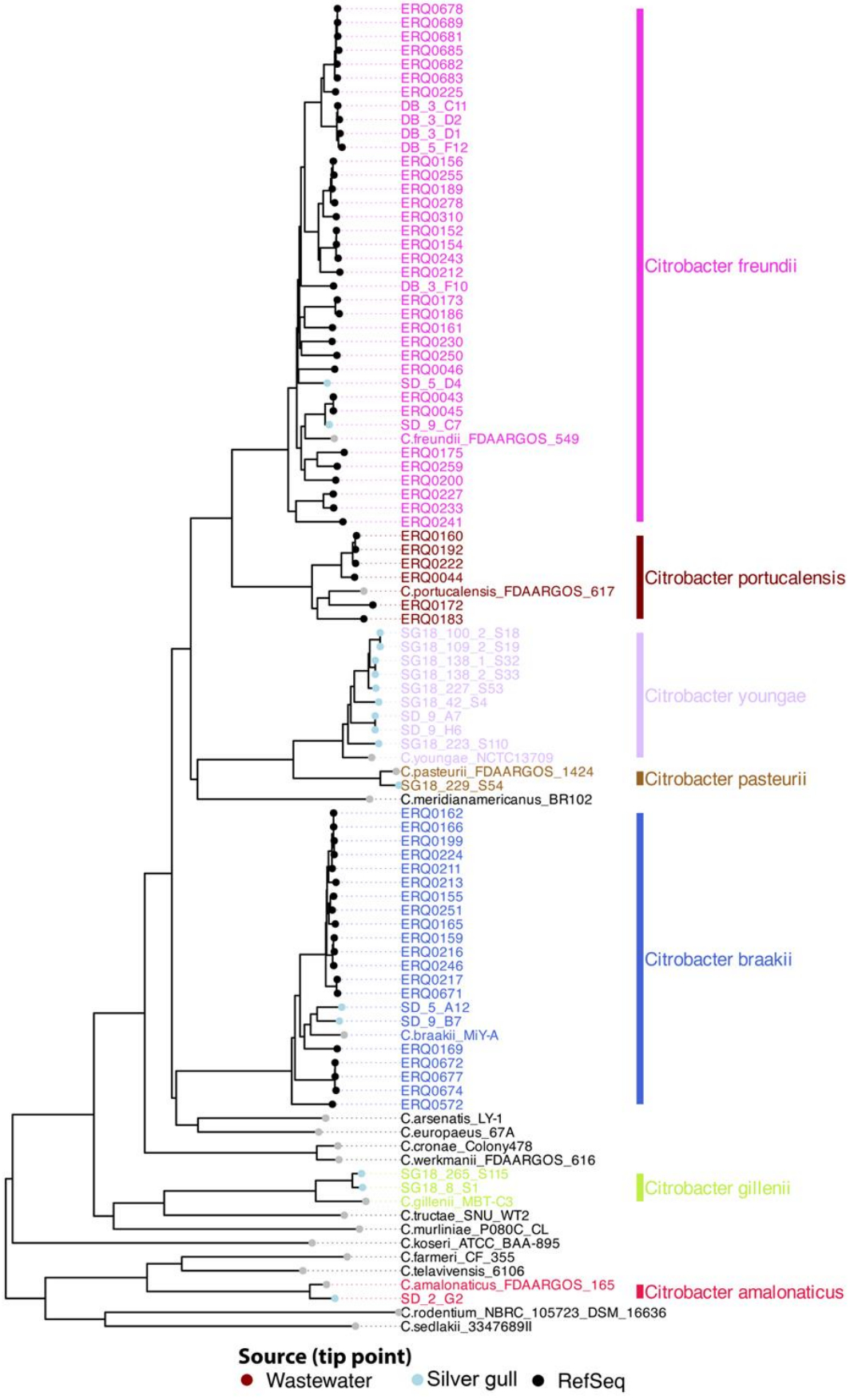
ERQ0678
ERQ0689
ERQ0681
ERQ0685
ERQ0682
ERQ0683
ERQ0225
DB_3_C11
DB_3_D2
DB_3_D1
DB_5_F12
ERQ0156
ERQ0255
ERQ0189
ERQ0278
ERQ0310
ERQ0152
ERQ0154
ERQ0243
ERQ0212
DB_3_F10
ERQ0173
ERQ0186
ERQ0161
ERQ0230
ERQ0250
ERQ0046
SD_5_D4
ERQ0043
ERQ0045
SD_9_C7
C.freundii_FDAARGOS_549
ERQ0175
ERQ0259
ERQ0200
ERQ0227
ERQ0233
ERQ0241
ERQ0160
ERQ0192
ERQ0222
ERQ0044
C.portucalensis_FDAARGOS_617
ERQ0172
ERQ0183
SG18_100_2_S18
SG18_109_2_S19
SG18_138_1_S32
SG18_138_2_S33
SG18_227_S53
SG18_42_S4
SD_9_A7
SD_9_H6
SG18_223_S110
C.youngae_NCTC13709
C.pasteurii_FDAARGOS_1424
SG18_229_S54
C.meridianamericanus_BR102
ERQ0162
ERQ0166
ERQ0199
ERQ0224
ERQ0211
ERQ0213
ERQ0155
ERQ0251
ERQ0165
ERQ0159
ERQ0216
ERQ0246
ERQ0217
ERQ0671
SD_5_A12
SD_9_B7
C.braakii_MiY-A
ERQ0169
ERQ0672
ERQ0677
ERQ0674
ERQ0572
C.arsenatis_LY-1
C.europaeus_67A
C.cronae_Colony478
C.werkmanii_FDAARGOS_616
SG18_265_S115
SG18_8_S1
C.gillenii_MBT-C3
C.tructae_SNU_WT2
C.murliniae_P080C_CL
C.koseri_ATCC_BAA-895
C.farmeri_CF_355
C.telavivensis_6106
C.amalonaticus_FDAARGOS_165
SD_2_G2
C.rodentium_NBRC_105723_DSM_16636
C.sedlakii_3347689II
Citrobacter freundii
Citrobacter portucalensis
Citrobacter youngae
Citrobacter pasteurii
Citrobacter braakii
Citrobacter gillenii
Citrobacter amalonaticus
Source (tip point)
Wastewater
Silver gull
RefSeq

Figure 23: A phylogeny of 96 Citrobacter isolates generated using mashtree. The tree, which is midpoint rooted, comprises 77 Australian environmental Citrobacter genomes and 19 representative type strain genomes sourced from RefSeq (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/Citrobacter). Clades comprised of Citrobacter species from the internal study are labelled and coloured species-wise, with the origin of a given genome indicated on the tip point. Species which were not identified within the gull or wastewater sourced Citrobacter spp. are shown in black font with their species identities and strain names shown as tip labels.

Table 6: *Citrobacter* spp. summary for the Australian wastewater and silver gull collection (n=77).

Species	Silver gull (n=17)	Wastewater (SA) (n=55)	Wastewater (NSW) (n=5)	Total (n=77)
C. amalonaticus	1 (5.9%)	-	-	1 (1.3%)
C. braakii	2 (11.8%)	19 (34.5%)	-	21 (27.3%)
C. freundii	2 (11.8%)	30 (54.5%)	5 (100%)	37 (48.1%)
C. gillenii	2 (11.8%)	-	-	2 (2.6%)
C. pasteurii	1 (5.9%)	-	-	1 (1.3%)
C. portucalensis	-	6 (10.9%)	-	6 (7.8%)
C. youngae	9 (52.9%)	-	-	9 (11.7%)

6.5.2 Resistance traits, ARG and MGE carriage

In the Australian *Citrobacter* spp., the most common plasmid replicon was IncFII (n = 58/77), followed by IncFI (n = 35/77), IncX5 (n = 17/77), IncP6 (n = 4/77), IncM2 (n = 4/77) and IncY3 (n = 3/77) (Figure 24). Replicon typing of the F plasmids revealed that various combinations of the FIIY_3 and FIB_70 alleles were most prominent in both *C. freundii* and *C. braakii*, with *C. braakii* carrying Y3:A-:B- (n = 3/77) and F-:A-:B70 (n = 9/77) subtype plasmids, while *C. freundii* carried Y3:A-:B70 (n = 5/77), Y3:A-:B- (n = 2/77), Y3:A10:B70 (n = 1/77) and a single K14:A-:B- plasmids. A wide variety of insertion sequences were identified across the *Citrobacter* spp., totalling 92 different elements. IS*26*, a key mobilisation element involved in the construction and transfer of complex AMR regions, was found in 34 isolates, including *C. braakii* (n=15), *C. freundii* (n=14) and *C. portucalensis* (n=5) (Figure 24). Similarly, IS*Ecp1* was identified in 4 isolates. The class 1 integron integrase *intI1* (n = 18/77) was found in *C. braakii* (n = 9/77), *C. freundii* (n = 8/77) and *C. portucalensis* (n=1). A single *C. freundii* isolate from wastewater carried the class 2 integrase gene *intI2* (Figure 24). All isolates encoding *intI1* in our study were

from wastewater, and it was observed that wastewater isolates were rich in ARGs irrespective of their species (again, note CRE selective media were used for wastewater isolates, while non-selective media were used for silver gull isolates).

In total, 79 ARGs were identified throughout the collection conferring resistance to multiple classes of antibiotics (Figure 24), with critically important antibiotic (CIA) resistance genes observed in 74/77 (96%) isolates and 26/77 (34%) isolates were MDR. On average, isolates sourced from Silver gulls carried genotypic resistance to one class of antibiotic, most commonly extended-spectrum β-lactams (13/17; 76%). By contrast, isolates sourced from wastewater carried ARGs conferring resistance to an average of three classes of antibiotics (most commonly extended spectrum β-lactams [100%], aminoglycosides [23/60; 38%] and sulphonamides [23/60; 38%]). Note that significance tests were not performed due to strain selection methodology between collections involving antibiotics. One *C. portucalensis* isolate (ST264; ERQ0172) from SA wastewater, carried ARGs conferring resistance to nine classes of antibiotics (both 21 ARGs in total; Figure 24). The ST264 *C. portucalensis* isolate carried more than 3-fold more ARGs than the other five *C. portucalensis* isolates (ST1107, ST1096 and ST166) in this collection (21 vs average 6). Additionally, three ST1110 *C. braakii* isolates (ERQ672, ERQ0677, ERQ0674) carried genotypic resistance to eight classes of antibiotics (both 20 ARGs in total; Figure 24), suggesting that ST1110 may constitute a novel MDR lineage identified in this study.

