# GENOMIC COMPARATIVE ANALYSIS OF AUSTRALIAN ENVIRONMENTAL *ENTEROBACTER* SPP. ISOLATES

**by**
**Zein Alabidine Maatouk**
**Bachelor of Biomedical Science**


Supervisors: Dr. Mehrad Hamidian

Co-supervisor: Prof. Steven Djordjevic

Submitted in partial fulfilment of the requirements for the degree of
Bachelor of Science (Honours)

School of Life Sciences
Faculty of Science
University of Technology Sydney
2024

# Keywords

Antimicrobial Resistance, Environmental *Enterobacter* species., Comparative Genomic Analysis, Acquired Resistance, One Health approach

# Abstract

The global spread of antimicrobial resistance (AMR), driven by excessive antibiotic misuse and lack of regulatory stewardship, is a major threat to public health, with many pathogens now displaying resistance to last-line antibiotics, such as carbapenems. The genus *Enterobacter*, particularly members of the *Enterobacter cloacae* complex (*Ec*C), represents a significant AMR concern, as these bacteria are both widespread in the environment and increasingly responsible for healthcare-associated infections. Despite the urgent need to understand environmental sources of AMR and their potential impact on clinical settings, current research is largely focused on clinical isolates, leaving critical gaps in knowledge on the environmental reservoirs of resistance within *Enterobacter* spp.

This study aimed to address these gaps by analysing the whole genomes of 31 environmental *Enterobacter* isolates from influent wastewater (IWW) in South Australia. We sought to identify species diversity, annotate antimicrobial resistance genes (ARGs), and investigate genetic linkages between environmental and clinically significant strains within a One Health framework. Genomic analyses included phylogenetic approaches, ARG and virulence gene annotation, and the identification of mobile genetic elements (MGEs) that facilitate gene transfer.

Our findings highlighted significant diversity among the isolates, spanning multiple *Ec*C species and other *Enterobacter* species. Notably, we identified a range of clinically relevant ARGs, including colistin (*mcr-9.1*), carbapenem ($bla_{\text{GES-5}}$), and third generation cephalosporins ($bla_{\text{SHV-12}}$) resistances, carried primarily on Inc-type plasmids such as IncF and IncHI2, which are commonly associated with multidrug resistance. These plasmids showed high genetic similarity to globally distributed plasmids involved in clinical outbreaks, reinforcing the role of environmental reservoirs in the AMR crisis. Furthermore, we identified novel mobile elements, including *dif* modules, previously only reported in *Acinetobacter baumannii*, expanding our understanding of genetic mechanisms driving resistance across bacterial genera.

These results emphasise the environmental reservoirs' role in facilitating AMR gene spread, with direct implications for public health and infection control. This study supports the One Health perspective, highlighting the interconnectedness of environmental and clinical AMR dynamics, and highlights the need for broader surveillance to address AMR within both environmental and clinical ecosystems.

# Table of Contents

# List of Figures

# List of Tables

# List of Abbreviations

| | |
|---|---|
| AMR | Antimicrobial resistance |
| ARG | Antimicrobial resistance genes |
| ANI | Average Nucleotide Identity |
| AN | Accession Number (GenBank) |
| bp | Base Pair |
| *bla* | Beta lactamase |
| BLAST | Basic Local Alignment Search Tool |
| CARD | Comprehensive Antibiotic Resistance Database |
| CR*Ec* | Carbapenem-Resistant *Enterobacter cloacae* |
| DNA | Deoxyribonucleic acid |
| *Ec*C | *Enterobacter cloacae* Complex |
| ESBL | Extended Spectrum Beta Lactamases |
| HGT | Horizontal Gene Transfer |
| IWW | Influent wastewater |
| Inc | Incompatibility (Plasmid classification system) |
| IS | Insertion sequence |
| iTOL | Interactive Tree of Life |
| MALDI MS | Matrix-assisted laser desorption/ionization mass spectrometry |
| MDR | Multidrug resistant |
| MGE | Mobile genetic element |
| MLST | Multilocus Sequence Typing |
| NCBI | National Centre for Biotechnology Information |
| *orf* | Open Reading Frame |
| ST | Sequence Type |

| | |
|---|---|
| UTI | Urinary Tract Infection |
| VF | Virulence Factor |
| WGS | Whole genome sequencing |
| WHO | World Health Organisation |

# Statement of Original Authorship

## Student Statement

The work in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

I have submitted a draft(s) of my thesis to my supervisor(s) with sufficient notice and have received feedback.

Final word count: 12,384

[Note: Thesis word count is 15,000 (10%), excluding abstract, table of contents, list of tables, list of figures, tables, figures and their legends, references, and appendices.]

Signature: 

Date: 01/11/2024

## Supervisor Certification

This is to certify that the work entitled **Genomic comparative analysis of australian environmental *Enterobacter* spp. isolates** is a piece of research work done by Zein Maatouk under my guidance and supervision for the degree of Honours.

Name of Supervisor(s): Dr. Mehrad Hamidian

Signature(s): 

Date: 01/11/2024

# Acknowledgements

Firstly, I'd like to acknowledge my primary supervisor, Dr. Mehrad Hamidian, and attribute my success to his continuous support throughout the progression of my Honours year. Mehrad often times knew me better than myself in certain aspects and could predict where I am going to be from weeks to months in advance. In doing so, he was able to shape a personalised style of teaching for me and invoke motivation within me that inspired me far beyond my limit. Mehrad had complete faith and trust in me from the start of it all, and for that I am completely grateful for, I couldn't have asked for a better supervisor. To say that Mehrad was just a supervisor to me would be a complete understatement, he is a complete role model to me. I have the tendency to close off and try do things all on my own when I am stressed, however Mehrad would combat this by asking for updates and always leaving his door open for a talk or a little nudge in the right direction when I was derailing. Mehrad has definitely helped me grow throughout this year, both academically and on a personal level.

I'd also like to thank Dr. Veronica Jarocki, although she was not a direct supervisor to me, she oftentimes helped me throughout this project via assembly processes and was eager to provide her insights into my project. This involved monthly meetings to update on my project, guidance throughout bioinformatics approaches and feedback to all of my assessment tasks. I'd also like to extend my acknowledgements to my co-supervisor, Prof. Steven Djordjevic, who has continuously made efforts to appear for my practice talks and official seminars, and his feedback throughout all my assessments.

Furthermore, I would like to acknowledge the PhD students of the Hamidian Lab – Liam Tobin, Jonathan Koong and Eradah A Sabah. This team has made me feel a part of the lab since the beginning. Going into Honours, I didn't feel that my work would be as valuable as others due to my limited knowledge in the field. However, my voice was heard and valued by all in the team, and as such, my inspiration for the topic deepened – likely contributing to my success throughout the year. Additionally, I'd like to thank Liam for his continuous guidance for my difficulties throughout the course, and his willingness to answer all my questions, despite his own busy schedule. The Hamidian Lab actively supported my practice presentations and official seminars, showing genuine care for my contributions. For that, I am grateful.

# Chapter 1: Introduction

## 1.1 Global Expansion of Antimicrobial Resistance

Since the introduction of Penicillin in 1928, antibiotics have been vital to treating bacterial infections and advancing the field of medicine [3]. However, the rise of antimicrobial resistance (AMR) has compromised our ability to manage and treat critical infections in clinical settings. The statistics are alarming and rising, with an estimated 4.95 million deaths associated with AMR and ~ 1.27 million of these being directly attributed to drug-resistant infections, disproportionately affecting low- and middle-income countries (LMIC) [4]. The economic burden of AMR is also alarming at an estimated $2.2 billion annually in the US alone [5]. The threat of AMR is heightened by the reduced effectiveness of 'last resort' antibiotics due to a broad spread of bacteria equipped with antibiotic resistance genes (ARGs), such as carbapenemases [6].

Australia monitors and regulates antibiotic usage in the human health sector and has comparatively stringent antibiotic stewardship [7]. Nevertheless, AMR in Australia was associated with 6700 deaths in 2019, 1600 of which were directly attributable to AMR [8]. This escalating crisis is predicted to take 10 million lives per year globally and leave an economic burden between $142-283 billion in Australia by 2050 [4].

The World Health Organization (WHO) has deemed AMR one of the greatest threats to human health and has systematically categorised "priority pathogens" that pose the highest risk of causing AMR infections in humans [9]. Of the WHO priority pathogens, those subcategorised into "Critical Pathogens" are the top priority. Ranking first among the Critical Pathogens are carbapenem-resistant and/or third-generation cephalosporin-resistant Enterobacterales [9].

## 1.2 The genus *Enterobacter* spp.

The *Enterobacter* genus of the Enterobacterales family consists of approximately 22 facultative anaerobic Gram-negative bacilli species [10]. Seven of these species have been grouped within the clinically prominent *Enterobacter cloacae* Complex (*Ec*C), due to high genetic and phenotypic homology (at least 60% DNA-DNA homology of each other) [10]. *Enterobacter* spp. comprises opportunistic pathogens adept at colonising many environments, including but not limited to water, sewage, soil, or as commensal microflora in the intestinal tract [10].

*Enterobacter spp*. have a high capacity to develop AMR and are a member of ESKAPE-E pathogens [11]. ESKAPE-E pathogens are a group of bacteria that are a leading cause of hospital-acquired infections and are a global threat to human health [11]. Recently, the WHO reported *Enterobacter spp.* (carbapenem-resistant), as having high mortality rate, and low preventability and treatability rate [9].

The *Ec*C stands out in the genus due to its severe clinical outcome, such as bacteraemia (5%), UTIs (4%), pneumonia (5%), and postsurgical peritonitis (10%), as well due to its high capability for AMR dissemination [12]. As of 2019, the impact of *Ec*C was quantifiably significant, accounting for 5% of all AMR-related deaths [4]. Additionally, *Enterobacter* species contribute resistance to clinically significant last-line treatment options, such as carbapenems and colistin.

## 1.3 Clinically Significant Drug Class Resistances

### 1.3.1 Carbapenem Resistance

Carbapenem resistance is a critical public health issue, particularly among Enterobacterales, which have developed mechanisms to evade carbapenems, one of the most potent antibiotic classes [13]. Carbapenems are a type of $\beta$-lactam antibiotic with broad-spectrum activity against Gram-negative pathogens [14]. They function by inhibiting penicillin-binding proteins, essential for bacterial cell wall synthesis [15]. However, the global surge in carbapenem resistance is attributed to multiple bacterial mechanisms that inactivate or evade these antibiotics [14].

The primary mechanism of carbapenem resistance in Enterobacterales involves the production of carbapenemase enzymes, which hydrolyse carbapenems and other $\beta$-lactams, rendering them ineffective [16]. These enzymes include KPC (*Klebsiella pneumoniae* carbapenemase), NDM (New Delhi metallo-$\beta$-lactamase), and OXA-48-like carbapenemases, commonly encoded on mobile genetic elements such as plasmids and transposons that facilitate their spread across species and regions [17-19]. Additional resistance mechanisms include alterations in outer membrane proteins, reducing drug entry, and overexpression of efflux pumps, which actively expel antibiotics from the cell [20].

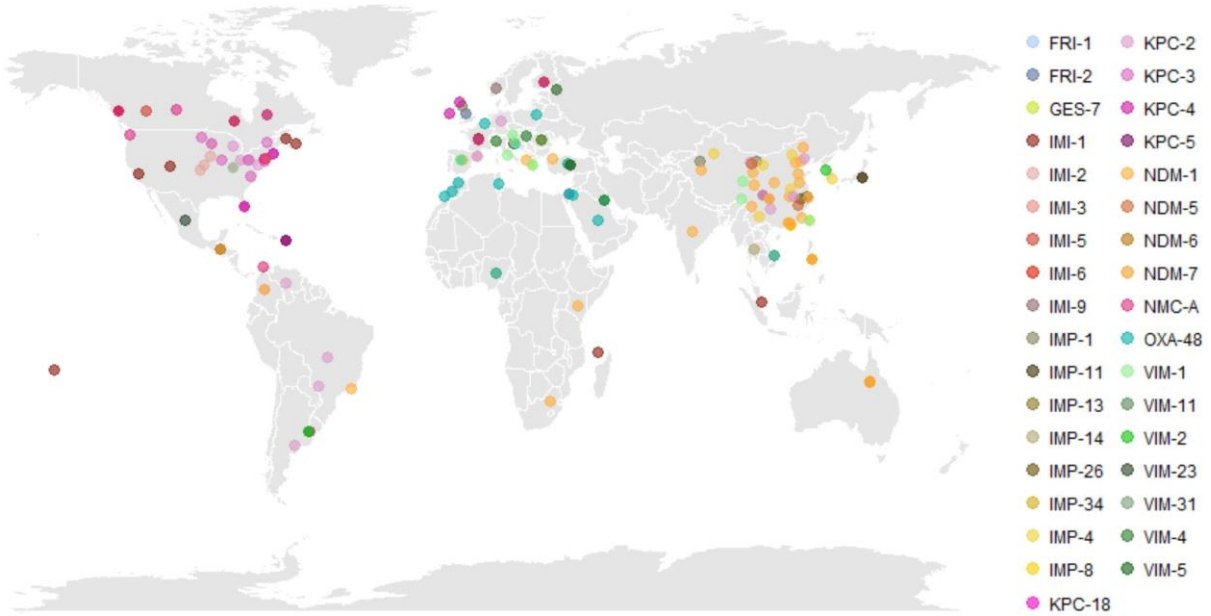



**Figure 1.** Global distribution of carbapenem-resistant *Enterobacter cloacae* complex. Different colour dots represent varying carbapenemases distributed on the map. Taken From [21]

Carbapenems are considered one of the most reliable drugs for treating bacterial infections, the emergence and spread of resistance to these antibiotics constitutes a major public health concern [12]. Carbapenem-resistant Enterobacterales, including carbapenem-resistant *Enterobacter* spp., complicate treatment options and limit effective therapies, leading to increased morbidity and mortality. This resistance pattern reinforces the importance of monitoring, infection control, and developing novel therapeutic strategies to control the spread of highly resistant pathogens (see Figure 1).

### 1.3.2 Colistin Resistance

Colistin, a polymyxin antibiotic, is a cationic antimicrobial peptide initially discovered in 1947. Colistin usage is significantly toxic to the body, particularly to the renal and neurological systems, however it has been shown to be an effective treatment to bacterial infections [22]. More recently, colistin has been used as a last-line treatment reserved for extensively drug-resistant Gram-negative bacteria, including *Enterobacter* spp. Its mechanism of action involves binding to lipid A in the bacterial outer membrane's lipopolysaccharides (LPS), disrupting membrane integrity and causing cell death [23]. This interaction displaces stabilising divalent cations like $Mg^{2+}$ and $Ca^{2+}$, increasing membrane permeability and ultimately leading to cell lysis [24].

Colistin resistance is significant because colistin remains one of the few effective treatments for infections caused by carbapenem-resistant Enterobacterales. The development of resistance in *Enterobacter* spp., raises serious concerns, especially in healthcare settings where colistin-resistant strains can lead to treatment failures in severe infections like bloodstream and respiratory tract infections. Colistin resistance in *Enterobacter* spp. is often facilitated by the mobile colistin resistance (*mcr*) gene, particularly *mcr-9*, which has been identified in various species within the *Enterobacter cloacae* complex (*Ec*C) [25, 26]. This gene encodes a phosphoethanolamine transferase that modifies the LPS structure, decreasing colistin's binding affinity to the bacterial membrane [27].

*Enterobacter* species are known for both intrinsic and acquired resistance capabilities, with colistin resistance further complicated by species-level genetic variation. Regulatory genes, particularly in the arnBCADTEF cassette, *phoPQ*, and *mgrB*, affect lipid A modification levels and colistin susceptibility [25]. Studies indicate that these genetic variations contribute to both resistance and heteroresistance, where subpopulations within a culture exhibit variable resistance levels to colistin [25]. This unique resistance pattern complicates detection and treatment, highlighting the necessity of continuous monitoring and research into important resistance mechanisms within *Enterobacter* spp.