The overall high rate of genotypic extended-spectrum β-lactam resistance in both Silver gull and wastewater isolates was attributed to the presence of *bla*$_{CMY}$ genes in 96% of the collection. We identified 16 *bla*$_{CMY}$ variants and these appeared to be species associated. For example, *bla*$_{CMY-101}$ was only found in *C. braakii* (17/21; 81%), *bla*$_{CMY-48}$ only in *C. freundii* (18/37; 49%) and *bla*$_{CMY-157}$ only in *C. youngae* (n=9; 100%). *Citrobacter* spp. are known to carry chromosomal *bla*$_{CMY}$ genes and the association different *bla*$_{CMY}$ alleles with different *Citrobacter* spp. was a notable observation. However, we noted that the aforementioned three novel ST1110 *C. braakii* isolates from SA carried two *bla*$_{CMY}$ genes – *bla*$_{CMY-83}$ and *bla*$_{CMY-2}$. The former was found to be chromosomal, but the latter was located on identical 53,366 bp contigs in each isolate, adjacent to IS*Ecp1* and proximal to a plasmid transfer region (*tra* genes). Furthermore, the contigs had ≥ 99.98% identity and 99% coverage to 96 IncC plasmid sequences (average length 174308 bp) from a range of *Enterobacterales* deposited to NCBI (most commonly *Salmonella enterica subsp.* [n=30], and *Klebsiella pneumoniae* [n=25]). These *C. braakii* isolates were three of four isolates identified as carrying IncC plasmid replicons in this collection

(Figure 24). Additionally, all three members of this sequence type carried multiple copies of *bla*$_{CMY}$, suggesting they may carry both chromosomal and plasmid-mediated AmpC β-lactamases. A single *C. freundii* ST481 from wastewater was also observed to carry both a chromosomal and an IS*Ecp1*-associated *bla*$_{CMY}$, although a contig break in the beta-lactamase sequence prevented both allele and context identification.

Other CIA resistance genes identified in this collection were three carbapenemases (*bla*$_{IMP-4}$, *bla*$_{NDM-1}$ and *bla*$_{KPC-2}$), one ESBL gene (*bla*$_{SHV-12}$), and one colistin resistance gene (*mcr-9*), all of which were identified in isolates from wastewater. The *bla*$_{IMP-4}$ and *bla*$_{KPC-2}$ genes were identified in four novel ST1105 *C. freundii* isolates (DB_3_C11, DB_3_D1, DB_3_D2 and DB_5_F12) sourced from NSW wastewater, all four of which carried *intI1*. The singular instances of *bla*$_{NDM-1}$ and *bla*$_{SHV-12}$ were identified in the previously mentioned highly resistant ST264 *C. portucalensis* isolate (ERQ0172), as was *mcr-9*. The *mcr-9* gene was also identified in *C. freundii* isolate (DB_3_F10; ST indeterminable due to missing *arcA* allele) sourced from NSW wastewater. A section on a preliminary investigation into the genetic context of these CIA resistance genes is provided below.

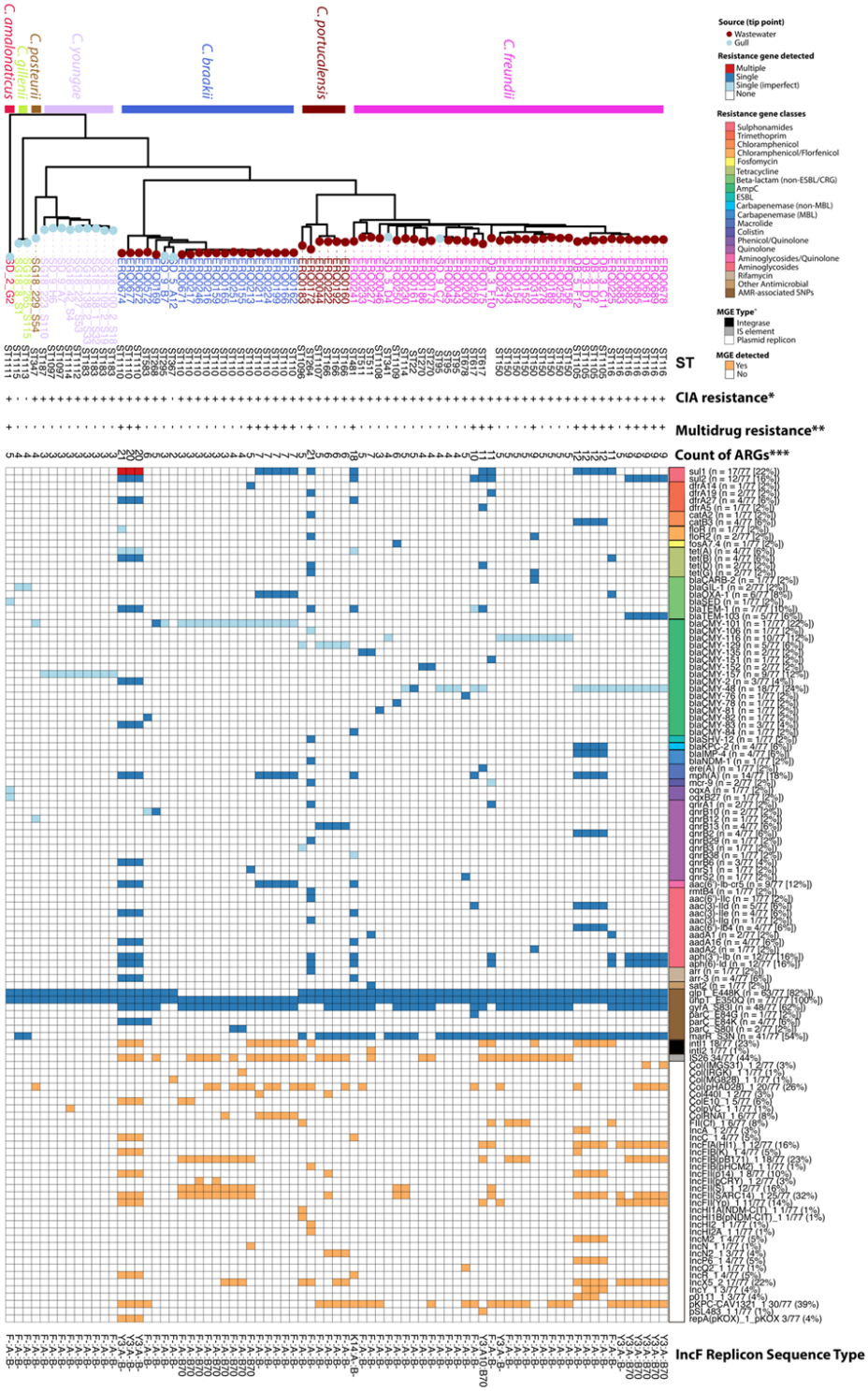

Figure 24: A phylogeny of the 77 genomes under investigation, generated using mashtree. The tree is midpoint-rooted, tip labels are coloured based on the species identity and the tip points are coloured based on the source from which the sample originates. The panel below shows carriage of antibiotic resistance genes and mobile

genetic elements. Coloured boxes shown beside gene names indicate the antimicrobial resistance class (or combination thereof) or mobile genetic element (MGE) type. CRG – Carbapenemase resistance gene, * - Carriage of genes conferring resistance to critically important antimicrobials (see methods), ** - Carriage of genes conferring resistance to three or more classes of antibiotics (see methods), *** - Count of antibiotic resistance genes (also includes AMR-associated SNPs), ^ - *intI1*/*intI2* included, though not strictly MGEs.6.5.3 Preliminary analysis of CIA resistance gene context in *Citrobacter spp.*

The *bla*$_{IMP-4}$ gene, carried by four novel ST23 *C. freundii* wastewater isolates, was situated within a class 1 integron structure: ΔIS*26*-*intI1*-*bla*$_{IMP-4}$ (carbapenem resistance)-*qacG2* (detergent resistance)-*acc(6')-Ib4* (gentamicin resistance)-*catB3* (chloramphenicol resistance)-*sul1* (sulfonamide resistance). The contigs carrying this class 1 integron exhibited high quality alignments to the *bla*$_{IMP-4}$-encoding IncM2 plasmid pEI1573 (NC_019368.1) first described in a clinical *Enterobacter cloacae* from an Australian hospital in 2012 [464].

The four *bla*$_{IMP-4}$ carrying *C. freundii* were the only isolates in this collection to carry IncM2 plasmid replicons. Plasmid alignments demonstrated that a pEI1573-like plasmid is present in these *C. freundii* isolates and is likely the genetic context for the *bla*$_{IMP-4}$ carrying class 1 integron structure as well as many of the other mobilised ARGs identified in these isolates (Figure 25A). Fourteen other plasmid sequences from GenBank, predominantly (13/14) from Australian clinical and environmental samples (*C. freundii* = 2*, Enterobacter* spp. = 5, *Klebsiella* spp. = 4, and one each from *Serratia marcescens, Leclercia adecarboxylata* and *E. coli*) were similarly identified as being highly related to pE11573 (> 90% coverage and 100% identity). Details on isolation and AMR carriage of these plasmids is presented in Supplemental Table 3. The same four novel ST23 *C. freundii* isolates also carried *bla*$_{KPC-2}$. The contigs containing this carbapenemase gene had the highest match score (>99% coverage at >99.99% identity) to an IncP6 plasmid pKPC2_045523 (CP032895.1) from *Enterobacter kobei* sourced from a human sample in China, 2017. Twenty-four highly similar plasmid sequences (>93% coverage, >99.8% identity) to pKPC2_045523 were identified, including those originating from *E. coli* (n=3), *E. cloacae* (n=4), *C. freundii* (n=3), and *Klebsiella spp.* (n=11) [Appendix 20]. In addition to carrying IncM2 plasmid replicons, the four *C. freundii* in question were also the only isolates in this collection to carry IncP6 replicons and plasmid alignments indicate the presence of a plasmid similar to pKPC_045523 (Figure 25B).

This data suggests the capture and simultaneous stable acquisition of these two important AMR-encoding plasmids by these related *C. freundii* isolates has occurred and that IncM2 plasmids are distributed widely in *Enterobacteriaceae* isolates from wastewater.

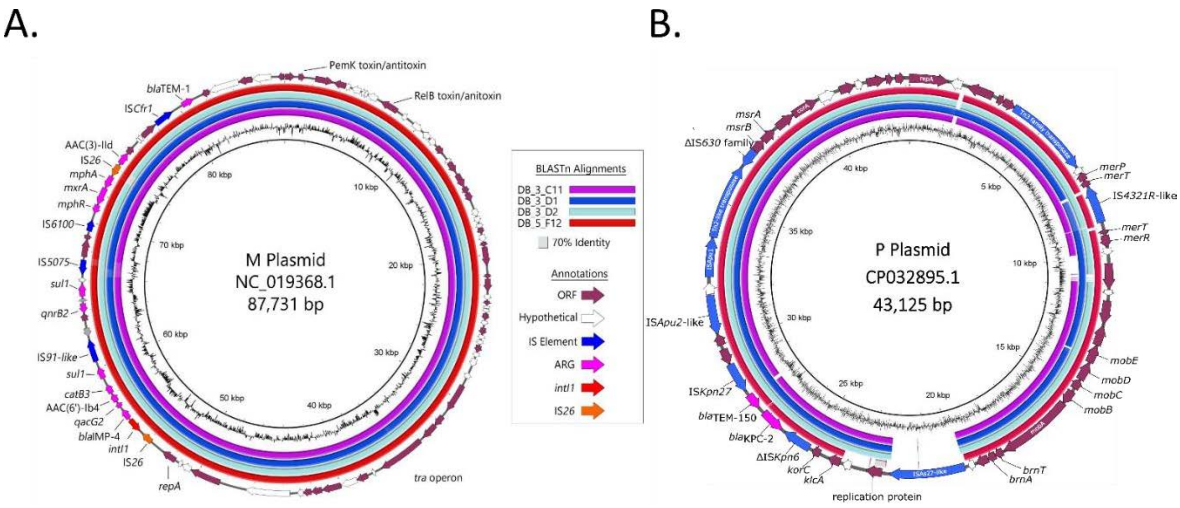

Figure 25: Putative AMR plasmids present in four ST1105 C. freundii isolates from NSW wastewater. A) Whole genome sequence alignments to reference E. cloacae IncM plasmid NC_019368.1 (pEI1573) encoding blaIMP-4. B) Whole genome sequence alignments to reference E. kobei IncP6 plasmid CP032895.1 (pKPC_045523) encoding blaKPC-2. Both A) and B) demonstrate high nucleotide sequence homology across all four ST1105 isolates to the two reference plasmids.

It was not possible to precisely ascertain the genetic context of the CIA resistance genes carried by the MDR isolate *C. portucalensis* ERQ0172 from SA wastewater as the contigs harbouring them were small (2845 bp for $bla_{\text{NDM-1}}$, 4679 bp for $bla_{\text{SHV-12}}$, and 2625 bp for *mcr-9*). Nevertheless, investigations into the context of $bla_{\text{NDM-1}}$ did allow for the identification of similar structures (100% BLASTn coverage and identity) observed as chromosomal insertions in several *P. aeruginosa* sequences, including strain JUNP13 (LC635759.1) from a clinical sample in Nepal, 2021; strain PSE6684 (CP053917.1) from a human urine sample in Korea, 2019 and strain PA790 (CP075176.1) from a human urine sample in India, 2019.

The other *mcr-9* gene, identified in a novel ST202 *C. freundii* isolate, was located on a 26,144 bp contig (contig00075) and found to be associated with IS*903B*. The *mcr-9*-carrying contig also contained arsenic and copper resistance genes and most of the contig (24,016 bp) represented a conserved region identified in several *Enterobacteriaceae* chromosomes as well as in IncHI2 plasmids (selection presented in Figure 26A). Notably, our comparative analysis suggests that the conserved region is

often adjacent to additional ARGs and/or metal resistance genes. The novel ST202 *C. freundii* isolate does not carry an IncHI2 plasmid replicon, suggesting a chromosomal *mcr-9* locale; however, the other *mcr-9*-carrying *C. portucalensis* isolate was the only isolate to carry an IncHI2 plasmid replicon (IncHI2:ST1) in this collection and plasmid alignments to the three IncHI2 plasmid examples provided in Figure 26 (also all IncHI2:ST1), indicated the presence of much of the same gene cargo (Figure 26B). Future long-read sequencing is needed to confirm these initial observations.

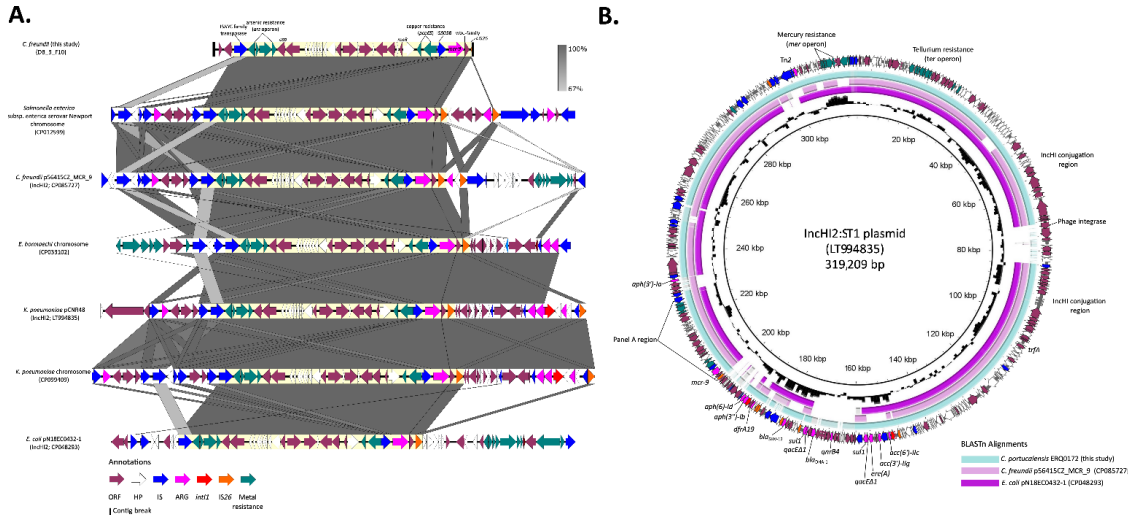

Figure 26: Genetic context of *mcr-9.* A) Comparison of contig carrying *mcr-9* in *C. freundeii* wastewater isolate to a selection of *Enterobacteriaceae* chromosomes and IncHI2:ST1 plasmids. Conserved region highlighted in yellow. B) Plasmid alignments to *mcr-9*-carrying *K. pneumoniae* IncHI2:ST1 plasmid (pCNR48), including *C. portucalensis* wastewater isolate from this study.

6.5.4 Putative virulence traits

Using the virulence factor database (VFDB), we identified a total of 12 virulence-associated genes (VAGs) among the 77 isolates under investigation (Appendix 22) and noted some species exclusivity (Appendix 22). For example, four *C. freundii* strains ST150 from SA wastewater carried *astA*. We noted only *C. braakii* carried the most VAGs of any species under study, including Vi polysaccharide encoding operon *vex*ABCDE, found in 16/21 isolates (21%); *tivE* (21%, n=16) and *tivD* (19%, n=15); and *mekA* and *mrkB* (genes for degradation of short chain alkyl methyl ketones in *Pseudomonas veronii* MEK700) (6%, n=5 for both).

6.5.5 Investigation of potential One Health linkages

We analysed 970 publicly available *Citrobacter* spp. genomes to explore if STs we identified in our environmental isolates were restricted regarding their source or geographical associations. These additional *Citrobacter* genomes were collected across

28 countries over 37 years and were predominantly of human origin (446/970) but also included samples from a breadth of other hosts and environments including wild and domesticated animals, wastewater and more (Appendix 21 and Appendix 18).

Among the publicly available genomes, the most closely related to the Silver gull isolates were *C. freundii* ST95. While source agnostic phylogenetic distances among *C. freundii* ST95 varied greatly (range 13-1877 SNPs) the minimum pairwise SNP distance between Silver gull-sourced *C. freundii* ST95 and ST95 isolates from any other source was 474 SNPs (GCF_013425705, human, 2016). Similarly, we performed pairwise SNP analysis on the wastewater genomes from the study collection and their closest relatives from the RefSeq collection. Among these genomes the most closely related cross-source pairs were those of *C. freundii* ST110 (range 161-1,563 SNPs) (Figure 27), including a strain from wastewater in the United Kingdom which showed between 161 and 200 SNPs with five strains collected from South Australian wastewater (while supplementary SNP analysis using a reference-based approach identified 148-208 SNPs between these strains), despite these strains being collected approximately 13,000km apart.

Overall, internal isolates were not found to cluster with isolates from public databases below the defined threshold of 100 SNPs. Therefore, while strains relatively closely related to both Silver gull and wastewater sourced *Citrobacter* spp. were identified, evidence indicates that these transmissions are unlikely to have occurred over epidemiologically relevant timelines.

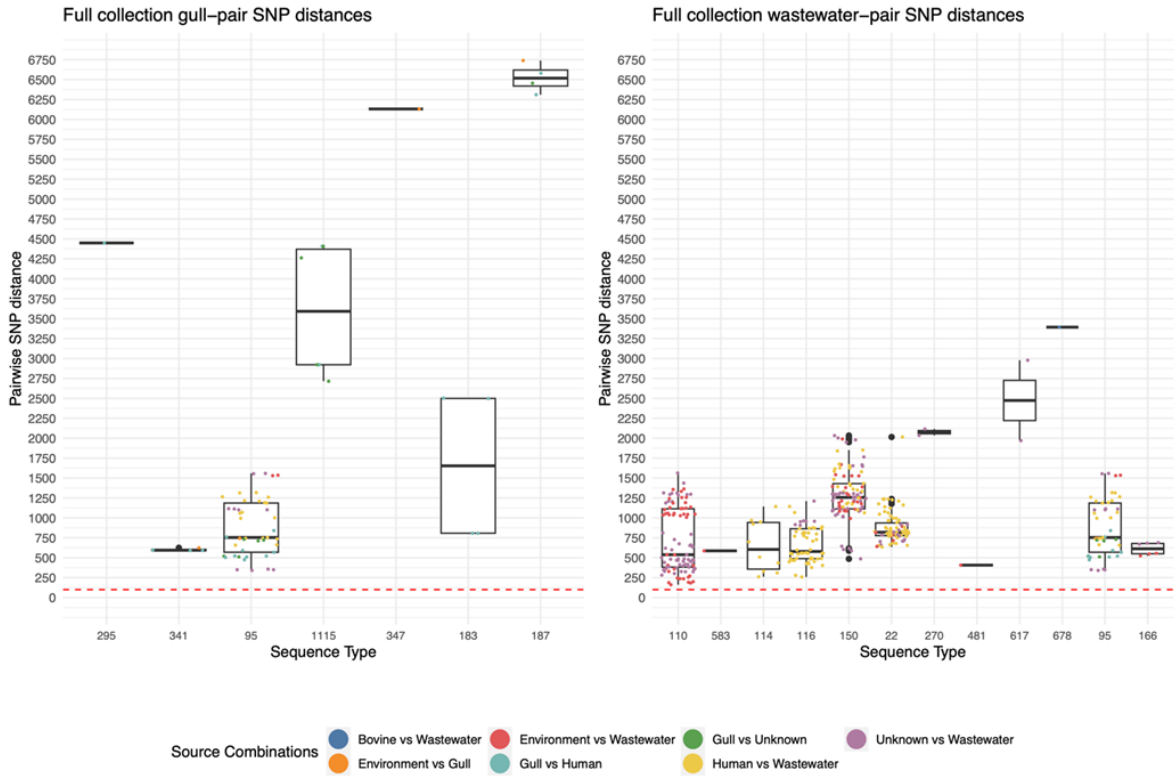

Figure 27: Pairwise phylogenetic distances between A) Silver gull-sourced strains, and B) wastewater-sourced strains, and members of the same sequence type. Each point indicates a pair of strains which is coloured by the combination of their source attributions, with the y coordinates indicating the pairwise SNP distance between these strains and the position on the x-axis indicating the ST to which the pair belongs. Note that data points are jittered (spread slightly across the x-axis within each sequence type) to prevent overplotting of data. Note also that only pairs containing at least one strain from the study collection were included.