## 1.4 Mechanisms of Antimicrobial Resistance Acquisition and Spread

### 1.4.1 Mutations

Mutations, or changes in an organism's genetic material, play a crucial role in the development of AMR. Point mutations involve the alteration of a single nucleotide in the DNA, while missense mutations lead to a single amino acid change in a protein. A recent study by Dong in 2023 highlights how point and missense mutations can alter drug-binding sites, driving AMR [28]. Nonsense mutations, on the other hand, introduce a premature stop codon that may truncate proteins involved in antibiotic susceptibility. Frameshift mutations, caused by insertions or deletions that disrupt the reading frame, result in proteins that an antibiotic might

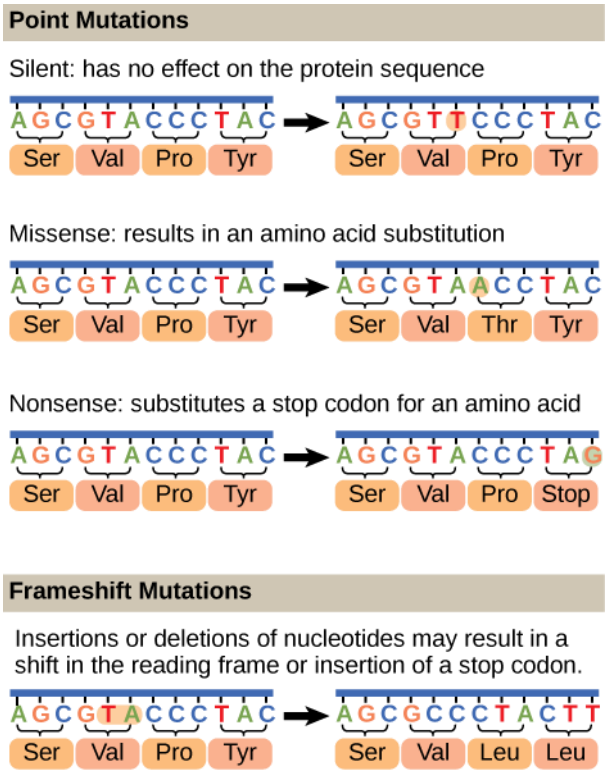



**Figure 2.** Types of mutations that can occur within genetic material potentially leading to AMR in bacteria. Diagram from [1]

not recognise. Such mutations can occur in genes related to antibiotic targets, efflux pumps, or enzymes that degrade antibiotics, collectively contributing to the emergence of AMR [4]. By modifying bacterial genetic structures, these mutations enable survival in the presence of antibiotics.

#### 1.4.1.1 Mutation Induced Quinolone Resistance

A prime example of mutational resistance can be found in *Enterobacter* spp. through resistance to fluoroquinolones. This is primarily due to mutations in protein-coding genes for the bacterial target enzymes DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) [29]. These enzymes play crucial roles in DNA replication and cell division [30]. When mutations occur in these genes, they reduce the binding affinity of quinolone antibiotics, effectively diminishing the drug's ability to inhibit bacterial growth [29, 30].

One of the most common mutations occurs at codon 83 in the *gyrA* gene, where a serine is often substituted with phenylalanine or tyrosine or threonine (Ser83Phe or Ser83Tyr or Ser83Thr), resulting in a significant reduction in drug binding [29]. Similarly, mutations in *parC*, often at codon 80, where serine is replaced by isoleucine (Ser80Ile), further enhance quinolone resistance [30]. These mutations alter the quinolone resistance-determining region, the area on these enzymes that quinolones target, leading to decreased drug susceptibility and, consequently, bacterial survival despite quinolone treatment.

### 1.4.2 Mobile Genetic Elements are the primary drivers of acquired resistance

Mobile genetic elements (MGEs) are DNA segments capable of moving within and between genomes. They are crucial in the horizontal gene transfer (HGT) of ARGs among bacteria, promoting the rapid dissemination of resistance across different species and environments.

### 1.4.2.1 Insertion Sequences

Insertion Sequences (ISs) are the simplest type of MGEs, consisting of small DNA segments, typically around 1-1.5 Kbp [31]. They have a genetic structure that enables independent insertion at different target sites within a host genome, either on the chromosome or within other MGEs.

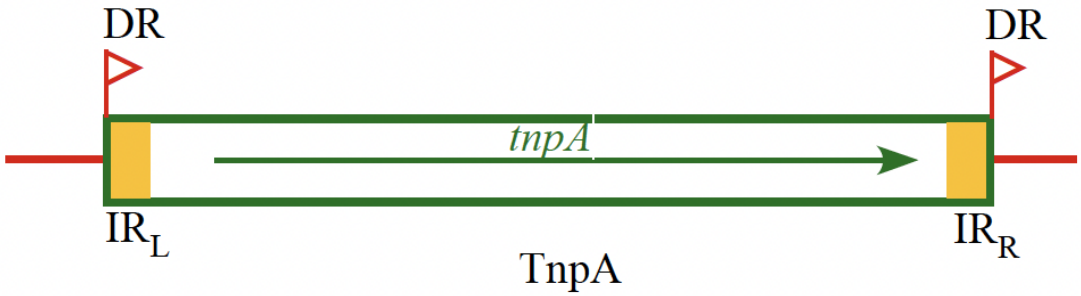



**Figure 3.** General Structure of an Insertion Sequence. Red line indicates a segment of DNA on which it has been inserted between. The yellow boxes represent the inverted repeats, left and right. Red flags on flanking ends indicate direct repeats generated by an IS typically upon insertion into a chromosome or plasmid.

Upon insertion, most IS elements generate short direct repeats (DRs) of the target DNA, which flank the IS and result from the cut-and-insertion mechanism facilitated by the transposase enzyme (*tnpA*) encoded by the IS [31]. IS elements also possess inverted repeats (IRs)—short, identical sequences (usually 10-40bp) located at both ends of the IS but in opposite orientations. These IRs are crucial for the mobility of the IS element, as they are recognised by the transposase, which binds to them to excise the IS from its original location and insert it into a new site within the genome [31]. Moreover, the IRs serve as boundary markers for the IS element, ensuring its complete transposition and accurate replication.

IS26, a member of the IS6 family, is noteworthy due to its involvement in the acquisition of ARGs. Commonly found in Gram-negative bacteria, IS26 is well documented for its significant role in the spread of last-line antibiotic resistance. For instance, IS26 has been notarised for its ability to amplify encoded carbapenem resistance via the $bla_{\text{OXA-1}}$ and extended spectrum $\beta$-lactamases (ESBLs) via $bla_{\text{CTX-M-15}}$ in a cohort study of carbapenem-resistant Enterobacterales [32].

### 1.4.2.2 Transposons

Transposons are MGEs akin to ISs in that they possess the intrinsic function to relocate within and across bacterial genomes. They are classed into three broad categories based on their transposition mechanisms [33]. Class 1 transposons, also known as composite transposons, exploit the transposition properties of flanking ISs to mobilise.

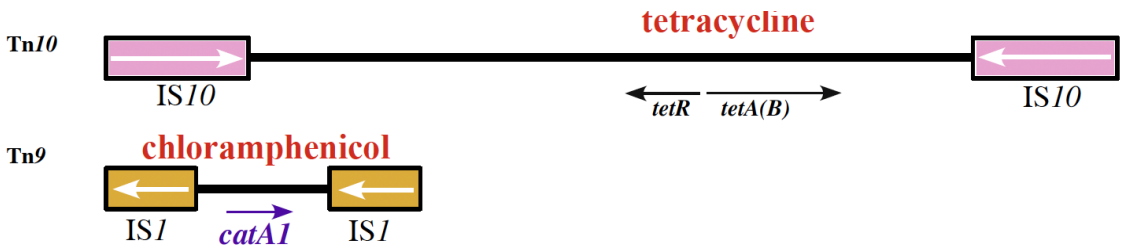



**Figure 4.** Illustration depicting common composite transposons found in Enterobacterales, Tn*10* and Tn*9*, providing drug class resistance to tetracycline and phenicols respectively. Both are shown with flanking ISs, indicating their mobility and transposon class.

Class 2 transposons are self-sufficient, carrying their own transposase and resolvase genes for independent translocation [34]. In contrast, Class 3 transposons encompass a broad group that does not fit into the first two classes of transposons (e.g. Tn*7* and transposons related to it such as Tn*6021*, Tn*6022*, Tn*6019*, etc.), however they encode their transposase, enabling their transfer between genomes [34].

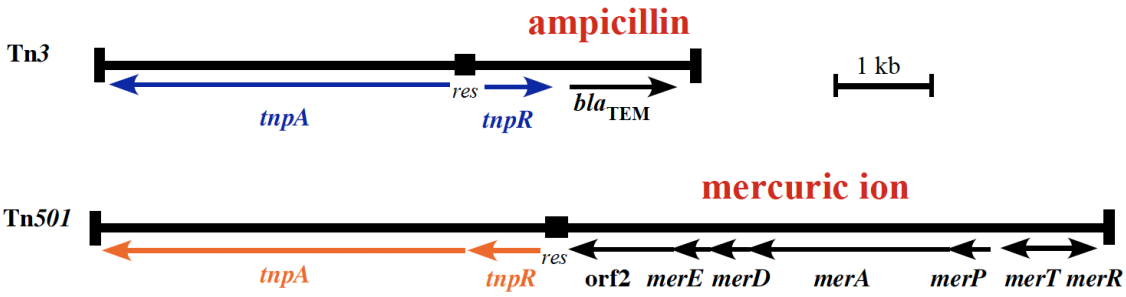



**Figure 5.** Class 2 transposons commonly found in Gram-negative bacteria, including *Enterobacter* spp. Tn*3* encodes for a narrow-spectrum beta-lactamase, conferring resistance to ampicillins and first generation cephalosporins. Tn*501* carries a mercury resistance operon, contributing to heavy metal resistance.

### 1.4.2.3 Integrons

Integrons are genetic elements that capture, organise and express gene cassettes, particularly ARGs. The integrase enzyme, encoded by the *intI* gene is responsible for recognising specific

recombination sites (*attI* and *attC*), facilitating the insertion or excision of gene cassettes, allowing the acquisition of new genes that provide selective advantages, i.e. AMR [35].

Unlike other MGEs, integrons are not intrinsically mobile, however, they are often found on plasmids and transposons or flanked by IS elements, which facilitate their transfer between cells and bacteria. They are classed into three different categories, based on their integrase gene and the types of gene cassettes they capture. However, class 1 integrons are most relevant in the dissemination of ARGs, as class 2 integrons often encode a non-functional *intI2* gene and class 1 integrons are uncommon [35].

Class 1 integrons contain a 5'-conserved region that contains the essential *intI1* gene, recombination site, and promoter (Pc) needed for capturing and expressing gene cassettes. The 3' conserved region carries resistance genes such as *sul1* and *qacEΔ1*, conferring resistance to sulfonamide [36].

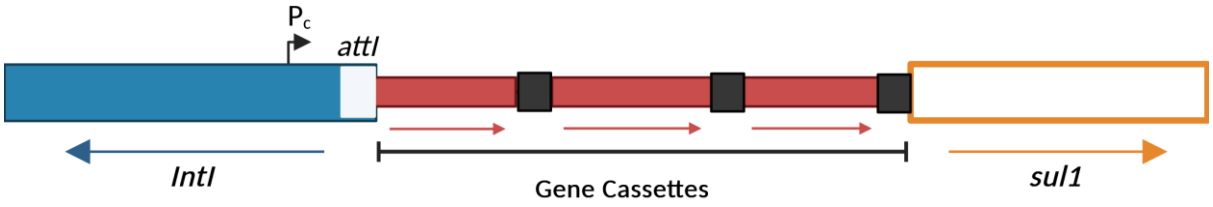



**Figure 6.** General Structure of Class 1 Integron. Created with Biorender.com.

#### 1.4.2.4 Plasmids

Plasmids are double-stranded extrachromosomal DNA units that can replicate and transfer between bacteria, either through bacterial conjugation or through mobilisation (co-transfer using large conjugative plasmid properties) [37]. Conjugative plasmids are often equipped with transfer genes, or *tra* genes, responsible for creating a mating pair formation complex, essential for establishing direct contact between bacteria [38]. Smaller, mobilisable plasmids, often lack essential *tra* genes and instead rely upon the conjunct mobilisation of larger conjugative plasmids as well as their own mobilisation genes to replicate and transfer, as per figure 7. However, both types of plasmids encode an origin of transfer (*oriT*) sequence and replication gene (*rep*), responsible for flagging where the transfer process of the plasmid begins and regulating the replication of the plasmid, respectively [37]. Plasmids are potentially the greatest threat to the dissemination of AMR, as they often carry all types of MGEs within their DNA, encoding resistance to a significant amount of drug resistance classes [39].

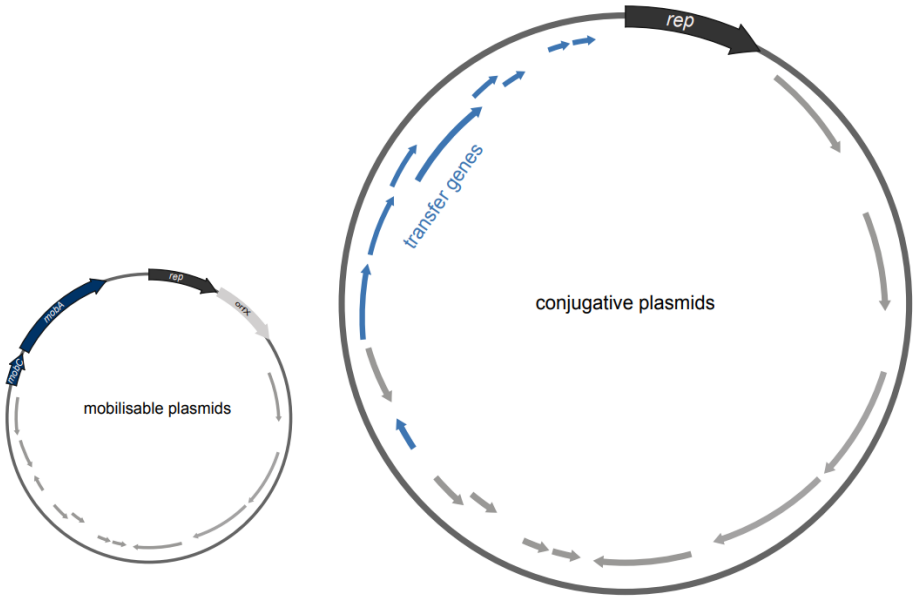



**Figure 7.** Mobilisable plasmids and conjugative plasmids. Mobilisable plasmids carry *mob* genes, whereas conjugative plasmids encode *tra* genes. Both encode a *rep* gene and oriT.

#### 1.4.2.4.1 Incompatibility Plasmid Replicon Typing Scheme

Inc-type plasmids, also known as incompatibility plasmids, are classified based on their replication and maintenance systems, which prevent plasmids of the same incompatibility (Inc) type from coexisting within a single bacterial cell [40]. This classification is crucial for understanding how plasmids drive the spread of ARGs among bacterial populations, including Enterobacterales. Each Inc group represents a distinct plasmid lineage with unique replication mechanisms, host ranges, and genetic cargo. These incompatibility systems determine plasmid compatibility within a cell, influencing the dynamics of HGT and the potential for developing multi-resistance profiles within bacterial communities.

Certain Inc types are closely associated with AMR in *Enterobacter* spp., each playing a distinctive role in ARG mobilisation. IncF plasmids, commonly found in Enterobacterales, are typically large, low-copy-number plasmids that inhabit multi-resistant strains. IncF plasmids frequently carry critical ARGs such as $bla_{\text{CTX-M}}$, which encodes ESBL enzymes, conferring resistance to third-generation cephalosporins or $bla_{\text{NDM-1}}$ conferring resistance to carbapenems. This plasmid type's adaptability and persistence make it significant in healthcare settings, where $bla_{\text{CTX-M/NDM-1}}$-harbouring IncF plasmids have been implicated in numerous multidrug-resistant infections [41-44].

IncI and IncN plasmids are medium-sized and highly transmissible, commonly carrying ARGs like *mcr-1*, which confers resistance to colistin which is a last-resort antibiotic used to treat carbapenem-resistant infections [45]. These plasmids play a major role in spreading resistance between different bacterial genera, especially within Enterobacterales, which are frequently encountered in hospital settings [46]. IncH plasmids are notable for their large size and broad host range, often associated with last-line resistance genes such as $bla_{\text{KPC}}$ (Klebsiella pneumoniae carbapenemase) and $bla_{\text{NDM}}$ (New Delhi metallo-$\beta$-lactamase), both of which provide resistance to carbapenems [19, 47]. The broad host range of IncHI plasmids allows them to transfer resistance genes across multiple bacterial species, posing a serious challenge in managing carbapenem-resistant infections in clinical environments.

IncQ plasmids are small, high-copy-number plasmids with a broad host range, which enhances their efficiency in disseminating ARGs, especially in environmental reservoirs. They are often implicated in carrying quinolone resistance genes such as *qnrS* and *qnrB*, which contribute to fluoroquinolone resistance [2, 48, 49]. Additionally, they have been reported in a clinical outbreak of carbapenem resistant *E. cloacae* via the $bla_{\text{GES-5}}$ in Canada in 2009 [50]. The high replication rate and mobilisability of IncQ plasmids make them particularly effective in environments like wastewater, where selective pressures favour the spread of resistance genes. Finally, IncA/C plasmids are widely associated with multidrug resistance in both clinical and agricultural settings. They carry a diverse range of ARGs, including $bla_{\text{CMY-2}}$ (a plasmid-mediated AmpC beta-lactamase conferring resistance to extended-spectrum cephalosporins) and *aadA* (conferring aminoglycoside resistance) [51]. In *Enterobacter* spp., these plasmids connect agricultural and clinical environments, facilitating cross-reservoir ARG transmission that impacts human health.