6.6 Discussion

Antimicrobial resistance (AMR) is a major public health concern, and environmental Gram-negative bacteria have been recognised as important reservoirs of clinically important antibiotic resistance genes [10,420,466]. *Citrobacter* is a common environmental bacterium and an opportunistic pathogen in humans and other animals, but little is known about the diversity and AMR potential of this genus in environmental isolates. In this study, we performed WGS on 77 *Citrobacter* isolates obtained from wastewater samples and Australian silver gull (which are known to frequent wastewater holding lagoons) and made several key observations: i) our phylogenetic analyses revealed a diverse *Citrobacter* population, with seven different species and 33 STs, 16 (48%) of which were novel; ii) the most common species in wastewater were *C. freundii* and *C. braakii*, while in Silver gulls, the most common species was *C. youngae*; iii)

wastewater isolates carried a heavy burden of ARGs; iv) several novel MDR lineages, including *C. braakii* novel ST218 which carried ARGs conferring resistance to eight to nine classes of antibiotics, and *C. freundii* novel ST23, which carried two carbapenemase genes, bla_{IMP-4} and bla_{KPC-2} were identified v) a MDR *C. porticalensis* isolate carrying bla_{NDM-1}, bla_{SHV-12} and *mcr-9* was identified and; vi) IncC, IncM2 and IncP6 are the likely vectors for most of the critically important mobilised ARGs. Our findings provide valuable insights into the diversity and AMR potential of *Citrobacter* in environmental samples.

Synanthropic birds, such as gulls, have been shown to carry AMR bacteria, with WGS revealing transmission chains linking humans, animals, and the environment (Wang et al., 2017; Wu et al., 2018; Dolejska and Literak, 2019; Cummins et al., 2020; Nesporova et al., 2020; Tarabai et al., 2021b; Wyrsch et al., 2022). Gulls, especially species feeding from anthropogenic sources, including wastewater, are regularly reported carrying ARGs of clinical importance for human and livestock [470–472]. Therefore, comparing *Citrobacter* isolated from wastewater and gull samples can provide valuable information on the transmission of resistant bacteria between different environmental compartments and potential risks to public health. Previous gull studies have mainly identified MDR *E. coli* isolates [457,473]. However, in the vast majority of these earlier studies, MDR isolates were cultured from cloacal swabs on antibiotic selective media. Here, our *Citrobacter* isolates from Silver gulls were recovered on non-selective media. We found that *Citrobacter* isolates from Silver gulls typically carried resistance due to chromosomal carriage of bla_{CMY} genes. While we found little evidence of other mobile resistance genes among these isolates, greater sampling of may shed light on the propensity of Silver gulls to carry MDR *Citrobacter* spp or those carrying genes conferring resistance to CIAs.

We did not detect any close relationships (≤100 SNPs) between Silver gull isolates and wastewater isolates, nor with isolates from either of these collections and the 970 *Citrobacter* genomes downloaded from public databases. While our study provides some insight into the relative low frequency of direct and indirect transmissions between gulls and wastewater and other sources, deep sampling of these microbial communities is required to elucidate any potential linkages between them. We did, however, identify strains of *C. freundii* collected from wastewater in the United Kingdom and in South Australia (from the present study) which shared a ST (ST110) differed by as few as 161 SNPs. This is unlikely to indicate a transmission pathway linking these two wastewater sources, however, and more likely to indicate the co-presence of this

bacterial lineage in human populations from which waste materials enter both such wastewater infrastructure. Note that while we were relatively conservative in our SNP distance thresholds, more research is needed to increase the understanding of SNP transmission thresholds in a One Health context, as most thresholds are designed to detect direct transmission events in epidemiologically relevant timelines (i.e. days or weeks).

While *Citrobacter* from Silver gulls had little to no mobilised ARG carriage, from wastewater we isolated three *Citrobacter* species – *C. braakii*, *C. freundii* and *C. portucalensis* - with considerable ARG carriage linked to specific plasmids. The former two species were also identified in Silver gulls, but with notably fewer ARGs. Of particular interest, in wastewater we identified a novel *C. freundii* MDR lineage, novel ST23, that carried two different classes of carbapenemase genes, *bla*$_{IMP-4}$ (class B) and *bla*$_{KPC-2}$ (class A). The *bla*$_{IMP-4}$ gene was situated within a class 1 integron structure (ΔIS*26-intI1-bla*$_{IMP-4}$*-qacG2-aacA4-catB3-sul1*). Plasmid replicon screening and alignments identified an IncM2 plasmid similar to pEI1573 as the likely vector for this integron. In Australia, *bla*$_{IMP-4}$ was first detected in Melbourne and Sydney in 2002 and has remained the most reported variant of *bla*$_{IMP}$ [474–477]. Consistent with our observation, *bla*$_{IMP-4}$ is most commonly present in a four-gene cassette integron array (In809): *bla*$_{IMP-4}$*-qacG-aacA4-catB3*, which is found widely disseminated across Australia and Asia [475,478,479]. This integron structure is most frequently found on IncHI2 and IncM2 plasmids in clinical settings [477,480] but among a broad collection of plasmid replicon types in gulls (Dolejska et al., 2016b; Tarabai et al., 2021).

IncM plasmids are an emerging threat due to their broad host range and increasing prevalence in MDR clinical, animal, and environmental isolates [128,129]. The IncM2 plasmid pEI1573 (similar to that found in our novel MDR *C. freundii* lineage) was first described in an Australian clinical *bla*$_{IMP-4}$ producing *Enterobacter cloacae* isolate in 2012 [482] and subsequently similar plasmids have been found in CPE (Carbapenemase-producing *Enterobacterales*) from Australian clinical and animal isolates [464]. While *Enterobacter* species are the predominant CPE in Australia [482], *bla*$_{IMP-4}$ producing *Citrobacter* isolates carrying IncM2 replicons have also been described in Australia [479]. Interestingly, in 2006, *bla*$_{IMP-4}$ was first identified in an Australian burns unit [476] in a patient known to be colonized with *bla*$_{IMP-4}$ *E. cloacae*. For approximately 10 years post this initial identification, *bla*$_{IMP-4}$ *Enterobacteriaceae*, most commonly *C. freundii* and *E. cloacae*, was consistently isolated from environmental wet area sampling

(e.g., sinks) from the same hospital despite targeted disinfection [483]. Indeed, these organisms were isolated in greater numbers post cleaning.

The authors hypothesised that the disruption of biofilms during cleaning underpinned this observation. However, it also highlights that multispecies biofilms could play a major role in the persistent exchange of promiscuous AMR plasmids across species, such as pEI1573, and provide a link between clinical and environmental reservoirs of AMR.

The novel *C. freundii* novel ST23 MDR lineage also carried bla_{KPC-2} which we linked to a IncP6 plasmid similar to pKPC2_045523 from *E. kobei* sourced from a human sample in China, 2017 (Accession: NZ_CP032895.1, unpublished). IncP plasmids are another broad host range plasmid type, known to carry genes involved in the degradation of xenobiotics and are commonly found in isolates from polluted environments [458,484]. IncP6 plasmids carrying bla_{KPC-2} have been previously described in clinical *C. freundii* [485] and *C. braakii* [486] isolates as well as in other species from sewage including *Pseudomonas*, *Klebsiella* and *Enterobacter* spp.[485,487]. *C. freundii* isolates have been reported carrying multiple bla_{KPC-2}-encoding plasmids [60] and both bla_{KPC-2} and bla_{NDM-1}[488,489]. Here we described a novel *C. freundii* lineage carrying both bla_{KPC-2} and bla_{IMP-4} on two broad host range plasmids highlighting the need for continued AMR surveillance in the environment and increased efforts to mitigate the spread of ARGs between bacteria.

Chromosomal AmpC β-lactamases (e.g., bla_{CMY} genes) are common in *Citrobacter* thereby complicating treatment for clinical isolates. We found bla_{CMY} genes in 96% of all our *Citrobacter* isolates and these were comprised of 16 variants that appeared restricted to species. The gene was only absent in *C. gillenii* and *C. amalonaticus* isolates, which were only identified in Silver gulls. The lack of bla_{CMY} genes in these two species has been described previously [490,491]. Notably, we identified a novel lineage of *C. braakii* – novel ST218 - that carried a chromosomal AmpC β-lactamase (bla_{CMY-83}) and a plasmid-mediated AmpC β-lactamase (bla_{CMY-2}). Chromosomal bla_{CMY} genes are usually regulated by the bacterial cell and expressed at low levels [492] Conversely, plasmid-borne bla_{CMY} genes are often associated with strong promoters (in this case IS*Ecp1*) and thus expressed at higher levels [493]. We mapped the location of bla_{CMY-2} in the *C. braakii* isolates to an IncC plasmid with ≥ 99.98% identity and 99% coverage to 96 IncC plasmid sequences deposited in NCBI. IncC plasmids have garnered much attention as prominent vectors for the dissemination of bla_{CMY} and

bla$_{NDM}$ genes across many *Enterobacteriaceae* [493]. In addition to *bla*$_{CMY,}$ *C. braakii* novel ST218 isolates carried class 1 integron integrases and ARGs conferring resistance to aminoglycosides, chloramphenicol, fluoroquinolones, fosfomycin, macrolides, sulphonamide, tetracyclines and trimethoprim. While there is limited knowledge of virulence determinants specific to *Citrobacter,* our *C. braakii* isolates carried *vex* genes (*vexABCDE*) known to encode the Vi capsule polysaccharide and provide higher potential for pathogenicity and adaption within humans [494–496]. Thus, we identified a novel lineage of *C. braakii* from wastewater with an extensive MDR profile and pathogenicity potential.

The third *Citrobacter* species identified in wastewater was *C. portucalensis*, a potentially underreported bacteria due to its repeated misidentification as *C. freundii* [61]. This species was only identified from NSW wastewater and the six representative isolates belonged to four STs (2 novel and 2 established). Five *C. portucalensis* isolates carried only two ARGs – *bla*$_{CMY-129}$ and *qnrB13* (quinolone resistance), however one ST264 isolate carried 21 ARGs conferring resistance to nine classes of antibiotics. The ST264 isolate, ERQ0172, was the only one to carry *bla*$_{NDM-1}$. NDM-positive bacteria are associated with high mortality rates [497] and there have been two previous reports of *C. portucalensis* strains carrying *bla*$_{NDM-1,}$ both human sourced from China [61,498]. This gene is most frequently carried by IncX3 plasmids and is generally associated with IS*Aba125* [497,499]. The contig on which *bla*$_{NDM-1}$ in ERQ0172 was situated was too small to determine its wider genetic context, however we did observe likely associations with resolved complex resistance structures, such as one from a clinical *Providencia rettgeri* isolate in China [500]. ERQ0172 did not harbour any IncX3 plasmid replicons, but it did possess an IncX5 replicon.

Future long-read sequencing is needed to determine to exact genetic context for *bla*$_{NDM-1}$ in this isolate. ERQ0172 was also one of two isolates that carried *mcr-9*, a contested colistin resistance gene [501]. While this gene is likely to only cause resistance under laboratory induced expression[502] , the gene was located on a highly conserved 24,016 bp region containing arsenic and copper resistance genes found in several *Enterobacteriaceae* chromosomes and IncHI2:ST1 plasmids, suggesting that this region is transferred horizontally, and that *mcr-9* may be co-selected by heavy metal resistance [92,503].

Our study has several limitations, namely that Silver gull isolates were not selected for any antibiotic phenotype while wastewater isolates were selected by growth

on medium containing antibiotics. This not only would have influenced ARG profiles but may also have biased the diversity of the *Citrobacter* wastewater population. Thus, comparisons between the two cohorts were constrained. Future studies utilising long-read sequence data would allow for more comprehensive comparisons of mobile genetic elements in these genomes, allowing for an enhanced understanding of the relevance of environmental *Citrobacter* species in the evolution and potential dissemination of AMR within a One Health context.

In conclusion, this study broadens our understanding on environmental *Citrobacter* populations and their AMR carriage, as well as the associations of mobile genetic elements with these resistance traits. Phylogenetic analyses identified many novel sequence types including three novel MDR lineages that carry carbapenemase-encoding genes and other clinically important resistance genes on broad host range plasmids. These findings emphasise the need for environmental AMR monitoring.

Chapter 7. General Discussion and Future Directions

This final chapter provides a summary of the major findings in relation to the thesis aims, and a discussion on the implications and contributions of this work to the epidemiological analysis of antimicrobial resistant bacteria using a One Health approach. Additionally, this chapter also addresses the limitations encountered during the study and proposes future directions, which could lead to further advancements in understanding and managing carbapenem resistance in environmental bacterial populations. It is important to highlight that although we applied conservative SNP distance thresholds, further research is necessary to enhance our comprehension of SNP transmission thresholds within a One Health context. Most existing thresholds are tailored to identify direct transmission events within epidemiologically relevant timelines, typically spanning days or weeks.

As previously mentioned, carbapenem resistance poses a severe and ongoing public health problem worldwide [504]. It is particularly prevalent among GNB in the Proteobacteria phylum [505]. The resistance can be intrinsic or mediated by horizontally transferred carbapenem encoding genes, facilitated by mobile genetic elements [354] such as plasmids (e.g. bla_{IMP-4} on an IncM plasmid and bla_{KPC-2} on an IncP plasmid, both in *Citrobacter freundii*) and GIs (as was the case for bla_{GES-5} in *Comamonas denitrificans*). Operons encoding for the metabolism of the heavy metals ("heavy metal resistance") copper and arsenic were observed on an IncP plasmid structure in *Comamonas denitrificans* also.

While the majority of AMR studies primarily centre around clinical isolates [10], it is important to note that numerous environmental bacteria exhibit innate resistance to carbapenems [506]. The improper use of antibiotics in human and veterinary medicine, as well as in agriculture, also plays a crucial role in the rise and dissemination of acquired carbapenem resistance among environmental bacteria [507].

Prior to this thesis, there was a lack of WGS data on carbapenem-resistant GNB from water bodies in Australia, particularly concerning opportunistic pathogens such as *Elizabethkingia*, *Comamonas*, and *Citrobacter* species. To address these gaps, the study utilised WGS data to investigate the genetic diversity of these species in water bodies, explore AMR transmission pathways, identify MGEs, and establish the genetic relationship between environmental and clinical carbapenem-resistant bacteria in

Australia. Consequently, this thesis offers valuable insights into the transmission dynamics of AMR in aquatic environments and its potential impact on human health.

7.1 Discussion of Aim 1: Genomic analysis of selected species

"Characterise the population structures of the three chosen bacterial genera through phylogenetic studies, genotyping, and single nucleotide polymorphism (SNP) analysis."

Carbapenem resistance poses a significant concern in healthcare settings by limiting treatment options [505]. However, many ARGs discovered in pathogens are known to have originated from the extensive and diverse genetic pool present in environmental bacteria [508]. Furthermore, metagenomic analyses have unveiled the exchange of ARGs between environmental and clinical bacteria [509,510]. Environmental MDR bacteria have been shown to harbor genes similar to those found in human pathogens and confer resistance to essential antibiotic categories such as aminoglycosides, amphenicols, β-lactams (including carbapenems), sulfonamides, and tetracycline [509,511]. Thus, understanding the diversity and distribution of carbapenem-resistant bacteria in the environment is essential to better manage and prevent their spread [354]. By integrating genotype data, metadata, and resistance phenotypes into phylogenetic analyses, we can gain insights into bacterial populations, identifying epidemiological links and gene acquisition trends, including ARGs [512].

This study, which focused on carbapenem-resistant isolates from water bodies, yielded some remarkable results. Out of 665 isolates initially sampled, we identified approximately 42 distinct genera. To cope with the dataset's vast species diversity, we employed a low-resolution phylogenetic analysis using PhyloSift [206]. This approach allowed us to visualize broader relationships between bacterial species, resulting in the identification of three major clades: *Flavobacteriales*, *Enterobacterales*, and *Pseudomonadales*. This broad overview is particularly crucial when dealing with a wide range of species simultaneously. Given the dataset's complexity, we strategically selected one representative genus from each Order (clade) for further analysis: *Elizabethkingia*, *Comamonas*, and *Citrobacter*. These genera were chosen based on their prevalence in the collection and their significance in global scientific literature (Table 7).

For an in-depth examination of the chosen genera, we utilised a variety of techniques for phylogenetic analysis, with a primary focus on generating trees through

SNV-derived alignments in specialized software. This method facilitated the identification of highly related isolates, potential discovery of novel species (see aim 4 below), and comprehensive assessment of the diversity present within each species.

Table 7: The three genera selected for in-depth genomic analysis.

	Elizabethkingia spp.	*Comamonas* spp.	*Citrobacter* spp.
Species	*E. miricola* (n=71), *E. anophelis* (n=16), *E. umeracha* sp. novel (n=7)	*C. denitrificans* (n=33), *C. testosteroni* (n=5), *C. aquatica* (n=1)	*C. freundii* (n=37), *C. braakii* (n=21), *C. youngae* (n=9), *C. potucalensis* (n=6), *C. gillenii* (n=2), *C. amanoliticus* (n=1), *C. pasteurii* (n=1)
Global context	Ubiquitous in the environment, particularly water.	Abundant in aquatic and soil environments, including wastewater.	Abundant in the environment and intestinal microbiota.
ARGs & MGEs	Naturally carbapenem-resistant (bla_{B}, bla_{GOB}) with novel ICE in 71% of aquatic environmental isolates.	Chromosomally acquired carbapenem resistance ($_{bla}$GES-5) and IncP1 plasmid carrying heavy metal resistance.	Plasmid-encoded carbapenem resistance (blaIMP-4 on IncM, blaKPC-2 on IncP).
Potential importance	Clinical pathogen, potential for petroleum bioremediation.	Clinical pathogen, potential in biotechnology.	Clinical pathogen, linked to MDR from other *Enterobacteriaceae*.

7.2 Discussion of Aim 2: Identifying critical genes and mobile elements

"Characterise antimicrobial resistance genes (ARGs) and virulence associated genes (VAGs), plasmid replicon sequence types and critical mobile genetic elements (MGEs) using bioinformatic methods to highlight any potential threat from environmental isolates in our study and compare these gene profiles to sequences in public databases."

A comprehensive analysis relating to this aim was conducted in each result chapter, yielding significant findings. Below is a concise summary of some of the major findings:

1. *Citrobacter* wastewater isolates carried multiple AMR genes, including bla_{IMP-4}, bla_{KPC-2}, *mcr-9*, on plasmids. IncM2, IncP, and IncHI2 plasmids were observed to disseminate multidrug resistance in *Citrobacter* spp. from Australian wastewater.
2. *Comamonas* spp. from various water sources, including wastewater influent, wetlands, and lakes, were found to acquire critical antibiotic resistance genes,

bla$_{GES-5}$, and *bla*$_{OXA}$, through genomic islands. Some *Comamonas* isolates also carried an IncP-1 plasmid encoding metal resistance.

3. *Elizabethkingia* spp. in aquatic environments exhibited natural resistance to carbapenems and cephalosporins, with the presence of *bla*$_{B}$, *bla*$_{GOB}$, and *bla*$_{CME}$ genes. Additionally, novel ICE elements were identified in 71.3% of the isolates, potentially contributing to genetic diversity in these bacteria.

Although reporting on ARGs and VAGs is common in genomic studies, this research aimed to go beyond that and establish links between ARGs, plasmid carriage and critical MGEs.

Significant findings from the study include the identification of various AMR genes, namely *bla*$_{IMP-4}$ and *bla*$_{KPC-2}$, in *Citrobacter* wastewater isolates. These genes were carried on IncM and IncP plasmids, respectively, which are known to play a key role in disseminating antibiotic resistance among bacterial populations [513] and have been associated with the spread of multidrug resistance in clinically important GNB such as *E. coli, K. aerogenes, Pseudomonas spp. and Sphingomonas* [514,515] responsible for various infections in humans [127]. The transfer of these plasmids has been observed across different genera [102] and in various clinical and environmental settings [516].

Interestingly, IncM plasmids are known to mobilise AMR within Australian clinical settings [517], and alignments of our isolates to a reference plasmid revealed a direct link between IncM plasmids in Australian environmental and clinical isolates. Additionally, the study identified the genetic context of the *mcr-9* gene in *C. freundii* from wastewater. The *mcr-9* gene was found associated with metal resistance genes and likely hosted on an HI2 ST1 plasmid along with other ARGs and a class 1 integron structure. HI2 ST1 plasmids have previously been identified as carriers of resistance genes conferring resistance to multiple classes of antibiotics [24] in clinically important GNB such as *E.coli*, *K. pneumoniae*, and *Enterobacter* spp. [518,519].