The variability of Inc-type plasmids in acquiring and mobilising ARGs is enhanced by their ability to capture drug class resistances via MGEs. This contributes to the global dissemination of ARGs, as shown by IncF and IncI plasmids that carry $bla_{\text{CTX-M}}$ and *mcr-1* genes, respectively. In *Enterobacter* spp., the diversity and mobility of Inc-type plasmids make them central to the horizontal transfer of ARGs, shaping resistance patterns in clinical and environmental settings alike. Understanding these Inc-type plasmids, along with the mechanisms underlying their HGT potential, is essential in tracking resistance patterns and developing strategies to mitigate the spread of resistance within and beyond healthcare environments.

### 1.4.2.5 XerC/XerD recombinase sites and *dif* modules

Recent studies on *Acinetobacter* plasmids have demonstrated their ability to exploit recombinase enzymes by mimicking chromosomal recombination sites recognised for cleavage and ligation [52]. These enzymes facilitate the movement of genetic material. Complementary alternating recombinase sites allow genetic material to be "cut" and "pasted" into various sections of a cell's genome or even transferred to another cell [52]. This mobile section of DNA is known as a *dif* module and is typically composed of one or more genes that drive bacterial survival, i.e. AMR. These genes are flanked by recombinase identification sites known as XerC and XerD. For the module to be mobile, these recombinase identification sites must alternate [53].

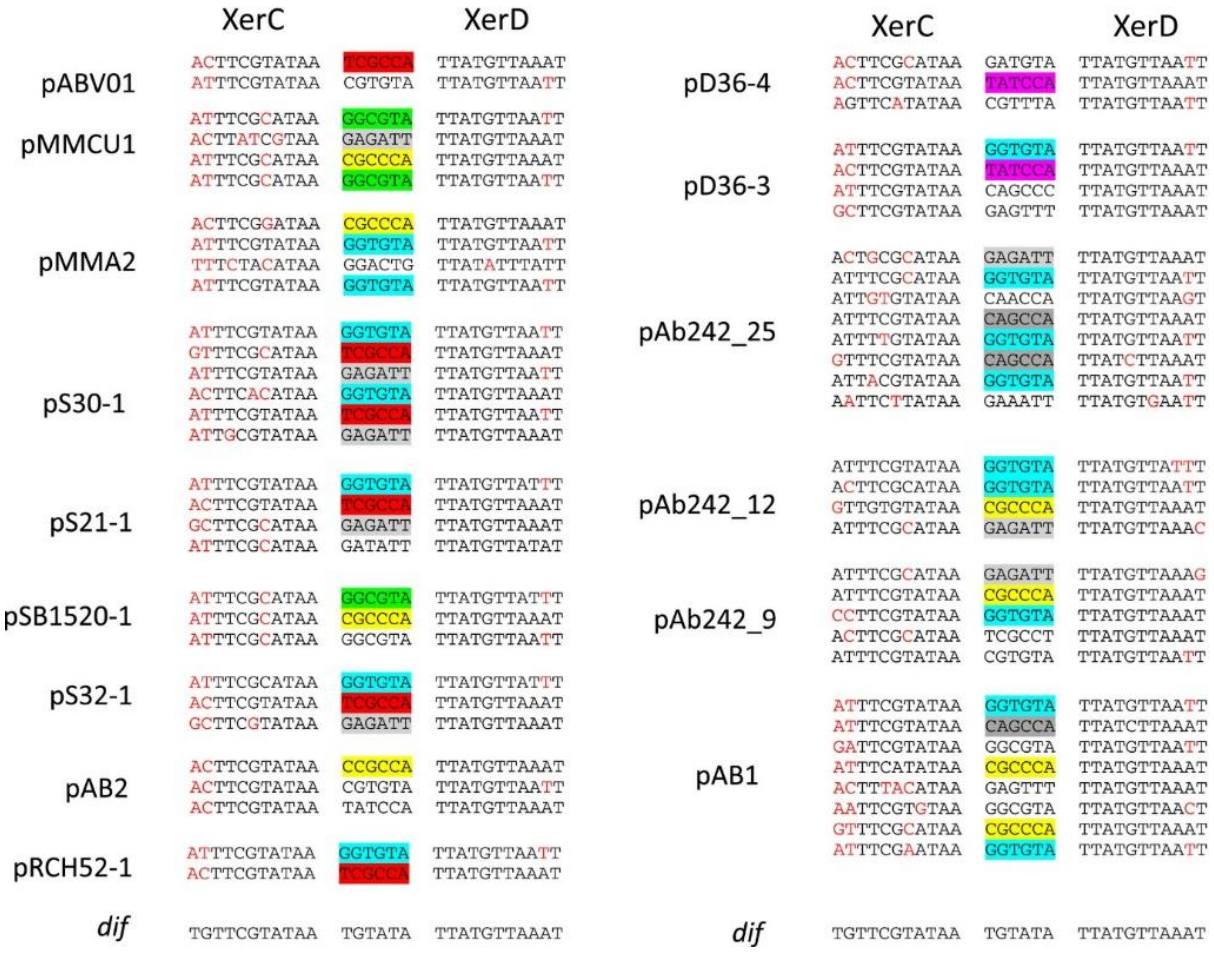



**Figure 8.** Alignment of multiple p*dif* sites from various Acinetobacter plasmids, compared against the chromosomal dif sequence from A. baumannii strain AB307_0294. Base differences from dif in the XerC or XerD binding sites are highlighted in red, with the majority being found in the XerC site. Taken from [53]

Instances of *dif* modules have been widely characterised across *Acinetobacter* between clinical and environmentally distributed strains, supported initially by Blackwell, 2017 and then Balalovski, 2020 in studies characterising the potentially novel mobilisation of these modules [52, 53]. However, this has not yet been recorded in literature for *Enterobacter* spp., until now.

### 1.5 Selective Pressures

The increase in AMR among Gram-negative bacteria, including *Enterobacter* spp., is primarily driven by several key environmental factors that exert selective pressures. These pressures include poor antibiotic stewardship, the overuse and misuse of antibiotics in agriculture (i.e. livestock), and the resultant contamination of antibiotic residues from clinical waste [54, 55]. Such conditions enable bacteria with resistant traits to survive, thrive, and multiply (see Figure 9). A recent European study has concluded that the influence of these selective pressures on AMR development could be even greater than previously assumed [56]. This highlights the urgent need for comprehensive antibiotic stewardship and tighter controls on antibiotic use across all sectors to mitigate the spread of resistance.

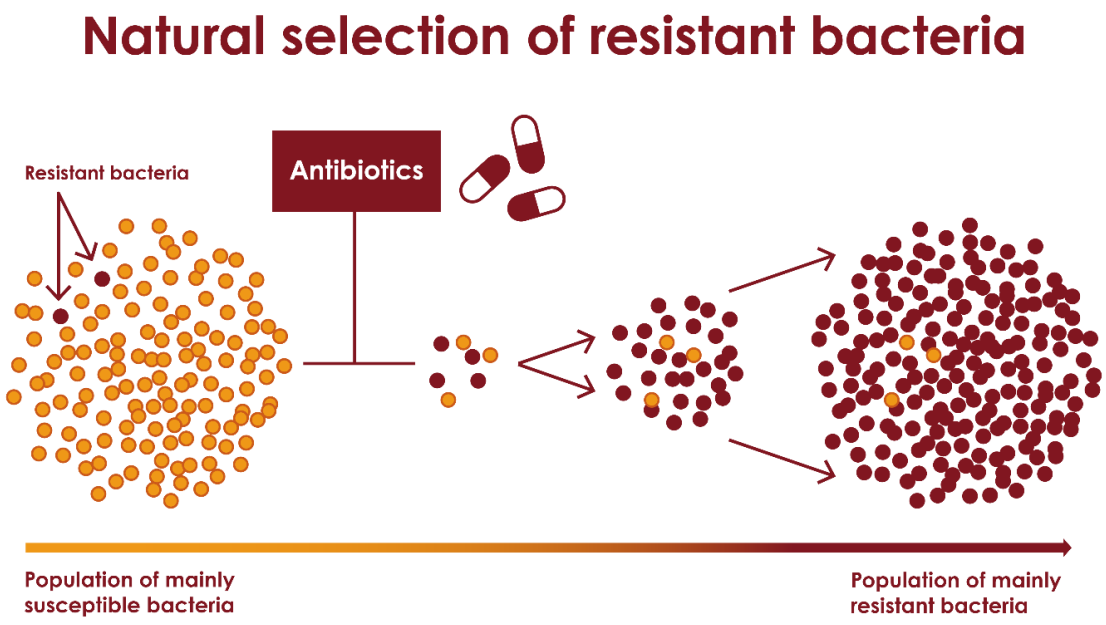



**Figure 9.** Illustration of generation of resistant bacteria in the environment. Orange circles indicate susceptible bacteria and red circles indicate resistant bacteria. Diagram from [57].

### 1.6 Multilocus Sequence Typing in *Enterobacter* spp.

Multilocus Sequence Typing (MLST) is a molecular typing method used to characterise bacterial strains by sequencing the internal fragments of seven 'housekeeping' genes (*dnaA*,

*fusA*, *gyrB*, *leuS*, *pyrG*, *rplB*, and *rpoB*)[58]. MLST provides a valuable tool for identifying genetic diversity, tracking the spread of AMR, and understanding the evolutionary relationships within the genus. By assigning sequence types (STs) based on allelic profiles of these housekeeping genes, MLST allows researchers to differentiate between closely related strains and to estabish phylogenetic relationships between isolates from different hosts and environments.

In *Enterobacter* spp., MLST has been instrumental in identifying specific lineages associated with heightened pathogenicity and AMR, particularly within the *Ec*C. Certain STs, such as ST78 and ST171, are frequently linked to hospital-associated infections and display multidrug-resistant profiles, including resistance to carbapenems and colistin [59, 60]. These high-risk lineages are often associated with MGEs like plasmids, transposons, and integrons, which carry resistance genes that can be horizontally transferred. Consequently, tracking these STs in clinical and environmental settings is crucial for understanding how AMR traits circulate and persist across ecosystems.

MLST also aids in tracing the spread of specific resistance determinants within *Enterobacter* spp. For instance, ST-based tracking has shown how environmental strains, often isolated from wastewater or soil, may share sequence types with clinical isolates, suggesting genetic exchange and adaptability between environments [61]. This is particularly relevant under the One Health framework, where linking STs across human, animal, and environmental sources can highlight potential reservoirs of AMR and inform interventions (see section 1.8).

### 1.7 Gaps in Knowledge

The presence of ARGs in clinical isolates is well established, yet knowledge about their prevalence, diversity, and dynamics in environmental reservoirs, especially in non-clinical settings like wastewater, remains limited [62]. Recently, emerging studies have highlighted the role that various environmental niches, particularly influent wastewater (IWW), may play as reservoirs of AMR through the mobilization of ARGs by MGEs [63-65]. Therefore, additional research is essential to deepen our understanding of the role environmental strains play in the acquisition and spread of resistance genes, a mechanism that frequently leads to the emergence of novel resistance clones.

## 1.8 One Health Framework

The One Health approach integrates the environmental, animal, and human health sectors to achieve optimal health outcomes by linking these interconnected areas (Figure 10). Pathogens with natural environmental reservoirs, such as *Enterobacter spp.*, are recognized as indicators of AMR dissemination across ecosystems [66]. This is largely due to environmental samples, particularly IWW, which may act as reservoirs, reflecting the AMR situation at both clinical and societal levels. Bacteria residing in IWW are exposed to a wide range of selection pressures such as pharmaceuticals (e.g. antibiotics), personal care products, pesticides, heavy metals, biocides, and other organic and inorganic compounds, as well as physicochemical properties, such as temperature and pH [67]. All of which effectively drive the selection of MDR bacteria (Figure 10).

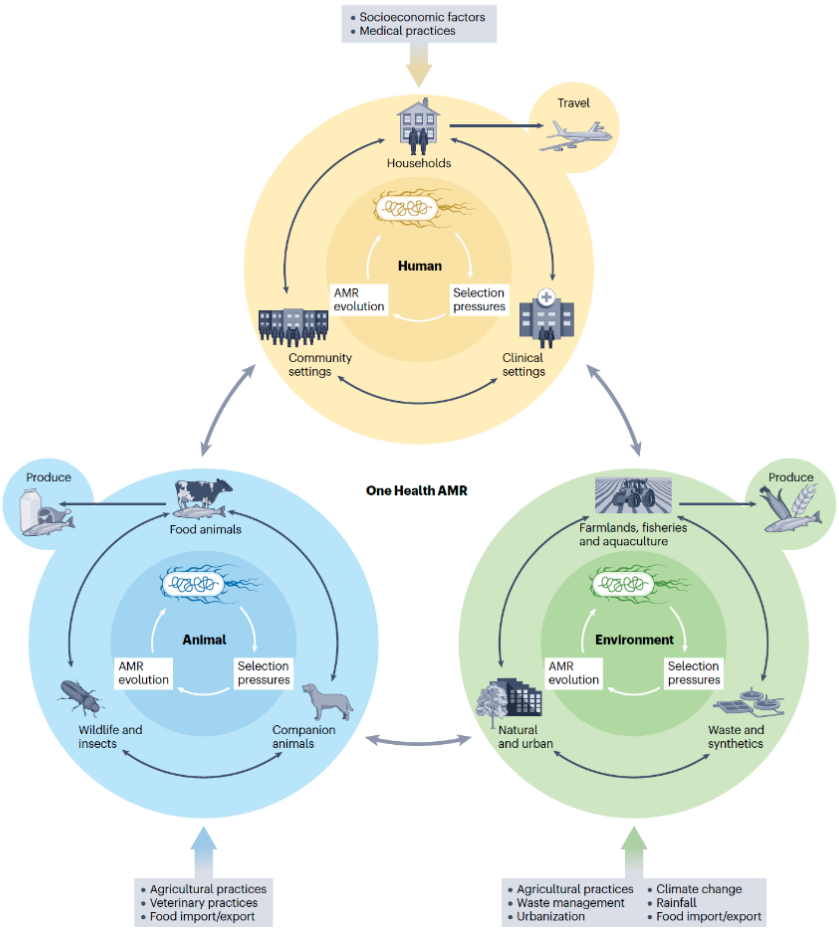



**Figure 10.** One Health Framework. Grey box indicate selection pressures. Diagram from [66]

By examining IWW samples, we can more effectively track the environmental spread of AMR and connect these findings to inform clinical responses aimed at combating this threat to human health. Thus, the One Health approach is essential, given the rapid evolution of resistant

bacteria, as the development of new antibiotics often leads to the emergence of novel resistances.

## 1.9 Research Questions and Aims

In addressing AMR within *Enterobacter* spp., integrating a One Health framework is essential, as the interconnectedness of human, animal, and environmental health influences both the development and spread of bacterial resistance [66]. This comprehensive approach is particularly crucial for *Enterobacter*, where understanding the genetic transfer of resistance determinants between clinical and environmental isolates is vital [68].

Research on *Enterobacter* has primarily focused on clinical isolates. Our preliminary analysis of *Ec*C assemblies in the PATRIC database shows that only 5% of 7,763 entries were environmental, with none of these environmental samples originating from Australia [69]. Studies on environmental *Enterobacter* are rarely conducted outside healthcare-associated settings, thereby failing to fully capture the genus's environmental interactions and impact. Hence, a clear need exists for a comprehensive understanding of *Enterobacter* strains persisting outside human or animal hosts and, more acutely, whether these environmental strains participate in a genetic exchange with clinical counterparts. Critical insights into these exchange events between clinical and environmental strains still require further precision.

The overarching question surrounding this project is to fill this gap in knowledge through the guise of - Do environmental *Enterobacter* isolates share genetic homology with clinical and globally distributed strains of *Enterobacter* spp. that have acquired antimicrobial resistance and virulence determinants? We expect environmental *Enterobacter* species to have a high genetic homology to clinical and global strains.

To test this, we have devised a set of aims:

**1.** Assemble the genome sequences of 31 environmental putative *Enterobacter spp*. isolates.

**2.** Determine the diversity of the environmental *Enterobacter* isolates using phylogenomic approaches.

**3.** Annotate AMR and virulence genes within each assembly of isolate.

**4.** Determine the genetic context of antibiotic resistance and virulence determinants to investigate the link between clinical and environmental strains using the One Health approach.

# Chapter 2: Methodology

## 2.1 Describing the isolates

Thirty-one environmental *Enterobacter spp.* isolates were studied in this project. All the isolates were recovered by collaborators from the Future Industries Institute at the University of South Australia, from IWW. Initial speciation of these isolates identified them as *E. cloacae* via the matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS). MALDI-MS is a technique that rapidly identify bacteria by first ionising the colony, firing the ions through a detector, and then measuring the time it takes for all ions to reach the detector [70]. DNA was extracted from each isolated and sequenced via the Illumina MiSeq short-read platform [71].

## 2.2 Whole genome sequencing

Short-read sequencing using the Illumina MiSeq platform was performed at the UTS Next Generation Sequencing Facility, while long-read sequencing with the Oxford Nanopore MinION was conducted at the Garvan Institute of Medical Research [71, 72]. After completing both sequencing methods, the raw read files were provided in FASTQ format.