While *Comamonas* spp. are generally considered to have low virulence, they have been responsible for adverse health conditions in many healthy individuals and have even led to fatalities in patients with underlying medical conditions [74]. In the past, treating infections associated with this species was relatively manageable; however, the emergence of antibiotic resistance in certain strains raises concerns about future challenges. Notably*, C. kerstersii* strain 8943 was previously noted harboring various

resistance genes, such as *tetA* (gene encode for tetracycline resistance), *strA, strB (*streptomycin resistance*)*, *sul1*, *sul2* (sulfonamide resistance) *bla*$_{OXA-1}$ (class D beta-lactamase resistance), *catB3* (chloramphenicol resistance), and *floR* (florfenicol/chloramphenicol resistance). An isolate of *C. thiooxydans*, causing a urinary tract infection, was found to carry the *bla*$_{IMP–8}$ gene, conferring resistance to carbapenems, alongside a novel class D beta-lactamase gene, *bla*$_{OXA}$, and the *aac(6')-Ib-c* gene for aminoglycoside resistance. This isolate also exhibited a range of efflux pumps within its genome [55]. In 2022, another *C. thiooxydans* isolate was identified, carrying a plasmid-mediated *bla*$_{IMP–1}$ gene [82], further highlighting the evolving complexity of antibiotic resistance within this bacterial species.

Our *Comamonas* strains harboured chromosomally acquired genes *bla*$_{GES-5}$, *bla*$_{OXA}$, and aminoglycoside resistance (*aadA*), all situated on putative GIs. The GIs containing *bla*$_{GES-5}$ and *bla*$_{OXA}$ were found exclusively within this particular Australian collection of *C. denitrificans*. Our *C. denitrificans* carried an IncP-1 plasmid housing genes associated with xenobiotic degradation and responses to oxidative stress.

Some studies found that *Comamonas* strains possess plasmids linked to the degradation of xenobiotic aromatics [520,521]. Notably, plasmids such as pI2 and pTB30 enable the breakdown of 3-chloroaniline [522]. Another plasmid, pCNB1, aids the conversion of 4-chloronitrobenzene in *Comamonas* sp. strain CNB-1. A recently identified plasmid, pBHB [523], in *Comamonas* sp. strain 7D-2, was involved in breaking down the herbicide bromoxynil [524]. All these plasmids belong to the IncP-1β [522]. These findings underscore the significance of plasmids in supporting xenobiotic degradation pathways within *Comamonas* strains. These observations also raise the possibility that diverse environmental pollutants may inadvertently cross select for *Comamonas* spp. that carry antimicrobial resistance.

Similar to our findings in *Citrobacter* and *Comamonas*, the characterisation of ARGs in *Elizabethkingia* also revealed diverse genes conferring carbapenem resistance. Among the *Elizabethkingia* species studied (*E. anophelis*, *E. miricola*, and *E. umeracha* sp. novel), multiple known alleles and novel variants of *bla*$_{B}$, *bla*$_{GOB}$, and *bla*$_{CME}$ genes were identified. Our data revealed an essential pattern of resistance across all the tested isolates, with marked resistance demonstrated against carbapenems, cephalosporins, and diverse penicillins, encompassing carboxypenicillin and monobactam. The diversity of resistance profiles based on distinct combinations of *bla*$_{B}$, *bla*$_{GOB}$ and *bla*$_{CME}$ genes

underscores the intricate nature of resistance mechanisms. Notably, exclusive resistance to piperacillin/tazobactam was observed in our *E. miricola* isolates possessing the specific $bla_{\text{B-26-like}}$/$bla_{\text{GOB-19-like}}$/bla_{CME} combination. Additionally, the emergence of cefepime resistance in two *E. anophelis* isolates, characterized by distinct bla_{B} and bla_{GOB} variations along with the common $bla_{\text{CME-1}}$ gene, further exemplifies the complex interplay of genetic determinants influencing AMR patterns in these bacterial strains. These findings contribute to a deeper understanding of resistance dynamics and underscore the importance of continued surveillance and exploration of resistance mechanisms in clinical settings. Furthermore, the *Elizabethkingia* isolates harbouring these ARGs were discovered to contain ICEs, which are mobile genetic elements capable of integrating into a host genome and replicating during chromosomal replication and cell division [145,268].

However, the study also acknowledges limitations in the availability and accuracy of existing databases and suggests complementary approaches, such as long-read sequencing analysis, to enhance the reliability of MGE identification.

7.3 Discussion of Aim 3: Finding novel species

"Identify possible novel species or uncommon species from the environmental water sourced and contribute to public database."

This aim was explored across three result chapters. In Chapter 4, a novel species named *Elizabethkingia umeracha* was identified with unique metallo β-lactamase and extended-spectrum cephalosporin gene alleles, providing insights into antibiotic resistance mechanisms, and enhancing our understanding of genetic diversity within the *Elizabethkingia* genus. In Chapter 5, the addition of 33 newly sequenced *Comamonas denitrificans* isolates to the public database enriched our knowledge of this species' genetic diversity, offering valuable resources for ecological, evolutionary, and microbial genomics research. Finally, in Chapter 6, the discovery of multi-drug resistant *Citrobacter portucalensis,* a newly reclassified species, in wastewater samples raised concerns about accurate species identification and antibiotic resistance in environmental samples. Our data on novel species or uncommon species from the environmental water sources contribute to a better understanding of microbial diversity, antibiotic resistance transmission, and public health implications.

The microbial diversity observed in our water samples, with around 40 genera and numerous species, suggests a rich array of microorganisms in the sampled

environments. This diversity hints at a complex ecological network with potential interactions and dependencies among various organisms. Genomic analyses identified specific genera like *Elizabethkingia* spp., *Comamonas* spp., and *Citrobacter* spp., suggesting future research on the potential associations with mammalian or other hosts. That research may be needed to estimate specific host reservoirs. The varied sources, including wastewater, dams, and wetlands, indicate potential diverse environmental reservoirs. Understanding the ecological niches and conditions supporting these genera in different environments is crucial for estimating environmental reservoirs.

Addressing these challenges requires ongoing research, technological innovation, and comprehensive strategies to mitigate the spread of antibiotic resistance in both clinical and environmental contexts. Nevertheless, despite challenges, efforts to identify and contribute novel and rare species to public databases remain crucial for advancing scientific knowledge and biodiversity conservation.

7.4 Discussion of Aim 4: Comparisons to public data

"Compare the dataset generated by this project with WGS data accumulated in public database repositories, sourced from humans, swine, poultry, and wild bird populations, and used to examine how bacteria and the mobile genetic cargo they carry may move through these hosts."

Aim 4 was primarily addressed in Chapter 4 and 6. Chapter 4 focused on three collections comprising 94 *Elizabethkingia* isolates from Australian aquatic environments, 27 isolates from Australian clinical samples and hospital environments, and 27 international strains from GenBank. The isolates were derived from various sources, including the environment (n = 102), humans (n = 42), Anopheles gambiae (n = 2), and one isolate each from Zea mays (corn) and a frog. *Elizabethkingia* spp. are emerging pathogens primarily classified as environmental bacteria, with water bodies serving as one of their reservoirs [525]. The transmission of *Elizabethkingia* spp. is frequently associated with contaminated water sources [304]. Previous studies on the *Elizabethkingia* genus have predominantly focused on clinical isolates [254–256] and those obtained from hospital environments (Burnard et al., 2020). Therefore, it is crucial to identify closely related environmental *Elizabethkingia* strains to clinical isolates to better understand transmission and pathogenesis, especially given the limited knowledge regarding this intrinsically carbapenem-resistant bacterium [526].

As stated in the manuscript, our study revealed that *E. anophelis* isolates from wetland and dam samples showed a close genetic relationship to a group of Australian clinical *E. anophelis* strains causing bloodstream infections, with a variation of around 36 SNPs. Furthermore, they were about 42 SNPs from two *E. anophelis* isolates obtained from sinks in a Queensland hospital [72]. This finding of genetic relatedness of environmental and clinical isolates can help elucidate whether the environment serves as a reservoir for carbapenem resistance genes, contributing to the persistence and spread of drug-resistant strains. This information is crucial for guiding infection prevention and control strategies in healthcare settings and beyond. It also raises important questions about the origins and dissemination of drug resistance. Healthcare facilities also serve as hotspots for the acquisition and transmission of resistant strains, underscoring the need for improved infection control measures. Assessing the influence of environmental versus clinical evolution and dissemination will greatly assist in tracking the spread of pathogens.

Chapter 6 of the study focuses on the presence of the bla_{IMP-4} gene in *Citrobacter* isolates, carried on an IncM2 plasmid. This plasmid was first identified in 2012 within an *Enterobacter cloacae* isolate encoding bla_{IMP-4} [482]. The same plasmid has since been found in CPE from clinical and animal sources [464]. Notably, the historical lineage of bla_{IMP-4} dates back to 2006 when it was initially detected in an Australian burns unit [476], where a patient carried the bla_{IMP-4} gene within *E. cloacae.*

The enduring presence of bla_{IMP-4}-positive *Enterobacteriaceae* over the course of more than a decade within hospital environment samples is a pivotal observation. Despite dedicated disinfection protocols, these resistant bacterial strains, primarily identified as *C. freundii* and *E. cloacae*, have displayed remarkable persistence. This raises thought-provoking questions about the mechanisms underlying their ability to withstand and persist through disinfection processes [483]. The notion of persistent colonisation in specific settings, such as sinks and wet areas, suggests the possible existence of protected niches or biofilm communities where these resistant strains can thrive.

In retrospect, Chapter 4's limitation lies in the inclusion of only two sources at the national level: environmental isolates from South Australia and clinical isolates from Queensland. Despite this constraint, the study supplemented its analysis with global clinical data to explore genetic relationships. The research provides valuable insights

into the genomic attributes and connections between clinical and environmental *Elizabethkingia* isolates, underscoring water bodies as reservoirs and the importance of investigating transmission routes and mobile genetic elements. Further studies could focus on related environmental isolates from hospital settings in South Australia or natural environmental samples in Queensland to deepen our understanding.

The work presented in Chapter 6 suffers from the same limitations as Chapter 2 (e.g., unable to fully assemble MGEs from short-read data), so the process of incorporating long-read sequence data is suggested. This approach would enable more comprehensive comparisons of mobile genetic elements, especially plasmids encoding AMR. It has the potential to enhance the understanding of the significance of environmental *Citrobacter* species in the evolution and potential dissemination of antimicrobial resistance, particularly within the context of One Health.

7.5 Future perspectives and directions

As part of the OUTBREAK One Health surveillance initiative, the thesis involved collecting water samples from various sources, followed by culturing on carbapenem selective media. This process facilitated the isolation and whole genome sequencing (WGS) of 665 isolates, representing approximately 40 genera and numerous species. The thesis focused on in-depth genomic analyses of three key genera—*Elizabethkingia* spp., *Comamonas* spp., and *Citrobacter* spp.—selected for their prominence in the sample collection and global significance, as substantiated by a comprehensive literature review in Chapter 2.