## 2.3 Quality control and assembly

Prior to genome assembly, quality control measures were applied to ensure the sequencing data was suitable for assembly. FASTQC was used to evaluate the quality of the raw reads, and Trimmomatic was employed to trim low-quality bases and remove adapter sequences, thereby ensuring that only high-quality reads were retained for assembly [73, 74]. The filtered reads were then assembled using Shovill, which incorporates SPAdes and other supporting packages [75, 76]. Following assembly, CheckM (v1.2.3) was used to assess the completeness of the genome and detect potential contamination from other bacterial or genetic material [77]. These steps ensured that Aim 1, which involved assembling the short-read data of 31 isolates using multiple software tools, was successfully completed.

To achieve the most accurate genome assembly, a hybrid approach combining long-read and short-read data were employed using Trycycler for two selected isolates [78]. This method leverages the length and continuity of long-read sequencing with the high accuracy of short-read data. Trycycler integrates outputs from multiple independent long-read assemblers, such

as Flye, Raven, and Miniasm, to produce a consensus long-read assembly [79-81]. In the final polishing stage, the high-accuracy short reads were used to refine the assembly, correcting errors in the long-read consensus and resulting in a more accurate, finalised hybrid assembly.

### 2.4 Phylogenetic analysis

Aim 2 was accomplished by generating detailed phylogenies of the genomes of 31 environmental isolates, in addition to reference sequences (RefSeq) of *Enterobacter* species from the NCBI database, to aid in identifying the species and subspecies of the IWW isolates. Several iterations of phylogenies were constructed, with reference to contextual studies on *Enterobacter* species.

The phylogenetic analyses were conducted using a combination of software tools. Panaroo was used to cluster the genomes into clades based on relatedness and to create a core genome alignment [82]. This alignment was filtered for recombination using Gubbins with default parameters, and the output was then analysed with IQ-TREE [83]. IQ-TREE employs a robust stochastic algorithm to deduce maximum likelihood phylogenetic trees [84]. Additionally, ModelFinder was used to determine the most appropriate partitioning scheme, and ultrafast bootstrap approximation provided reliable branch support values. The resulting phylogenetic trees were visualised and annotated using the Interactive Tree of Life (iTOL) platform [85].

Initially, phylogenetic trees were generated solely for the environmental isolates. RefSeq genomes of known *Ec*C) species, including *E. asburiae, E. ludwigii, E. mori, E. cloacae, E. kobei, E. roggenkampii*, and *E. hormaechei*, were subsequently added. This helped confirm the identity of 11 strains, although the results were unclear for another 11 isolates due to genetic divergence. To resolve this, six diversified *E. asburiae* genomes were added.

The identification of 9 remaining isolates was inconclusive. To clarify this, genomes of additional *Enterobacter* species (e.g., *E. nimipressuralis, E. wuhouensis, E. huaxensis, E. cancerogenus, E. hoffmannii*, etc.) were incorporated. Further iterations involving diversified *E. ludwigii* and *E. cloacae* genomes.

### 2.5 Genome annotation

The thirty-one genome assemblies were annotated using Prokka, an automated pipeline designed to rapidly identify and label coding sequences [86]. The options --compliant and --addgene were used to ensure standardised annotation and the inclusion of relevant gene features. The purpose of annotating the genome assemblies was to identify genes and genomic

structures, particularly elements such as MGEs and ARGs, to enable further investigation. Prokka identifies genes by comparing sequences against major databases, including the NCBI Bacterial Antimicrobial Resistance Reference Gene Database, UniProtKB, ISFinder, and others.

To detect specific ARGs, virulence factors (VFs), and plasmids of interest, ABRicate, a BLAST-based tool, was used with default NCBI BLAST parameters, which require >85% gene coverage and >85% nucleotide identity [87]. ABRicate screens sequences against multiple relevant databases (see Table 1&2) to identify genes associated with antibiotic resistance, virulence factors, and plasmid presence.

**Table 1.** A list of programs to be used for bioinformatics approach

| Program | Description | Reference |
|---|---|---|
| ABRicate | Screens DNA sequences for the presence of known AMR genes. | [87] |
| Prokka | Used for annotating bacterial genomes (coding sequences, rRNA, tRNA, etc.) | [86] |
| MLST | Used for typing multiple 'housekeeping' genes to assign isolates of a microbial species to a sequence type (ST). | [88] |
| Panaroo | Used for pan-genomic analysis, involving the comparison of genomes to multiple members of a bacterial species. | [82] |
| IQTree | Designed to infer evolutionary relationships among sequences or species and form a phylogenetic tree. | [84] |
| BLAST | Used to compare an amino acid or nucleotide sequence to identify regions of similarity. | [89] |

## 2.6 Contextual comparative analysis

Comparisons were made between clinical *Enterobacter* assemblies from the NCBI database and our thirty-one environmental isolates, focusing on identifying shared ARGs, VFs, and MGEs. BLAST results were used to examine sequence homology, particularly in conserved regions associated with MGEs such as plasmids, transposons, and integrons. High sequence identity in these regions suggested shared gene transfer events between environmental and clinical strains.

The arrangement of genes within MGEs was analysed to compare the genomic structure of shared ARGs and VFs, further supporting the identification of common dissemination pathways. Recombination hotspots, such as integron recombination sites, were identified, pointing to possible mechanisms for gene acquisition and spread.

**Table 2.** A list of databases used for ARGs, contextual studies and utilised by ABRicate.

| Database | Description | Reference |
|---|---|---|
| TnCentral | A resource related to transposons (mobile genetic elements) and their impact on bacterial genomes. | [90] |
| MEGARes2.0 | A comprehensive database for acquired antibiotic resistance genes, aiding in the identification of resistance determinants. | [91] |
| ISFinder | A platform dedicated to studying insertion sequences (IS) within genomes, including visualisation tools. | [92] |
| CARD | The Comprehensive Antibiotic Resistance Database provides information on antibiotic-resistance genes and their associated proteins. | [93] |
| VFDB | The Virulence Factor Database catalogues virulence genes found in pathogenic bacteria. | [94] |
| ResFinder | A platform for identifying acquired antibiotic resistance genes in bacterial genomes. | [95] |
| PlasmidFinder | A tool to detect plasmids (extrachromosomal DNA) in bacterial isolates. | [96] |
| PubMLST | A resource for Multi Locus Sequence Typing (MLST) from assembled genomes or read sets. | [97] |
| NCBI | A comprehensive database for biological information, including genomic data and literature references | [98] |
| Custom Databases | Custom databases were made for the specific screening of resistance mechanisms (i.e. *dif* modules and *sil*-operon) | - |

BLAST results were cross-referenced with databases like ISFinder and PlasmidFinder to classify specific MGEs and their role in the movement of resistance genes. Phylogenetic analysis of MGEs was performed to trace their evolutionary history and identify HGT events. Furthermore, custom databases were utilised for the screening of the novel MGE, *dif* modules and for the screening of the silver resistance operon. This analysis provided insights into how environmental reservoirs might contribute to the spread of ARGs and VFs to clinical settings, aligning with the One Health perspective.

# Chapter 3: Results

## 3.1 Draft sequencing of thirty-one environmental isolates of *Enterobacter* spp.

Our study determined and analysed the genome sequence of thirty-one *Enterobacter* isolates collected from South Australian influent wastewater. All 31 isolates were assembled using short-read sequence data, producing draft assemblies. The size of each genome ranged from 4,773,947 to 5,640,369 bp, similar in length to other draft and complete assemblies of *Enterobacter* genomes (4.75–5.69Mbp) [99, 100].

**Table 3. Draft genome assembly details (*n*=31)**

| Isolate | ST | Size (bp) | % G+C | Plasmid Reps |
|---|---|---|---|---|
| 148 | 339 | 5,540,028 | 55 | 10 |
| 153 | - | 5,168,651 | 55 | 6 |
| 158 | 122 | 5,136,046 | 55 | 4 |
| 163 | - | 4,805,542 | 53 | 7 |
| 167 | 520 | 4,856,683 | 55 | 5 |
| 177 | - | 5,107,647 | 55 | 6 |
| 178 | 910 | 4,991,585 | 55 | 2 |
| 181 | - | 5,120,281 | 55 | 6 |
| 182 | 339 | 4,773,947 | 56 | 9 |
| 184 | 807 | 4,910,626 | 55 | 3 |
| 185 | - | 5,187,099 | 53 | 4 |
| 190 | - | 4,966,471 | 53 | 8 |
| 191 | - | 5,112,609 | 54 | 6 |
| 193 | - | 5,029,109 | 55 | 5 |
| 196 | - | 5,008,957 | 55 | 6 |
| 197 | - | 5,640,369 | 53 | 8 |
| 208 | - | 5,074,164 | 55 | 4 |
| 209 | - | 5,458,464 | 50 | 7 |
| 214 | - | 5,296,901 | 56 | 9 |
| 215 | 422 | 4,984,284 | 53 | 6 |
| 220 | - | 4,965,733 | 56 | 8 |
| 234 | - | 4,923,867 | 53 | 5 |
| 237 | - | 4,872,042 | 53 | 3 |
| 242 | - | 4,744,344 | 56 | 5 |
| 257 | - | 5,096,444 | 55 | 4 |
| 329 | 807 | 4,841,971 | 55 | 4 |
| 350 | - | 5,085,508 | 55 | 4 |
| 354 | 339 | 4,867,522 | 55 | 5 |
| 366 | - | 4,800,922 | 55 | 4 |
| 368 | - | 5,079,927 | 55 | 3 |
| 375 | 339 | 4,942,156 | 56 | 5 |

Four of these 10 isolates were assigned to ST339, and two were assigned to ST807, suggesting they are a part of the same clonal complex. The remaining 21 isolates not assigned STs had novel alleles on at least one of the seven housekeeping genes used to assign STs, suggesting a potentially novel or under-characterised genetic lineage within the species.

In draft assemblies, the number of plasmids present was approximated using the number of plasmid replication initiation genes within each genome. In total, 171 plasmid replication initiation proteins (*rep*) genes were observed, ranging from 2 to 10 plasmids, with an average of 5.2 per isolate. This suggests that these environmental *Enterobacter* isolates carry a variety of plasmids, potentially contributing to the spread of resistance traits across different bacterial populations (see section 3.4).

### 3.2 Metadata of publicly available *Ec*C shows neglect of environmental studies

Among the 7,763 publicly available *E. cloacae* assemblies on GenBank, isolates have been sourced from 97 countries spanning six continents. Assessing the metadata of these assemblies, 5% of these samples were environmentally sourced from the publicly available environmental samples; none were Australian samples. From this, it was evident that there is a clear lack of focus on environmental studies revolving around *Enterobacter* spp. Hence, this study is distinct in its focus on environmental *Enterobacter* isolates from South Australian wastewater. Unlike the predominantly clinical datasets available globally, our sampling of environmental isolates addresses a critical gap, offering insights into the role of aquatic environments in disseminating AMR.

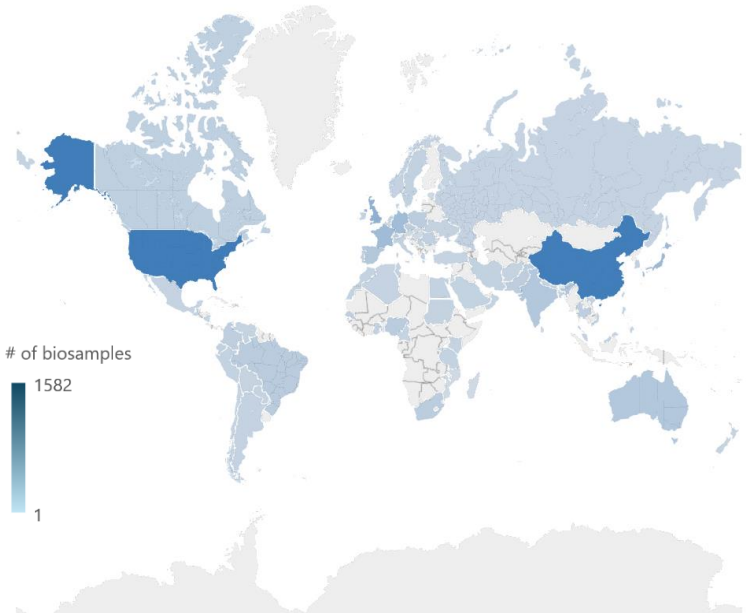



**Figure 11.** Geographical distribution of publicly available *E. cloacae* assemblies from GenBank. (as per 31/05/24).

### 3.3 The thirty-one isolates mainly belonged to *Enterobacter cloacae* Complex (*Ec*C)

To study the phylogenetic relationships and confirm the genus and species designation of genomes analysed in this project, a phylogenetic tree using core genome alignment was constructed using 44 *Enterobacter spp.* known reference genomes available on GenBank (as of 31/05/2024). From all of our genomes, 23 of 31 tightly clustered around five known species. These included *E. asburiae* (*n*=11), *E. roggenkampii* (*n*=5), *E. kobei* (*n*=3), *E. mori* (*n*=3) and *E. soli* (n=1). Five of these did not cluster within any known *Enterobacter spp.* However, they still showed a high genetic homology to *Enterobacter* spp. as they still clustered within relative proximity to strains of the *Ec*C and non-*Ec*C. This suggests that they possibly were diverged from the *Ec*C and may be a very diverged strain of *E. ludwigii*, as they shared an ancestral Class C *β*-lactamase *ampC* gene variant ($bla_{\text{ACT-12}}$) with them. Hence, they may be novel *Enterobacter* spp.

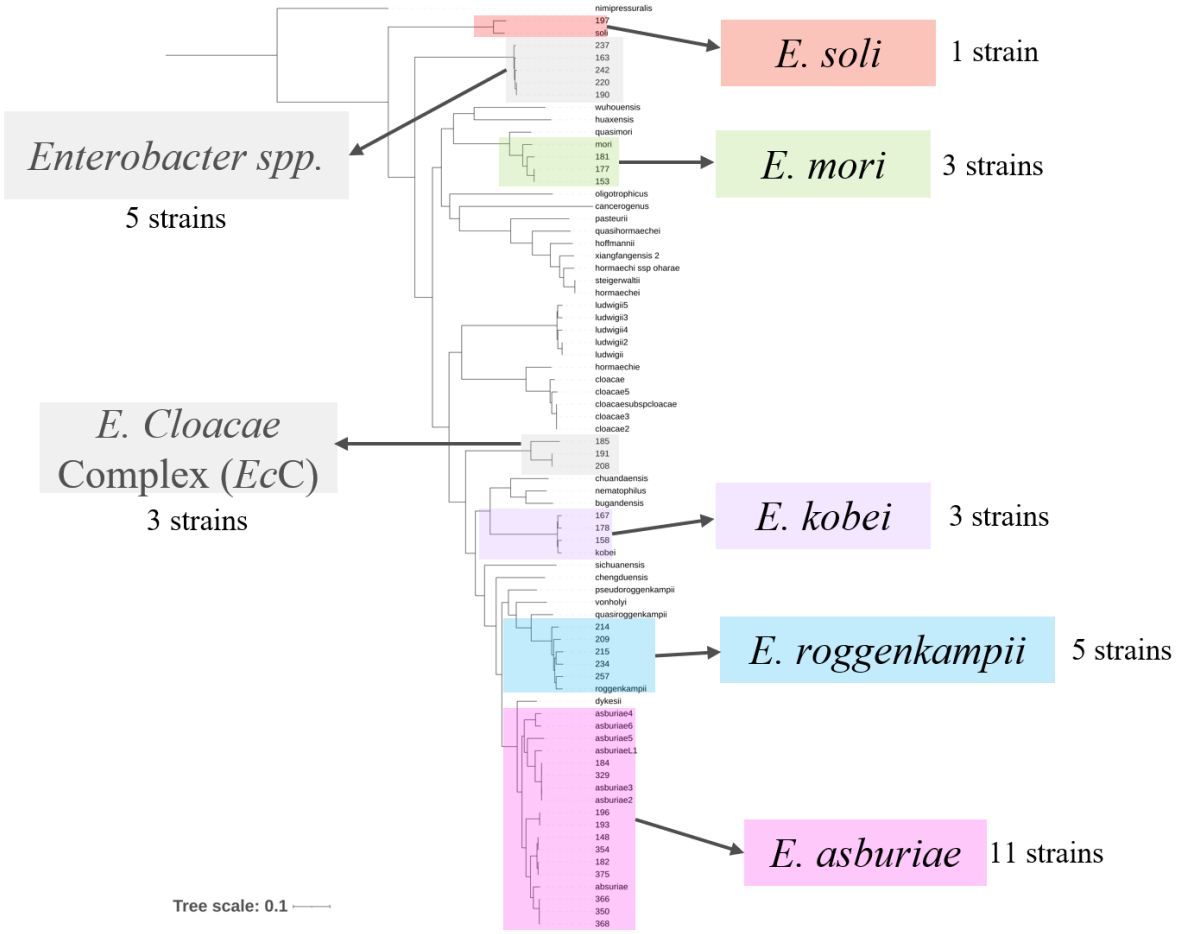



**Figure 12.** Phylogenetic Tree with our 31 environmental *Enterobacter* isolates aligned against all *Enterobacter spp.* reference sequences. Coloured boxes indicate isolates that were precisely determined. Grey boxes indicate isolates that were non-specified. (see Appendix A for RefSeqs and additional genomes used for this core-genome alignment)

Another three of these did not cluster to any specific reference genomes but were part of the *Ec*C clade, consequently they may be novel *E. cloacae* complex species. The presence of potential novel species indicates a genetic diversity that warrants further investigation, especially in the context of environmental AMR reservoirs. Furthermore, the clustering of isolates around known species suggests common genetic backgrounds, which may be linked to shared environmental niches. Therefore, we investigated significant genetic features, such as ARGs, MGEs (i.e. plasmids) and VFs in this study to establish this linkage.

### 3.4 Antibiotic resistance gene repertoires in *E. asburiae* & *E. roggenkampii* genomes

The analysis of ARGs across the environmental *Enterobacter* isolates revealed significant variability in resistance profiles, as illustrated in the heatmap (Figure 13). Notably, *E. asburiae* strain 184 and *E. roggenkampii* strain 209 exhibited higher ARG counts than other strains,

spanning multiple drug classes, including clinically significant last-line carbapenem and colistin resistance, ESBLs, macrolides, and sulfonamides. A recent review on colistin resistance of *Enterobacter* species (*n*=3246) has revealed that *mcr-9* was present in 20% of all *Enterobacter* isolates, as opposed to <2% of isolates for *E. coli* and *Klebsiella* [26]. Thus suggesting *Enterobacter* is a significant contributor to the dissemination of last-resort colistin resistance.

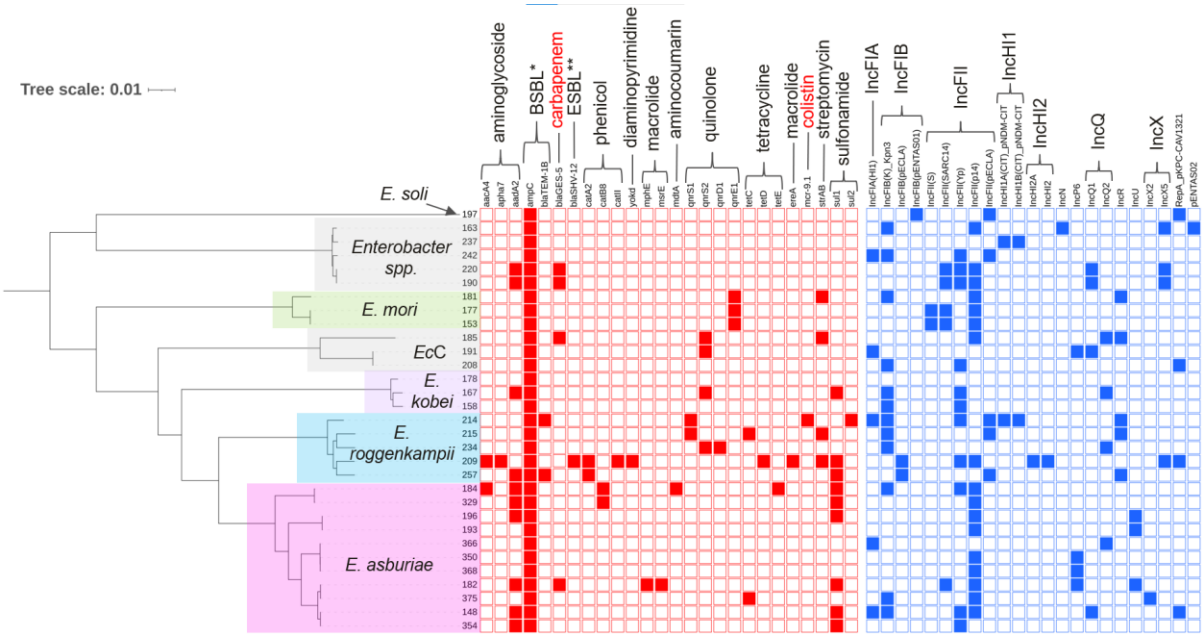



**Figure 13.** Antibiotic resistance gene and plasmid replicon profiles of our *Enterobacter* set. Drug class resistances that are red in the labelling are last-line antibiotics. Red heat map represents resistance genes, blue heat map represents plasmid replicons.
* Broad-spectrum beta-lactamase.
** Extended-spectrum beta-lactamase

Across the set, IncFIA/B and IncFII plasmids were quite widespread. These plasmid types have been associated with high variability and adaptability due to their large conjugative status and ability to acquire resistance to wide range of drug classes (see section **1.4.2.4.1**). IncF-type plasmids have been established in *Ec*C and Enterobacterales in disseminating ESBLs (such as $bla_{\text{CTX-M-15}}$) in clinical outbreaks in Bangui, Africa [101]. Additionally, IncQ plasmids were moderately spread throughout our isolates. IncQ plasmids are small mobilisable plasmids, known for their broad host-range, propagating to many bacteria species and conferring resistance to fluoroquinolones, carbapenems and streptomycins [2, 48, 50, 102].

### 3.5 Complete hybrid assembly of significant spreaders of AMR

**Table 4.** Complete genome assembly details

| Isolate | Size (bp) | % G+C | Plasmid Reps |
|---|---|---|---|
| 209-Chr | 4,797,255 | 55 | - |
| p209-1 | 307,020 | 47 | IncHI2/ IncHI2A |
| p209-2 | 116,706 | 52 | IncFIB |
| p209-3 | 90,196 | 55 | - |
| p209-4 | 74,988 | 52 | *repB* (R1701) |
| p209-5 | 62,378 | 46 | IncX5 |
| p209-6 | 5,349 | 46 | - |
| p209-7 | 4,572 | 48 | - |
| 184-Chr | 4,577,625 | 55 | - |
| p184-1 | 120,852 | 55 | IncFIB |
| p184-2 | 106,013 | 60 | - |
| p184-3 | 97,404 | 56 | - |
| p184-4 | 8,732 | 46 | - |

Due to the extensive resistance profiles of the *E. roggenkampii* (isolate 209) and *E. asburiae* (isolate 184) strains, they were selected for complete hybrid assembly to resolve the genetic context of their antibiotic resistance genes. A total of 11 complete plasmids were identified from the two *Enterobacter* genomes. The IncFIB replicon type was present in both genomes. Plasmid sizes varied from 4,572bp to 307,020bp, with an average size of 90,383bp. These resolved plasmids will be explored throughout the results.

### 3.6 Intrinsic Resistance

#### 3.6.1 Common Class C beta-lactamase found in all *Enterobacter*

AmpC *β*-lactamases are clinically relevant cephalosporinases encoded on the chromosomes found in many of the Enterobacterales family and others, mediating resistance to cephalothin, cefazolin, cefoxitin, and most penicillins [103]. All our *Enterobacter* strains encoded a class C *β*-lactamase (*ampC*) gene, identified as $bla_{\text{ACT}}$ and $bla_{\text{MIR}}$, which shared 85% identity and, thus, were grouped under *ampC*. Comparative analysis of the surrounding regions of *ampC* showed a similar genomic context across species examined here, with *ampR* directly downstream, regulating its expression. This suggests that the *ampC* gene in likely to be conserved across the genus. In many cases, these AmpC enzymes are inducible, resulting in hyper-expression by mutation, leading to resistance to broad-spectrum cephalosporins such as cefotaxime, ceftazidime and ceftriaxone indicating that these environmental strains can potentially be

highly resistant to broad spectrum beta lactamases under selective pressure [104].

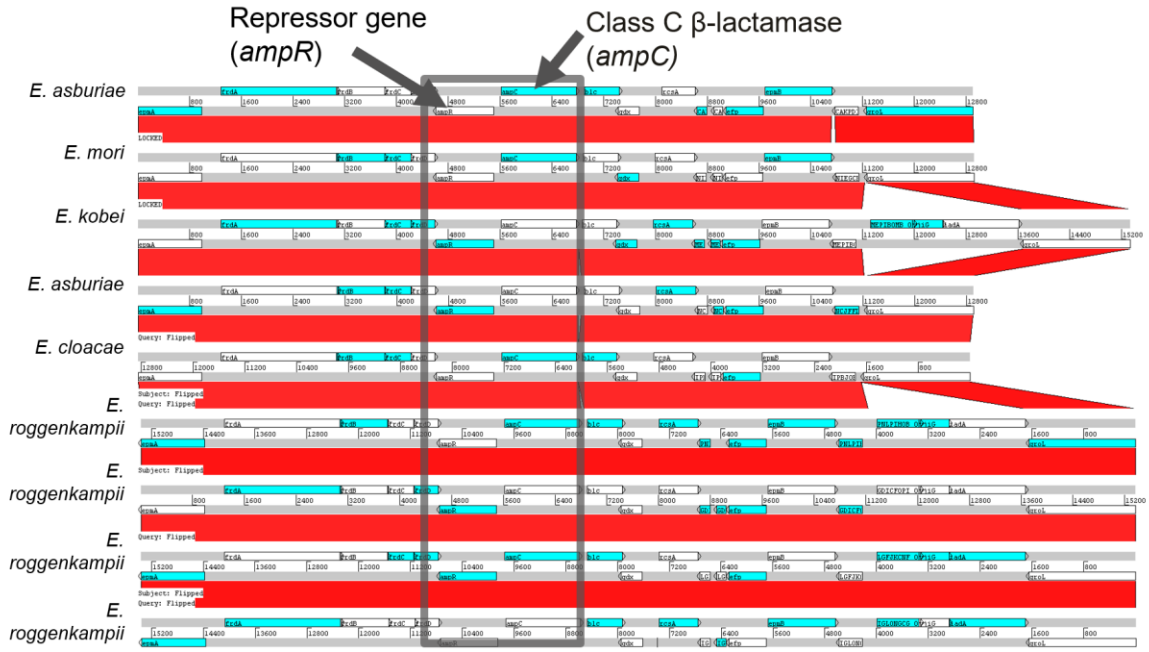



**Figure 14.** Chromosomal genetic context of *ampC* gene across our *Enterobacter*.

### 3.6.2 Resistance to quinolones due to intrinsic resistance determinants

All *Enterobacter mori* (n=3) strains chromosomally encoded the quinolone resistance gene, *qnrE1*. This was confirmed by searching for the resistance gene in the assembly and then blast-searching these contigs using the default parameters. Blast results showed a high percentage of coverage and identity compared to other chromosomal *E. mori* strains that were publicly available. The *qnrE1* gene in all three strains was identical within 10 Kbp flanking regions of the quinolone resistance, which was found within the chromosomal region of the *E. mori*. This is consistent with findings from Albornez 2017, describing *qnrE1* as originating from this subspecies [105].

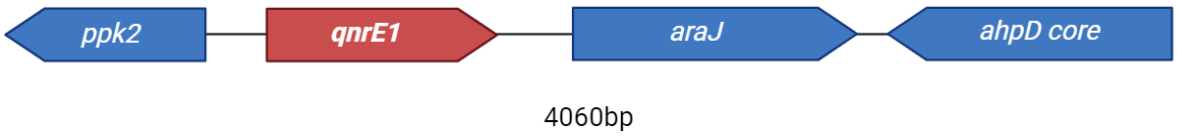



**Figure 15.** Chromosomal genetic context of quinolone resistance gene, *qnrE1*, found in *Enterobacter mori* strains. Creating using Biorender.com

Further analysis of the protein sequence of GyrA and ParC found within the chromosome revealed more information regarding quinolone resistance. As a serine substitution in GyrA at position 83 to threonine, isoleucine, or tyrosine resulted in quinolone resistance. A serine substitution in ParC at position 80 to isoleucine also resulted in quinolone resistance (see

section 1.4.1.1). Therefore, although isolates (208, 209 & 257) do not encode specific quinolone resistance genes as the other isolates, they are likely to confer resistance to quinolones due to their GyrA and ParC substitutions (Table 5).

**Table 5.** Quinolone Resistance present in our environmental *Enterobacter* spp.

| Predicted Species | Isolate No. | GyrA substitution | ParC substitution | *qnr* Gene | Predicted Resistance |
|---|---|---|---|---|---|
| *E. roggenkampii* | 209 | S83T | - | - | R |
| | 214 | S83T | - | S1 | R |
| | 215 | S83T | - | S1 | R |
| | 234 | S83T | - | S2/D1 | R |
| | 257 | S83I | S80I | - | R |
| *E. cloacae* | 185 | - | - | S2 | R |
| | 191 | - | - | S2 | R |
| | 208 | S83Y | - | - | R |
| *E. kobei* | 167 | - | - | S2 | R |
| *E. mori*[1] | 153 | - | - | E1 | R |
| | 177 | - | - | E1 | R |
| | 181 | - | - | E1 | R |

[1]*Enterobacter mori* strains were all present with chromosomal *qnrE1* resistance.

## 3.7 Acquired resistance antibiotic resistance genes.

### 3.7.1 Overview

The 31 environmental *Enterobacter* isolates showed diverse acquired resistance profiles. Firstly, *E. asburiae*, which was our largest set (*n*=11), exhibited significant resistance up to a maximum number of nine genes conferring resistance to carbapenems, aminoglycosides, and macrolides. In this set, six of the eleven strains acquired resistance via the class 1 integron, integrase, *intI1*, resulting in sulfonamide and aminoglycoside (*sul1* and *aadA2* genes) drug resistance (see section 3.7.4). Additionally, last-line antibiotic resistance to carbapenems ($bla_{GES-5}$) was found in strain 182. This strain also carried a novel MGE that was previously only described in *A. baumannii*, carrying the macrolide resistance genes, *msr-mph*(E) (see section 3.8.1).

Moreover, our second largest set (*n*=5), *E. roggenkampii*, had a diverse resistance profile, carrying a maximum of 13 ARGs, including ESBLs, colistin and fluoroquinolone resistance genes. In this set, two of the five strains acquired resistance via the class 1 integron, with both strains carrying aminoglycoside resistance via *aadA2* and *aacA4*. Strain 209 exhibited a complex resistance region, carrying a class 1 integron with six ARGs (diaminopyramidine, macrolide, aminoglycoside and diaminocoumarin), embedded within a potential composite

transposon. This transposon additionally encoded last-line colistin (*mcr-9.1*) resistance, an ESBL ($bla_{SHV-12}$) and streptomycin resistance (*strAB*) within the transposon, all of which were carried on a large conjugative plasmid (307,020 bp) (see section 3.7.6). Three of the *E. roggenkampii* strains (214, 215 & 234) encoded plasmid-mediated quinolone resistance on small mobilisable IncQ plasmids via the *qnrS2* and *qnrS1* genes (see section 3.7.7).

Although the remaining strains did not exhibit significant counts of acquired ARGs, their drug class resistance profiles were still important. For instance, two of the novel *Enterobacter* spp. isolates (190 and 220 strain) and one novel *Ec*C strain (185) encoded last-line carbapenem resistance ($bla_{GES-5}$), with the novel *Enterobacter* spp. strains carrying this on small mobilisable IncQ plasmids (see section 3.7.3).

**Table 6.** Summary of Acquired Resistance within our *Enterobacter* spp.

| Species (n=31) | Max NO. Resistance Gene(s) | Acquired Resistance Gene(s) | Drug Class Resistance |
|---|---|---|---|
| *E. asburiae* (*n*=11) | 9 | *aacA4*, $bla_{GES-5}$, *mph-msr*(E), *tetE*, *catB8*, *aadA2*, *sul1*, *mdtA* | **Carbapenem**, Aminoglycoside, Streptogramin, Macrolide, Tetracycline, Sulfonamide & Aminocoumarin |
| *E. roggenkampii* (n=5) | 13 | *aacA4*, *strAB*, *apha7*, ***bla*SHV-12**, $bla_{TEM-1B}$, *ereA*, ***mcr9-1***, *qnrS1*, *sul1*, *sul2*, *tetCD*, *dfrA19*, *catII*, *ereA2* | **Colistin, Third generation cephalosporins,** Aminoglycoside, Streptogramin, Macrolide, Tetracycline, Sulfonamide, Streptomycin, Fluoroquinolone, Phenicol |
| *E. kobei* (*n*=3) | 3 | *qnrS2*, *aadA2*, *sul1* | Fluoroquinolone |
| *E. mori* (*n*=3) | 2 | *strA*, *strB* | Streptomycin |
| *E. soli* (*n*=1) | 0 | - | - |
| Other *Enterobacter spp.* (*n*=8) | 4 | $bla_{GES-5}$, *aadA2* | Carbapenem, Aminoglycoside |

Furthermore, one *E. kobei* strain (167) encoded a class 1 integron conferring aminoglycosides and sulfonamides via the *aadA2* and *sul1* genes, respectively. This strain also encoded plasmid-mediated quinolone resistance on a small mobilisable IncQ plasmid, via the *qnrS2* gene, similar

to those found in the *E. roggenkampii* strains (214, 215 & 234). Additionally, *E. mori* strain 181 had streptomycin resistance through *strA* and *strB* genes, which appeared as a remnant of a known transposon, Tn*5393* (GenBank AN: M96392.1). This was consistent throughout all cases of streptomycin resistance occurring (i.e. strains 185, 209 and 215). One *E. soli* isolate lacked acquired resistance genes, which was expected as it is not a clinically relevant strain in regards to AMR [10].