Potential future work involves performing WGS on water samples from additional locations, including the other Australian states to begin with, to further explore carbapenem-resistant bacteria and the underlying mechanisms. Targeted exploration of these water-borne bacterial genera can enhance our understanding and management of antibiotic resistance spread, safeguarding water resources and public health [527].

Selecting isolates that encode key AMR genes and plasmid types for long-read sequencing, leading to the assembly and analysis of completed genomes, can provide a more comprehensive sequence assessment, advancing research on MGEs and antibiotic resistance spread [528].

Adopting a One Health approach emphasizes collaboration among relevant stakeholders and acknowledges that antibiotic resistance can spread between these

domains [43]. Future research should aim to highlight reservoirs of antibiotic resistance, helping with the implementation of antimicrobial stewardship programs to promote the responsible use of antibiotics in healthcare and agriculture. Reducing antibiotic usage can help minimize selection pressure for resistance.

Lastly, public health agencies should prioritise raising awareness about the significance of carbapenem resistance surveillance in environmental samples among healthcare professionals, the public, and environmental workers. This collective effort will contribute to better monitoring and control of the spread of antibiotic resistance, leading to improved public health outcomes.

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Appendixes

Appendix 1

Spreadsheet containing metadata of all isolates used in the study for Chapter 4.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc1.xlsx

Appendix 2

Spreadsheet containing assembly statistics of study isolates.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc2.xlsx

Appendix 3

Pairwise SNP matrices for *E. anophelis* and *E. miricola* isolates.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc4.xlsx

Appendix 4

New species identification by using 16S rRNA and *rpoB* sequence identities by ANIb, ANIm and GGDC.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc5.xlsx

Appendix 5

Gene presence/absence matrix generated by Roary.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc6.xlsx

Appendix 6

Scoary to calculate of genes present in *E. umeracha* sp. nov. isolates using a total of 1886 genes identified in the seven novel isolates.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc7.xlsx

Appendix 7

STRING identified functional enrichments of tryptophan biosynthesis, molybdenum and cofactor biosynthesis.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc8.xlsx

Appendix 8

MDS analysis on putative virulence factors identified this collection.

Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc9.xlsx

Appendix 9

AST data by MIC using EUCAST non-species and NCSI non-Enterobacteriaceae PK-PD breakpoints .

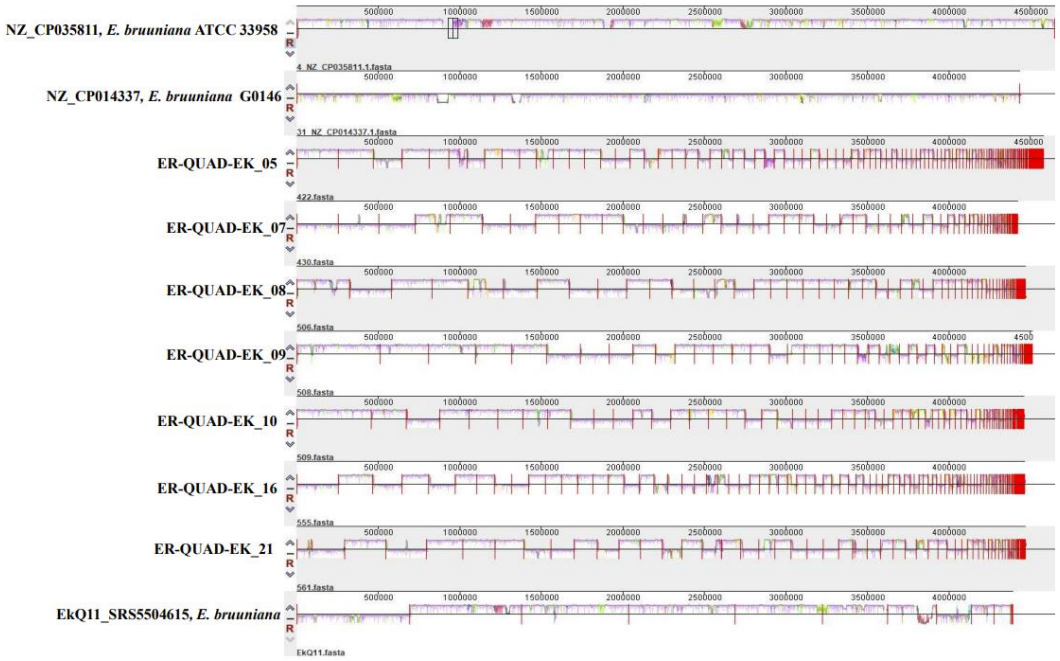
Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc12.xlsx

Appendix 10

Phage analysis on 94 *Elizabethkingia* genomes via Phaster.

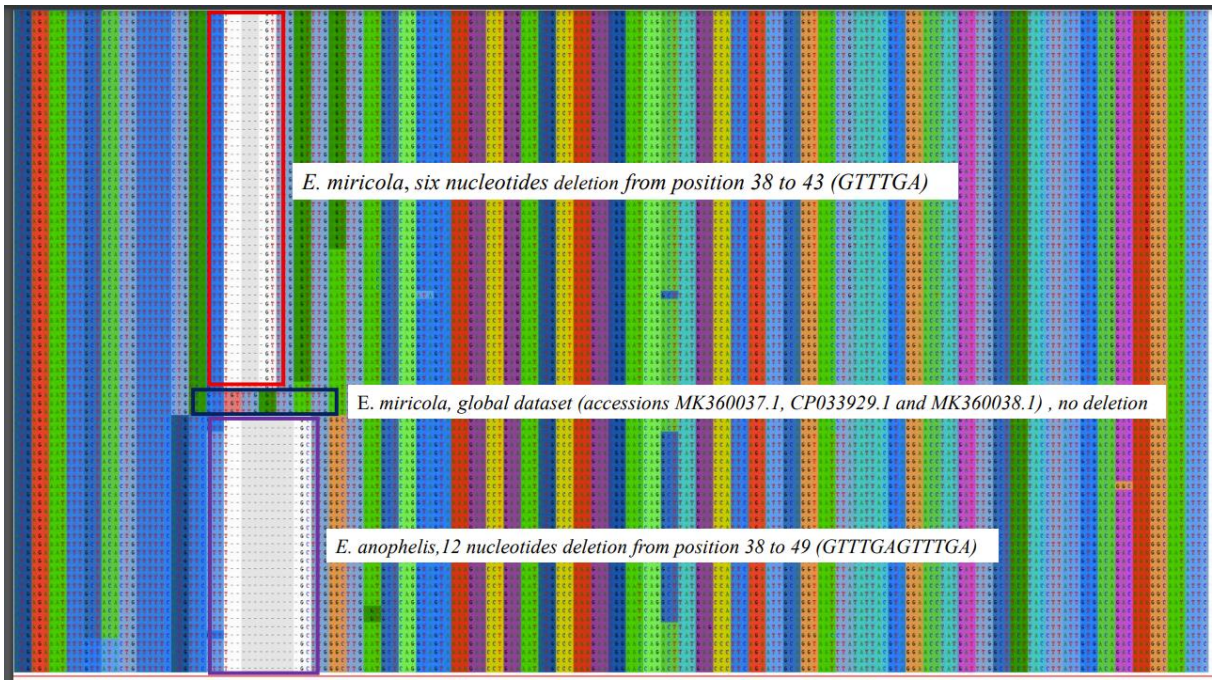
Accessible via link: https://ars.els-cdn.com/content/image/1-s2.0-S2666517421000638-mmc14.xlsx

Appendix 11



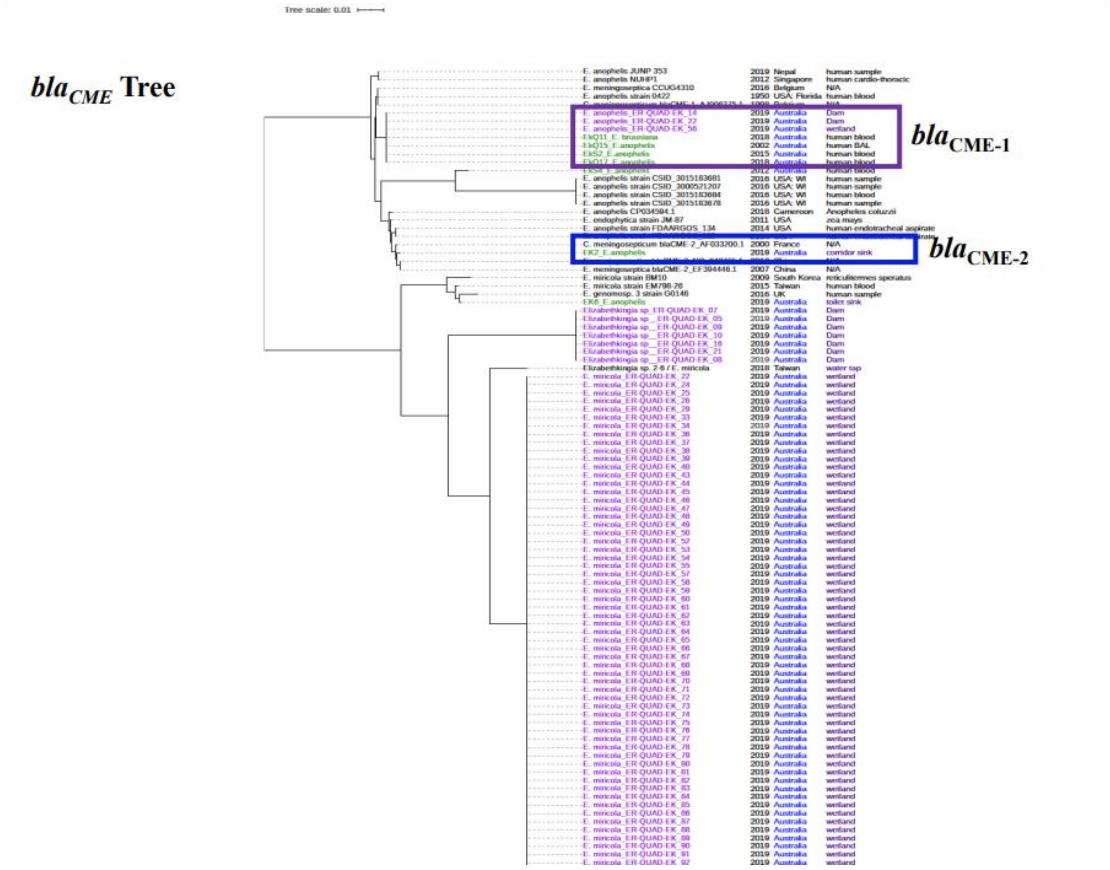
Appendix 11: A progressive Mauve analysis *of Elizabethkigia umeracha* sp.novel with all available reference sequences.