### 3.7.2 Plasmid Acquisition Drives AMR spread in *Enterobacter* spp.

We screened our plasmids from the two complete hybrid assemblies (strains 184 *E. asburiae* and 209 *E. roggenkampii*) against all our strains (see Table 7). This revealed selective plasmid sharing, with plasmids like p209-3 (90 K bp), across 15 of the 31 *Enterobacter* strains, including a variety of species *E. asburiae*, *E. kobei*, *E. mori* and other *Enterobacter* spp. These plasmids were not AMR significant as they did not encode any ARGs. Although screening of p209-3 against p184-1 revealed significant sharing of the plasmid backbone (p209-3 had 50% coverage of p184-1), through the coverage of the transfer region, but not the replicon gene – *repA*-IncFIB. BLAST searching of p209-3 against GenBank revealed complete plasmid backbone homology (i.e. complete coverage and identity of p209-3) to a 148 Kbp plasmid, pWP8-W19-CRE-01_2 (GenBank AN: AP022270.1), sourced from *Raoultella ornithinolytica* in Tokyo wastewater that carried the carbapenem ARG, $bla_{\text{GES-24}}$. This reinforces the notion that these plasmids have a broad host range across *Enterobacter* species and have the potential to acquire clinically significant ARGs.

Furthermore, p209-5, an IncX5 plasmid was found in four strains (163, 190, 193, and 220). Although this plasmid did not encode any ARGs, this plasmid type has been notarised for carrying and disseminating the carbapenem resistance gene, $bla_{\text{KPC-2}}$, originating from *K. pneumoniae* [106]. This gene has been recorded in other Enterobacterales, such as *Enterobacter* spp., *E. coli* and *S. marcescens*. Thus, this plasmid type can potentially encode and confer resistance to significant drug classes. Moreover, p184-2 was found in seven of the strains. All *E. mori* strains (153, 177 & 181), two *E. asburiae* strains (182 and 329), and two *E. roggenkampii* strains (234 & 257) encoded p184-2, which was involved with virulence, carrying the gene *entA* (see section 3.9 for more on virulence). This plasmid type being found across a variety of species, suggests its contribution to increasing the pathogenicity of *Enterobacter* through virulence determinants. This appeared to be the same case with p184-4,

which was found in one *E. asburiae* strain 182, two *E. mori* strains (153 & 177) and one *E. kobei* strain 158.

**Table 7.** Screening of Complete Hybrid Assembly (184 & 209) plasmids vs. our *Enterobacter*

| Expected Species ID/ Strain | p209 -1 | p209 -2 | p209 -3 | p209 -4 | p209 -5 | p209 -6 | p209 -7 | p184 -1 | p184 -2 | p184 -3 | p184 -4 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *E. asburiae* (148) | - | - | + | - | - | + | - | - | - | - | - |
| *E. asburiae* (182) | - | - | - | - | - | - | - | - | + | - | + |
| *E. asburiae* (184)[a] | - | - | +[b] | - | - | - | - | + | + | + | + |
| *E. asburiae* (193) | - | - | + | - | + | - | - | - | - | + | - |
| *E. asburiae* (196) | - | - | + | - | - | - | - | - | - | + | - |
| *E. asburiae* (329) | - | - | + | - | - | + | - | - | + | - | - |
| *E. asburiae* (350) | - | - | - | - | - | - | - | - | - | - | - |
| *E. asburiae* (354) | - | - | - | - | - | - | - | - | - | - | - |
| *E. asburiae* (366) | - | - | - | - | - | + | + | - | - | + | - |
| *E. asburiae* (368) | - | - | + | - | - | - | - | - | - | - | - |
| *E. asburiae* (375) | - | - | + | - | - | - | - | - | - | - | - |
| *Enterobacter* spp. (163) | - | - | + | - | + | - | - | + | - | - | - |
| *Enterobacter* spp. (190) | - | - | - | - | + | - | - | - | - | - | - |
| *Enterobacter* spp. (220) | - | - | - | + | + | + | - | - | - | - | - |
| *Enterobacter* spp. (237) | - | - | - | - | - | - | - | - | - | - | - |
| *Enterobacter* spp. (242) | - | - | + | - | - | - | - | + | - | - | - |
| *E. kobei* (158) | - | - | + | - | - | - | - | - | - | - | + |
| *E. kobei* (167) | - | - | - | - | - | + | - | - | - | - | - |
| *E. kobei* (178) | - | - | - | - | - | - | - | - | - | - | - |
| *E. mori* (153) | - | - | + | - | - | - | - | - | + | - | + |
| *E. mori* (177) | - | - | - | - | - | - | - | - | + | - | + |
| *E. mori* (181) | - | - | + | - | - | - | - | - | + | - | - |
| *E. roggenkampii* (209) [a] | + | + | + | + | + | + | + | +[b] | - | - | - |
| *E. roggenkampii* (214) | - | - | - | + | - | - | - | - | - | - | - |
| *E. roggenkampii* (215) | - | - | - | + | - | - | - | - | - | - | - |
| *E. roggenkampii* (234) | - | - | - | - | - | - | - | - | + | - | - |
| *E. roggenkampii* (257) | - | - | - | - | - | - | - | - | + | - | - |
| *E. soli* (197) | - | - | - | - | - | + | - | - | - | - | - |
| Novel *Ec*C (185) | - | - | + | - | - | - | - | - | - | - | - |
| Novel *Ec*C (191) | - | - | - | - | - | - | - | - | - | - | - |
| Novel *Ec*C (208) | - | - | + | - | - | - | - | - | - | - | - |

[a] Strains that were complete hybrid assembled and used for screening of their plasmids against all the isolates

[b] p184-1 and p209-3 are likely related due to significant sharing of plasmid backbone (50% coverage)

This plasmid encoded the virulence gene, *ompA*, contributing to the stability of the outer membrane of bacteria, biofilm formation, adhesion and invasion, and immune evasion. All of which indirectly contribute to antibiotic tolerance and persistence. These virulence plasmids being shared throughout our set, suggest the transmission of these plasmids to be significant to the pathogenicity of *Enterobacter*.

### 3.7.3 Carbapenem Resistance carried on small mobilisable IncQ plasmids

Carbapenem antibiotics are often considered the last line of defense against multidrug-resistant Gram-negative infections, making resistance to these drugs a critical public health concern (see section 1.3.1). Two environmental novel *Enterobacter* spp. isolates (strains 190 and 220) were found to carry carbapenem resistance on a small mobilisable IncQ1 plasmid similar to pCHE-A, previously identified in clinical outbreaks of *E. cloacae* in Canada. These findings underscore the potential for environmental strains to act as reservoirs for resistance genes that can be mobilised into clinical settings.

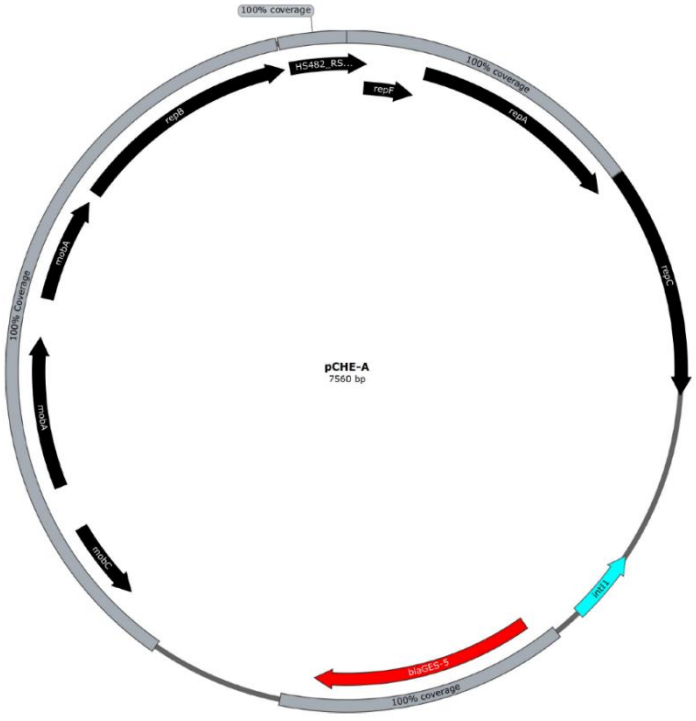



**Figure 16.** Plasmid pCHE-A: Carbapenem resistance identified in isolates 190 and 220 of the novel *Enterobacter spp*. Regions of grey around the plasmid signify a complete coverage of pCHE-A.

Both isolates carried an identical 9,232 bp plasmid, displaying 69% coverage and 99.18% identity to pCHE-A (7,560 bp, GenBank AN: NC_012006.1), which encodes the *bla*$_{\text{GES-5}}$ gene responsible for carbapenem resistance. The *bla*$_{\text{GES-5}}$ gene is known for its ability to hydrolyse carbapenems, conferring resistance that limits treatment options. Additionally, the plasmids included essential regions for mobilisation and replication, such as *repB*, *repA*, *repF*, *mobA*, and *mobC*, suggesting they can be efficiently transferred between bacteria through HGT. The presence of these genes highlights a

mechanism that facilitates the spread of resistance, which is particularly concerning given the plasmid's small size, allowing for easy transfer across different bacterial species (Figure 16).

### 3.7.4 Class 1 Integron – a hotspot to capture ARGs

Class 1 integrons are key MGEs that facilitate the acquisition of ARGs by serving as hotspots for capturing gene cassettes associated with AMR. Our genomic analysis identified the presence of class 1 integrons in nine of the 31 isolates, predominantly in *E. asburiae* (6 of 11 strains), *E. roggenkampii* (2 of 5 strains), and one *E. kobei* strain. The detection of these integrons was achieved by screening for the integrase gene, *intI1*, and confirming the presence of the 5' and 3' conserved segments (refer to Figure 6).

In all cases but *E. roggenkampii* strain 209, the integrons primarily encoded aminoglycoside resistance (*aadA2*), which was the first gene cassette captured. Additionally, two isolates (185 and 209) carried aminoglycoside resistance genes (*aacA4*) within their integrons. The most complex integron was identified in a fully assembled hybrid genome of *E. roggenkampii*, containing six ARG cassettes (see Figure 20).

Notably, the integrons were consistently located on plasmids across all isolates. In the complete hybrid assemblies, they were associated with IncFIB and IncHI2A plasmid types. While the exact plasmid types could not be resolved for draft assemblies due to limited genetic context, exploring GenBank using BLAST on contigs carrying key integron components indicated that all draft assemblies carried *intI1* on a plasmid. In the two complete hybrid assemblies, *intI1* was confirmed to be integrated within transposons, enabling the plasmids to ensure mobility and spread of AMR. This finding aligns with the known features of integrons, which are not inherently mobile and rely on other MGEs, such as transposons and plasmids, to facilitate the dissemination of resistance genes [35, 36].

### 3.7.5 Complete hybrid assembly of *Enterobacter asburiae* strain 184

Isolate 184, a complete hybrid-assembled strain, encodes four plasmids ranging in size from 8,732 to 120,852 bp. Its chromosomal size (4,577,625 bp) falls within the expected range for *Enterobacter*. Among the plasmids, only p184-1 was associated with acquired AMR, while p184-4 carried the virulence factor (*ompA*) and p184-2 encoded *entS* (see section 3.9). Plasmid p184-1 (120,852 bp) was particularly notable, containing six ARGs within a 41,911 bp resistance region, primarily located on a novel 23,906 bp transposon, Tn*6928*. This transposon

is mobilised by IS*Pa38* elements, a member of the Tn*3* family, flanking both ends. The presence of a class 1 integron, *intI1*, within Tn*6928* facilitates the capture and dissemination of resistance cassettes, including genes conferring resistance to aminoglycosides, phenicols, and other drug classes.

**Table 8. Properties of *E. asburiae* (184) strain complete hybrid assembled**

| Plasmid/ Chromosome | Plasmid Type | Size (bp) | AMR Gene(s) | Additional Feature(s) | Drug Class Resistance |
|---|---|---|---|---|---|
| Chromosome | - | 4,577,625 | *ampC* | - | 1st Gen Cephalosporin |
| p184-1 | IncFIB | 120,852 | *tetE, aacA4, aadA2, sul1, mdtA, catB8* | Tn*6928*, *intI1* | Tetracycline, aminoglycoside, sulfonamide, aminocoumarin & phenicol |
| p184-2 | - | 106,013 | - | *entS* | - |
| p184-3 | - | 97,404 | - | - | - |
| p184-4 | - | 8,732 | - | *ompA* | - |

Exploring GenBank, ISFinder and TnCentral using the full sequence of this transposon revealed that there were no publicly available sequences that covered this region in its entirety, and it has not been reported explicitly as a transposon by any published literature. Hence, it was named Tn*6928* (Figure 17).

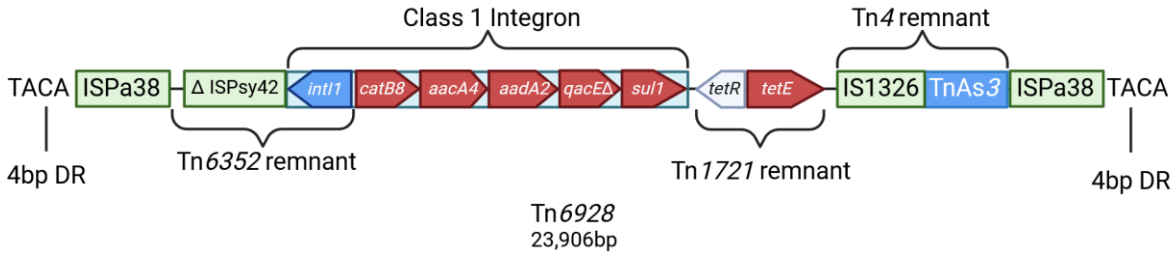



**Figure 17.** Novel transposon, Tn*6928*, found on p184-1. Resistance genes are marked in red. Insertion sequences are marked in green. Mobile genetic elements are marked in blue. Direct repeats on flanking ends of the transposon indicate insertion of this transposon.

The identification of Tn*6928* is significant as it highlights a potential mechanism for HGT, enabling the spread of multiple resistance traits. Such novel transposons, facilitated by IS elements, serve as hotspots for gene acquisition and recombination, contributing to the adaptability and survival of bacterial strains under antibiotic pressure.

Additionally, other sequences within the novel transposon, such as IS*1326* and TnAs*3*, appear to be remnants of Tn*4*, and the delta-IS*Psy42* aligns with sequences from Tn*6352*, indicating historical integration events. This suggests a dynamic history of genetic rearrangements that may have facilitated the acquisition of resistance genes over time. Similarly, the acquisition of tetracycline resistance (*tetE*) appears to be a remnant of Tn*1721*, a member of the Tn*3* family, further illustrating the mobility of AMR determinants across different bacterial populations (Figure 18).

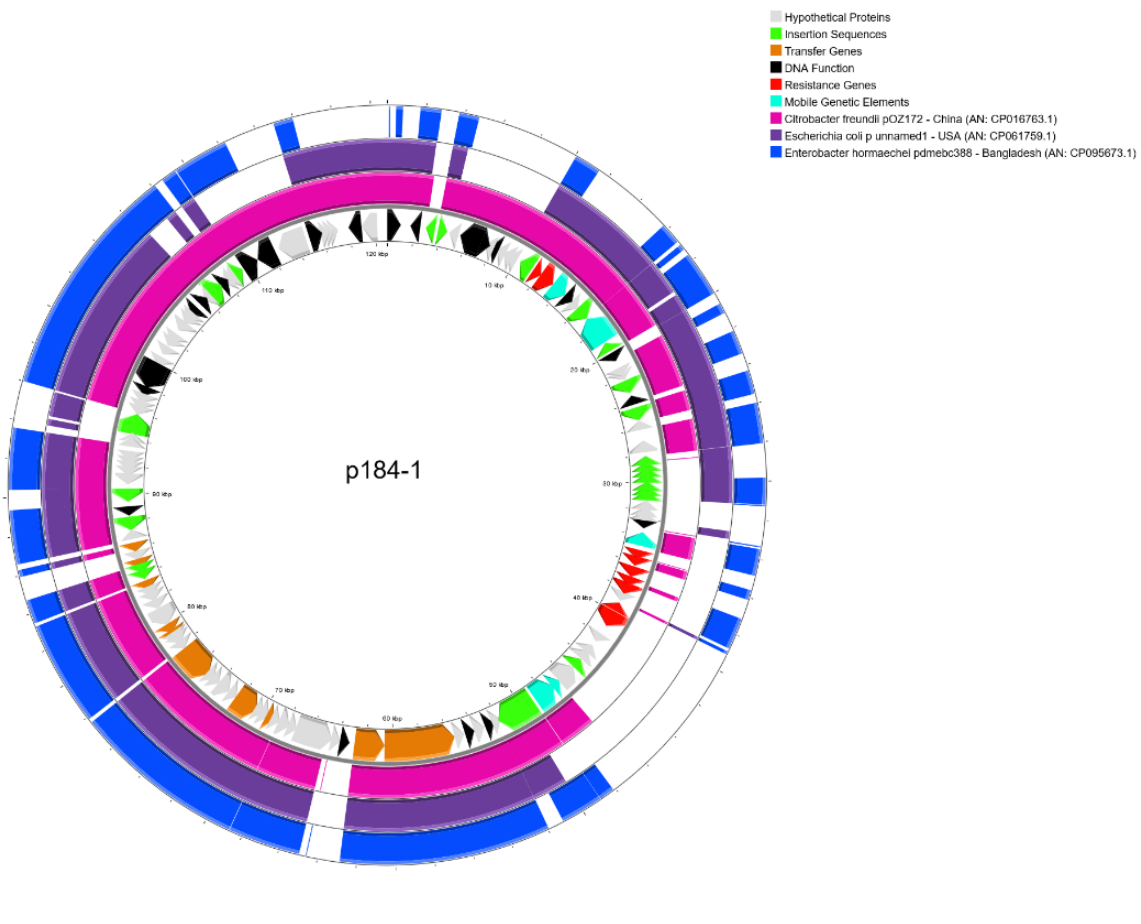



**Figure 18**. Plasmid, p184-1, aligned against similar clinically distributed plasmids, highlighting shared resistance features. Pink plasmid indicates pOZ172 sourced from *C. freundii* involved in a last-line imipenem (*bla*$_{\text{IMI-4}}$ gene) resistance outbreak in Hong Kong. Purple plasmid indicates p-unnamed1 sourced from *E. coli* involved in a foodborne outbreak of *E. coli* in US. Blue plasmid indicates pdkmebc388 sourced from carbapenem resistant *Enterobacter hormaechei* in a clinical outbreak in Bangladesh. These plasmids were selected for comparison in this diagram as they had the highest coverage of our resolved plasmid, thus indicate relatedness of these plasmid types. Innermost ring with genes labelled indicates p184-

1. Resistance genes marked in red, transfer genes in orange, blue genes indicate MGEs and DNA function genes marked in black. Picture drawn to scale using Proksee [107].

The identification of these MGEs on plasmids, particularly IncFIB-type plasmids like p184-1, is concerning because such plasmids are known for their ability to transfer between diverse bacterial hosts. The integration of resistance genes within these MGEs allows for their rapid spread within clinical settings and across environmental reservoirs, potentially enhancing the persistence of resistance in different ecological niches.

### 3.7.6 Complete hybrid assembly of *Enterobacter roggenkampii* strain 209

Our complete assembly of *E. roggenkampii* strain 209 revealed a 4,797,255bp chromosome and seven encoded plasmids ranging in size from 4,572bp to 307,020bp. However, only two of these plasmids were involved with AMR. In particular, p209-1 was an extensively drug-resistant plasmid carrying 12 ARGs within a large conjugative plasmid (307,020 bp) (Figure 20).

**Table 9.** Properties of *E. roggenkampii* (209) strain complete hybrid assembled.

| Plasmid/ Chromosome | Plasmid Type | Size (bp) | AMR Gene(s) | Additional Feature(s) | Drug Class Resistance |
|---|---|---|---|---|---|
| C | - | 4797255 | *ampC* | - | 1st Gen cephalosporin, monobactam |
| **p209-1** | IncHI2/ IncHI2A | 307,020 | ***bla*SHV-12**, *dfrA19*, ***mcr-9.1***, *strAB*, *tetD, aacA4, sul1, apha7, catII, ereA2, yokD, qacE*Δ | Class 1 Integron *intI1* | **Third generation cephalosporins**, **colistin** diaminopyrimidine, streptomycin, tetracycline, aminoglycoside, sulfonamide, phenicol & macrolide |
| **p209-2** | IncFIB | 116,706 | *apha7* | Tn*3, cop-sil* operon | Aminoglycoside |
| p209-3 | - | 90,196 | - | - | |
| p209-4 | *repB* (R1701) | 74,988 | - | - | |
| p209-5 | IncX5 | 62,378 | - | - | - |
| p209-6 | - | 5,349 | - | - | - |
| p209-7 | - | 4,572 | - | - | - |

Eleven of the ARGs were carried within a potential transposon in a complex resistance region, however the exact bounds of this transposon were difficult to characterise due to multiple

insertion events of the ISs (Figure 20). However, this complex resistance region was flanked by two IS*5*. Within this 32,430 bp region there was a significant amount of ARGs conferring resistance to last-line antibiotics, such as Colistin, and as well as an ESBL. These resistance genes were likely mobilised and captured via the IS26, a member of the IS6 family, which is flanking both of these genes, respectively.

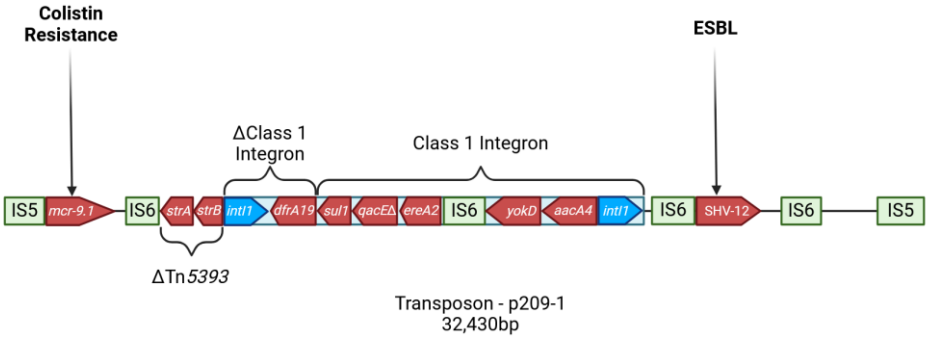



**Figure 19.** Complex resistance region on p209-1 (32,430 bp), hypothetically carried on a composite transposon. Resistance genes are marked in red. Insertion sequences are marked in green. Mobile genetic elements marked in blue.

This complex resistance region was shared with globally and clinically significant plasmids found in Denmark and Alaska. The class 1 Integron was encoded within this region, however there were two copies of *intI1*, potentially disabling it from further acquiring gene cassettes.

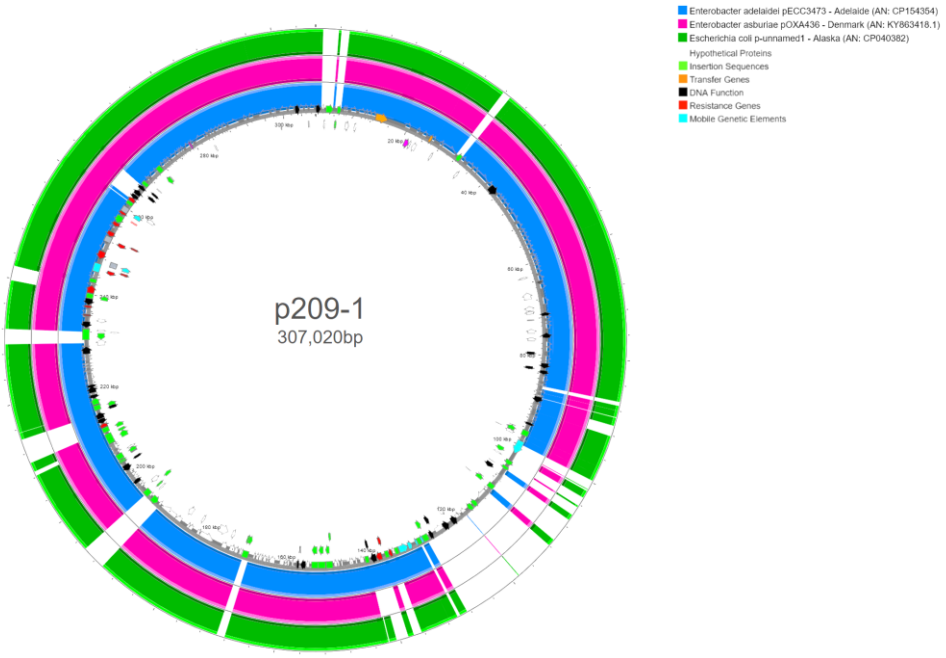



**Figure 20.** Plasmid, p209-1, aligned against three other plasmids that were clinically and globally distributed with significant resistance. Blue plasmid indicates a colistin (*mcr-9.1*) resistant plasmid, pECC3473, isolated from a novel species of *Ec*C – *Enterobacter adelaidei*.

Pink plasmid indicates carbapenem ($bla_{OXA-4}$) resistant plasmid, pOXA436, isolated from a clinical outbreak of *E. asburiae* in Denmark. Green plasmid indicates carbapenem ($bla_{KPC-2}$) resistant plasmid, p-unnamed1, isolated from a clinical outbreak of *E. coli* in Alaska. These plasmids were selected for comparison as they had the highest coverage of p209-1 and were clinically significant, suggesting global dissemination of our environmental strains. The innermost ring indicates p209-1. Resistance genes are marked in red, transfer genes in orange, blue genes indicate MGEs and DNA function genes are marked in black. Picture drawn to scale using Proksee [107].

Within the class 1 integron were 6 ARGs, conferring resistance to macrolides, aminoglycosides and diaminopyrimidines. Furthermore, streptomycin resistance (*strA* & *strB*) shared identity (34% query coverage) with the Tn*5393*, a member of the Tn*3* family, suggesting it is an ancestral remnant of this transposon.

### 3.7.7 Quinolone resistance was largely acquired via small mobilisable plasmids

Quinolone resistance in two of our isolates were shown to both carrying the *qnrS2* gene on identical plasmids to a small mobilisable IncQ2 plasmid pGNB2 (GenBank AN: DQ460733.1). Strain 185 *E. cloacae* exhibited 100% coverage and sequence identity to pGNB2, while strain 234 *E. roggenkampii* had 78.8% coverage, missing only the *orfI* gene from the accessory module. This aligns with the plasmid-replicon profile for these isolates as they both encoded the IncQ2 *rep* gene (figure 13). Both isolates carried the *qnrS2* gene, originally identified on pGNB2 from a bacterial community in a wastewater treatment plant in Germany. This plasmid's small size and IncQ-family backbone, including replication (*repA, repC*) and mobilisation (*mobC, orf1*) modules, allow it to transfer efficiently across diverse bacterial hosts, facilitating the spread of resistance traits via HGT.

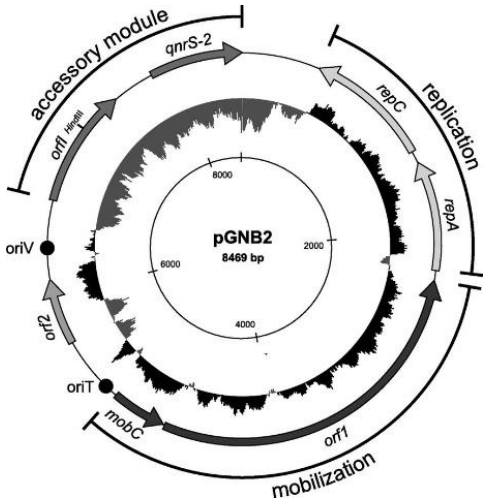



**Figure 21.** Plasmid, pGNB-2, carrying quinolone resistance, *qnrS2*, homologous to strains 185 and 234. Taken from [2]

The *qnrS2* gene encodes a protein that protects bacterial DNA gyrase and topoisomerase IV—key targets of quinolones—from inhibition, reducing susceptibility to various quinolones,

including nalidixic acid and fluoroquinolones like ciprofloxacin and levofloxacin. The origin of pGNB2 in an environmental reservoir highlights the potential for these settings to act as sources of resistance traits that may reach clinical pathogens. The modular nature of pGNB2's accessory elements, as seen in isolate 234, indicates flexibility in the plasmid structure, allowing it to adapt to various hosts and environments, enhancing its potential for widespread dissemination.

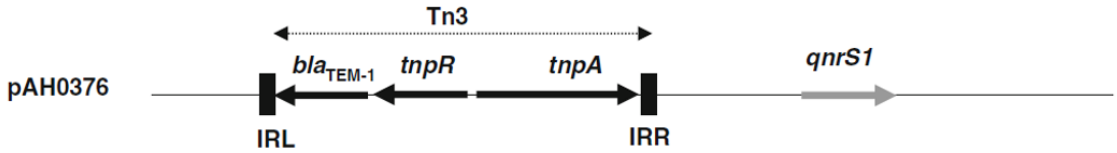



**Figure 22.** Plasmid, pAH0376, carrying quinolone resistance, *qnrS1*, notarised by its Tn*3* located upstream of it. Homologous to isolate 214. Adapted from [49]

Strain 214 *E. roggenkampii* carried *qnrS1* resistance on a small mobilisable plasmid similar to that sourced from a clinical outbreak in Japan, pAH0376 (GenBank AN: AB187515.1). This plasmid is ancestral and has been described thoroughly in literature in regards to plasmid-acquired quinolone resistance. Directly upstream of the resistance is a Tn*3* that encodes the narrow-spectrum beta-lactamase $bla_{TEM-1B}$.


**Figure 23.** Plasmid, p426_p3, carrying *qnrS2* – quinolone resistance. Homologous to strain 167 *E. kobei.* Taken from [108]

*E. kobei*, strain 167, carried *qnrS2* resistance on a small mobilisable IncQ2 plasmid sourced for IWW in Poland (figure 13), p426_p3. This was confirmed via its complete coverage and identity to the plasmid. Strain 167 encoded the key genes *mobC, mobA, repB, repA* and *repC* all required for the mobilisation and replication of this plasmid. This also aligns with the plasmid replicon profile of this isolate (Figure 13).

Strain 191, novel *Ec*C, encoded quinolone resistance, *qnrS2*, on a small mobilisable plasmid with complete coverage and identity to an IncQ2 plasmid, pHP18 (6388 bp, GenBank AN: KU644672.1). Strain 191 encoded all regions of mobilisation and replication, involving genes such as *repC, repA, mobA, mobC and orfA*. Additionally encoding the resistance region, involving fluoroquinolone resistance, via the *qnrS2* gene. The source of this plasmid's

resistance is theorised to be mobilised by a mobile insertion cassette, which encodes IS-like features including left and right inverted repeats and the generation of 5 bp direct repeats flanking these inverted repeats, allowing for the movement and insertion of the *qnrS2* gene.

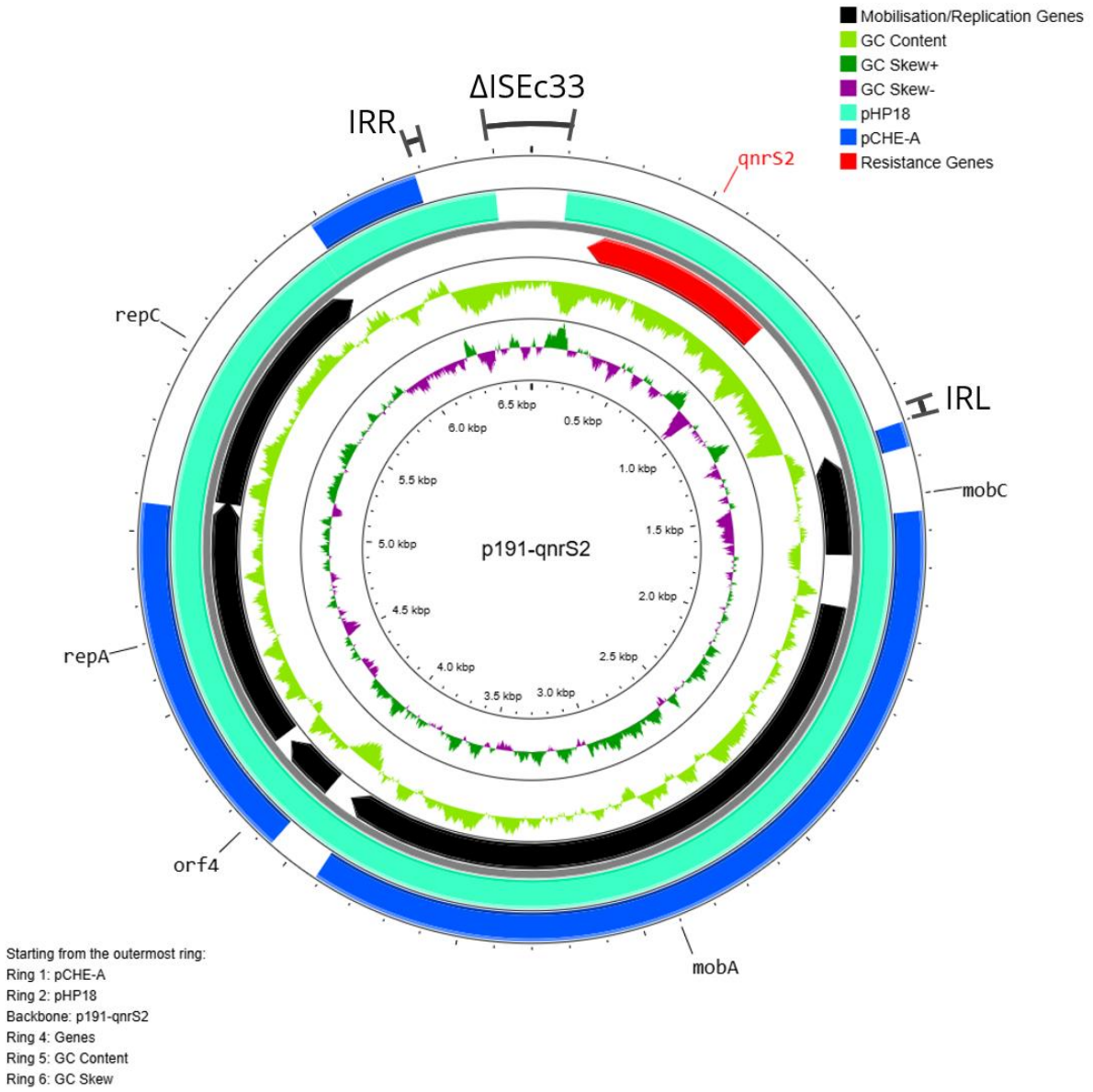



**Figure 24.** Plasmid, p191-qnrS2, aligned against similar plasmids, pHP18 and pCHE-A. Turqoise plasmid indicates pHP18, the original plasmid found in river water of China, which was completely covered by our novel *Ec*C strain 191. Dark blue plasmid indicates pCHE-A involved with carbapenem resistance. Genes in black indicate replication/mobilisation genes. Genes in red indicate resistance genes. GC content and skew indicated by inner green rings. Picture drawn to scale using Proksee [107].