Appendix 12



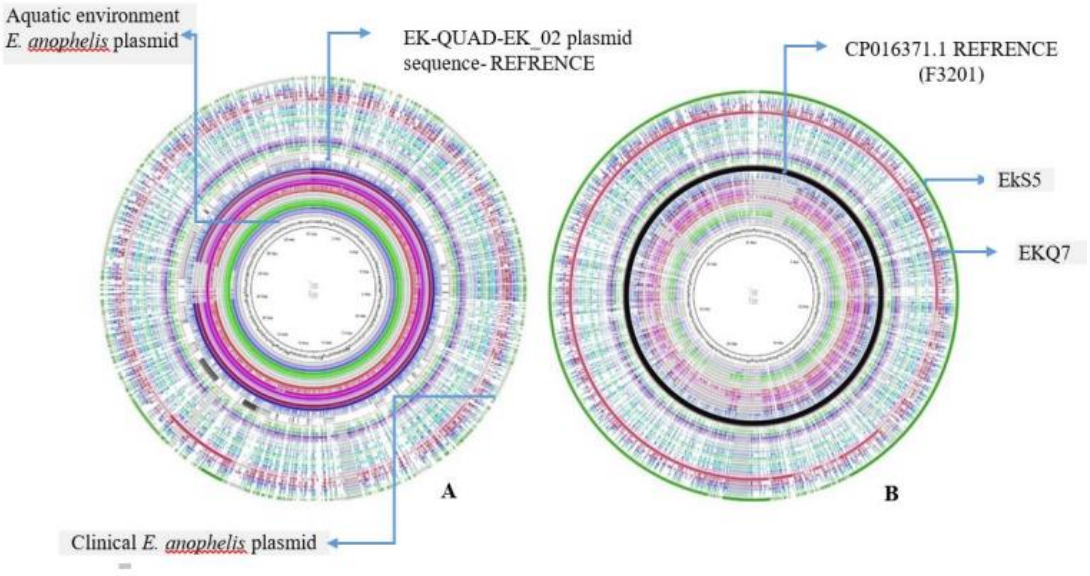
Appendix 12: MUSCLE alignments of all *Elizabethkingia* species carried bla_{GOB}, with distribution of several distinct signature deletions of 2–4 amino acids within the different alleles.

Appendix 13



Appendix 13: *bla*$_{CME}$ tree of *Elizabethkingia* isolates in our study.

Appendix 14



Appendix 14: Plasmid alignments of all *E. anophelis* from aquatic environment (n=16) and clinical isolates (n=22) to reference plasmid CP016375 using BRIG. A) plasmid maps and alignment of a representative plasmid from *E.anophelis* from Australian aquatic environment (EK-QUAD-EK_02) against all plasmids identified from *E.anophelis* in this study (aquatic environment and clinical isolates).Inner rings show Blastn alignment against related plasmid sequences (from inside to outside started by aquatic environment isolates followed in order by clinical isolates).Center histogram shows GC content.(B)Ananalysis buy using a plasmid reference from Genbank with accession CP016375 fromstrain F3201.

Appendix 15

Spreadsheet containing metadata of all isolates used in the study for Chapter 5

Accessible via link: https://journals.asm.org/doi/suppl/10.1128/aem.00646-22/suppl_file/aem.00646-22-s0001.xlsx

Appendix 16

Full assembly statistics

Accessible via link: https://journals.asm.org/doi/suppl/10.1128/aem.00646-22/suppl_file/aem.00646-22-s0002.xlsx

Appendix 17

BLASTn (ANIb), ANI average nucleotide identity MUMer (ANIm), and in silico DNA-DNA hybridization (DDH) analyses to identify *Comamonas denitrificans.*

Accessible via link:https://journals.asm.org/doi/suppl/10.1128/aem.00646-22/suppl_file/aem.00646-22-s0003.xlsx

Appendix 18

Metadata and accession numbers for isolates used in this study, including RefSeq genomes.

Accessible via link : https://figshare.com/account/projects/177588/articles/24080496

Appendix 19

Genomic data, including allelic variants for known and novel STs, for strains sequenced in present *Citrobacter* study

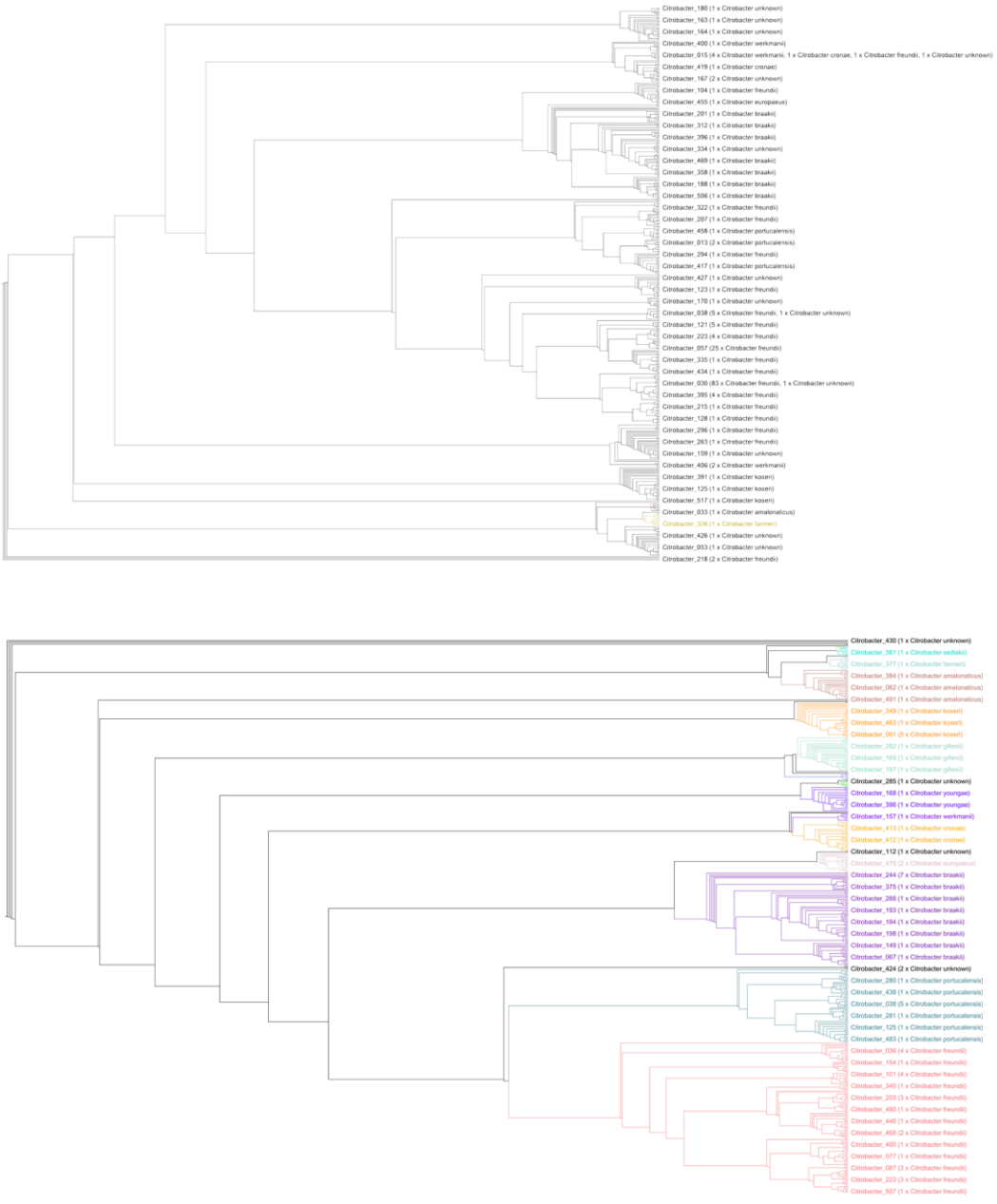
Accessible via link: https://figshare.com/account/articles/24082524

Appendix 20

Fourteen other plasmid sequences from GenBank highly related to pE11573 (> 90% coverage and 100% identity).

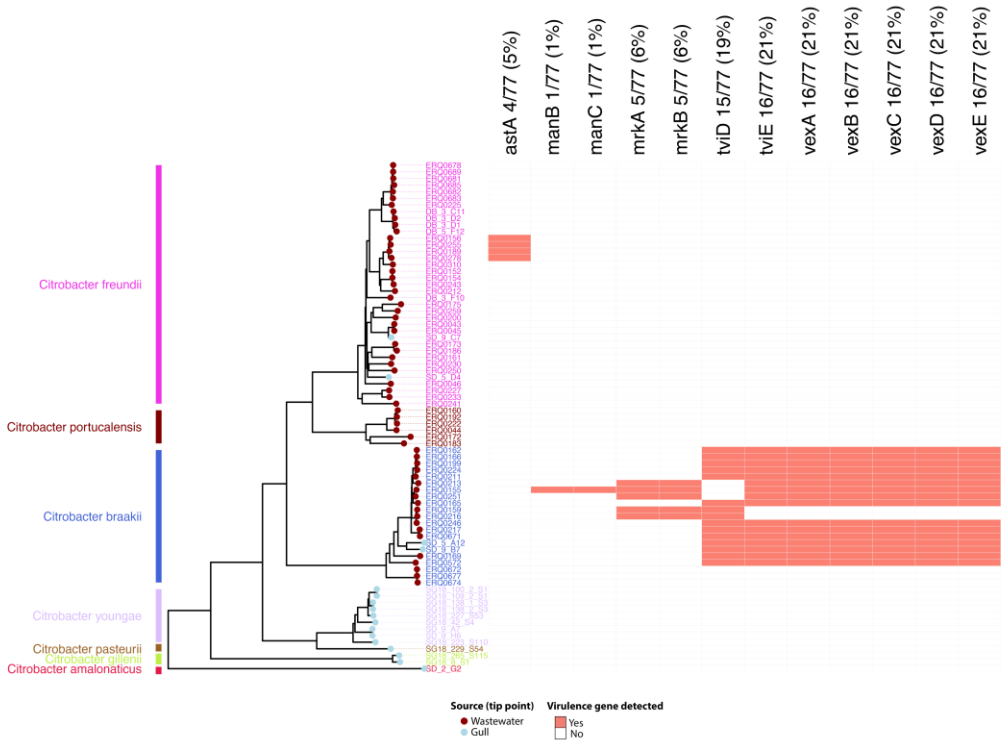
Accessible via link: : https://figshare.com/account/articles/24082533

Appendix 21



Appendix 21: Original and curated *Citrobacter* phylogenies used to speciate genomes from the *Citrobacter* study collection.

Appendix 22



Appendix 22: Virulence-associated genes (VAGs) among the 77 isolates from our *Citrobacter* study.