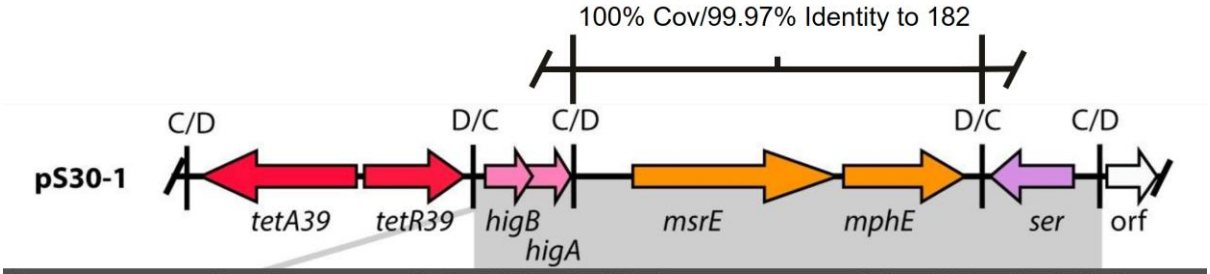
This mobile insertion cassette likely utilises the function of a transposase encoded within the genome of this isolate to mobilise, as it does not encode its own transposase. This IncQ2

plasmid is also related to the carbapenem (*bla*$_{GES-5}$) resistant IncQ2 plasmid, pCHE-A (Figure 16), due to its shared backbone (figure 24). This was evident in the predominant sharing of replication and mobilisation genes via the coverage of *repA*, *orfA*, and *mobA*, having ~50% coverage of this pHP18.

## 3.8 Other mobile genetic element found in *Enterobacter* spp.

### 3.8.1 *E. asburiae* strain encodes novel *dif* modules

The *E. asburiae* strain 182 was found to carry macrolide resistance genes, *msr-mph*(E) on a XerC/XerD recombination site. Screening of a custom database for *dif* modules revealed *E. asburiae* strain 182 encoding two *dif* sites on a particular contig. They were apart of a 2950 bp fragment size module, flanked by 28 bp sequences identical to those previously identified in *dif* modules reported in *A. baumannii* [52]. Our comparisons of the module found in strain 182 to those identified in *A. baumannii* in pS30-1 (GenBank AN: KY617771.1) were determined to be almost identical (see figure 25) This system, initially identified only in *A. baumannii*, demonstrates a broader role in spreading AMR genes across multiple genera, including *Enterobacter*. This is the first documented instance of such a module found in environmental *Enterobacter*, suggesting that *dif* modules plays a more significant role in disseminating AMR than previously understood.




| Sequence | *msrE-mphE* fragment size (bp) | Left p*dif* site: XerC-XerD | Right p*dif* site: XerD-XerC |
|---|---|---|---|
| pS30-1 | 2950 | ATTTCGTATAAGGTGTATTATGTTAATT | ATTTAACATAAAATTTCTTATGTGAAGT |
| 182 | 2950 | ATTTTATAATGGTGGGCTTTTGTTGTGT | ATTTAACATAAAATTTCTTATACGAAAT |

**Figure 25.** *msr-mph*(E) alignment of our sequence to *A. baumannii* strain plasmid, pS30-1. Amino acids marked in red indicate point mutations. Adapted from [52].

Given the significance of this only being reported in *A. baumannii*, and this being the first ever recorded instance of *Enterobacter* spp. encoding this *dif* module, it was decided to explore GenBank for further context. Upon exploring GenBank using our *dif* module as the search query, it was found that the *dif* module appeared in various other genera and species signifying the widespread nature of this novel MGE. For instance, it was found in *Klebsiella pneumoniae*, *Salmonella enterica*, *Escherichia coli*, *Proteus. mirabillis*, and *Morganella morgannii*. All of

these cases were from a variety of isolation sources as well, involving clinical isolates from blood, environmental cases such as municipal wastewater and animal sources such as wild birds (see table 10). Thus, suggesting the ability of these *dif* modules in disseminating resistance across a variety of Genera, species, clinical settings, environmental settings and animal settings. Further reinforcing the One Health framework utilised throughout this study to suggest the interconnectedness of this ecosystems. The mechanism, once thought to be restricted to *Acinetobacter*, is now shown to be more generic, primarily located on plasmids or chromosomes across multiple species, indicating its potential to facilitate broad HGT.

**Table 10.** Strains with complete coverage of *msr-mph*(E) *dif* module site from our *Enterobacter*

| Strain | Species | Country | Year | Source | Chromosome /Plasmid | Size (bp) | GenBank Accession |
|---|---|---|---|---|---|---|---|
| 692 | *E. hormaechei* | Lebanon | 2021 | Blood | pVIM-Exn692 | 196427 | OR497351.1 |
| FL23 | *E. kobei* | Brazil | 2023 | Recreational Coastal waters | pEkFL23_IncU | 31631 | OR098884.1 |
| A2-1 | *K. pneumoniae* | Japan | 2017 | Municipal Wastewater | pA2-4 | 86197 | LC508722.1 |
| 6911.28 | *K. pneumoniae* | Switzerland | 2018 | Blood | p2-6911.28 | 41470 | CP082997.1 |
| SGH0823 | *A. baumannii* | Singapore | 2008 | Endotracheal aspiration | pS30-1 | 18234 | KY617771.1 |
| D46 | *A. baumannii* | Australia | 2010 | Urine | pD46-4 | 208004 | CP048135.1 |
| 12-01738 | *S. enterica* | Germany | 2012 | Wild bird | pSE12-01738-2 | 177190 | CP027679.1 |
| ST_F0903R | *S. enterica* | China | 2020 | Human Faeces | pST_HI2_NDM-1 | 321025 | CP129631.2 |
| A1_181 | *E. coli* | US | 2019 | Human Faeces | p_unnamed1_KPC2 | 210031 | CP040068.1 |
| PG010208 | *E. coli* | Chile | 2020 | Cattle | pPG010208 | 135803 | HQ023861.1 |
| HBRJC7FQ | *P. mirabilis* | China | 2020 | Broiler | pHBRJC7 | 142092 | MK630213.1 |
| CCUG70746 | *P. mirabilis* | Sweden | 2013 | Human Faeces | pPmi70746_1 | 191769 | CP023274.1 |
| MM9291 | *M. morganii* | China | 2020 | Urine | pMM9291-NDM-1_257k | 257632 | CP149990.1 |
| MMAS2018 | *M. morganii* | China | 2018 | Swine | C | 4025805 | CP086203.1 |

### 3.8.2 Novel *dif* modules found across variety of *Enterobacter* species

Screening of a custom database of p*dif* sites against all *Enterobacter* genomes, revealed consistent *dif* sites across a variety of our species. It was found that 12 of the 31 strains encoded the XerC/XerD recombination sites, p*dif58* and p*dif211*. In each case of these *dif* sites, it was discovered that they encoded a GNAT family N-acetyl transferase gene and a conserved protein domain family (DUF1778) gene within a 939 bp *dif* module.

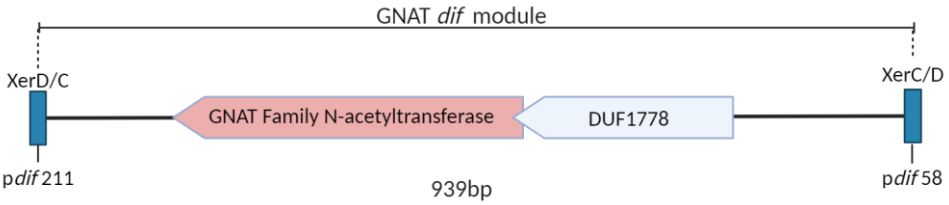



**Figure 26.** GNAT *dif* module. Creating using Biorender.com.

To confirm whether this region was inserted or apart of the chromosome, we aligned all cases of the *dif* module plus an additional 100 bp from each flanking module. The alignment was different for each case when observing the 100bp flanking sides. Additionally, a hypothetical sequence query that involved 100 bp from the left and right flanking regions was crafted. Then multiple BLAST searches of these hypothetical sequences against GenBank was completed. Results for these searches presented multiple continuous sequences with 100% coverage and 100% identity to the hypothetical sequence. The BLAST hits were significant to plasmids. Thus, suggesting that this sequence has likely been mobilised and inserted via the mechanism of *dif* modules and is likely carried on a plasmid.

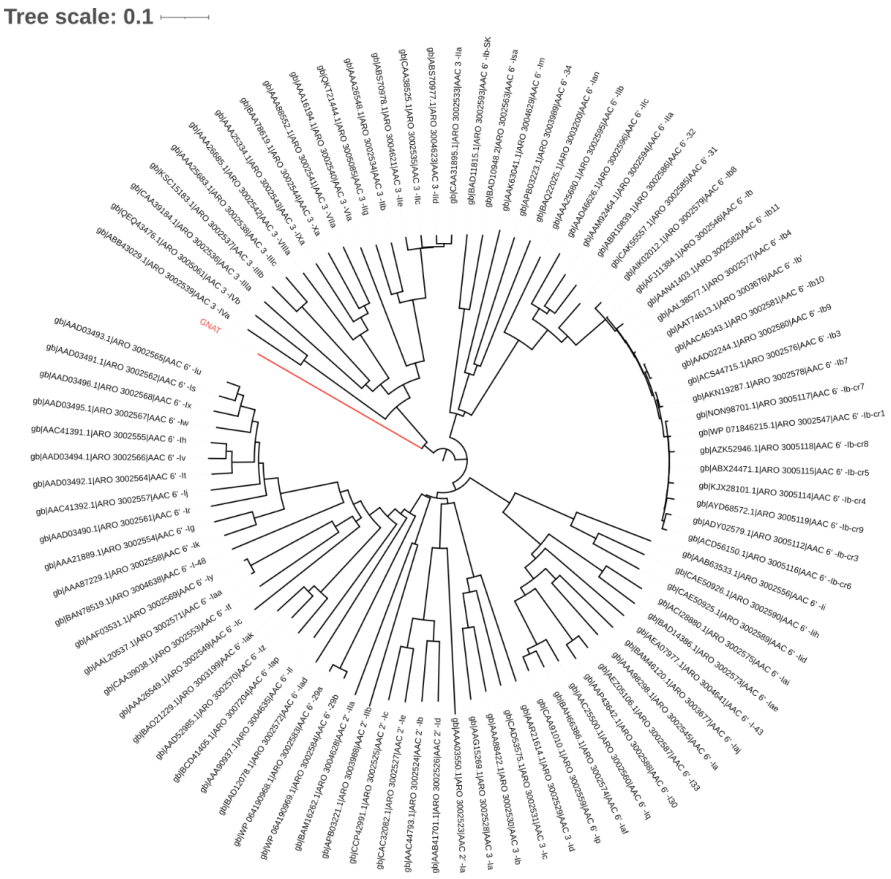



**Figure 27.** Phylogenetic Tree of GNAT Family N-Acetyl Transferase gene against all aminoglycoside resistance gene variants, AAC. The branch highlighted in red represents the GNAT gene.

We then searched the GNAT family N-acetyltransferase and DUF1778 by tblastn against the CARD database. The DUF1778 gene did not have any significant identities. However, GNAT had a 49% identity to the carbapenem resistance gene, OXA-9, and a 40% identity to the aminoglycoside resistance gene, AAC(3'). Hence, we filtered the CARD database for all OXA variants (*n*=918) and all AAC variants (n=90). Upon filtering for these genes, we used Clustal Omega to perform phylogenetic analysis on the GNAT gene against all the OXA and AAC variants. This was done to determine whether GNAT had ancestral origins or was connected to these drug-resistance genes. The AAC phylogenetic tree showed no relation of GNAT to AAC genes.

The phylogenetic tree of GNAT against all OXA variants did not clade specifically with most OXA variants, except for PJM-1. Suggesting that these GNAT and PJM-1 have an ancestral link. PJM-1 genes are a subclass of oxacillinase beta-lactamases. Further testing will be required to confirm the drug class resistance profiles of the GNAT family N-acetyl transferase. BLAST searching of this *dif* module has shown 100% coverage and 100% identity to a wide range of other species acquired via plasmids. Namely, *Citrobacter* spp., *Acinetobacter baumannii*, *Klebsiella pneumonia* and *Enterobacter* spp. Thus, suggesting its ability to spread amongst other Gram-negative bacteria.

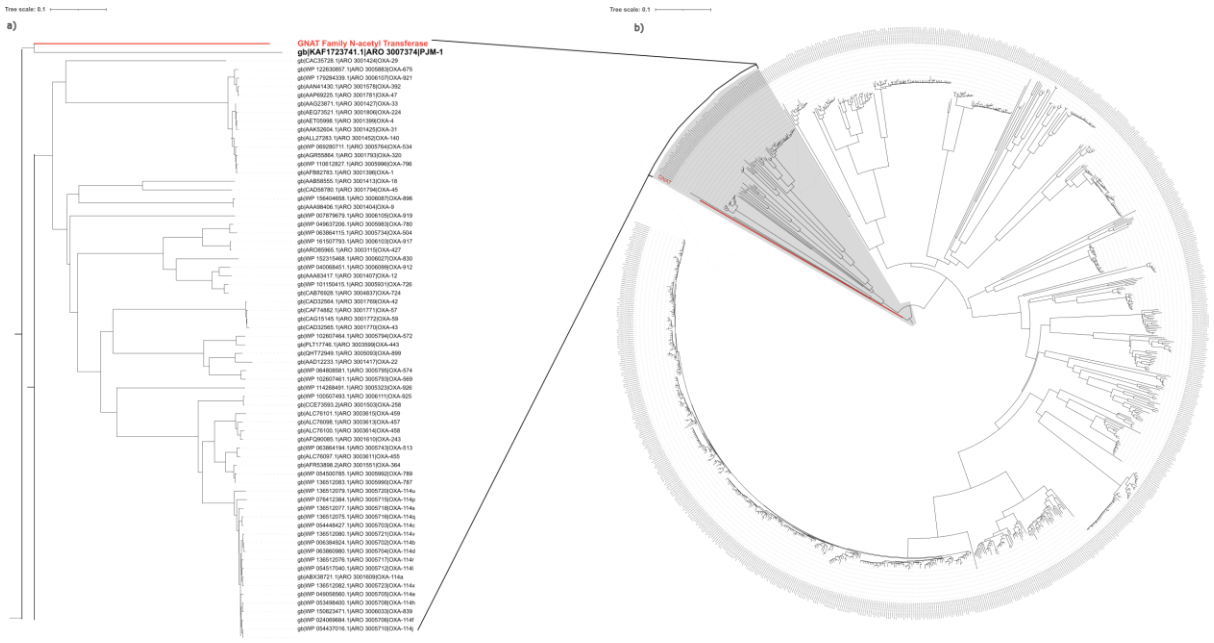



**Figure 28.** Phylogenetic Tree of GNAT Family N-Acetyl Transferase against all Oxacillinase beta-lactamases resistance gene variants, OXA. a) Tree expanded to show region relevant to

GNAT, which is highlighted in red. b) Full phylogenetic tree with 919 OXA variants against GNAT.

### 3.8.3 Heavy metal resistance found within *Enterobacter* spp.

In this study, we examined all our *Enterobacter* genomes for silver resistance genes using the *sil*-operon (12280 bp) as a query (GenBank accession number CP129241.1). It was observed that 15 out of 31 *Enterobacter* isolates encoded the *sil*-operon. However, in every instance of the *sil*-operon appearing in the contigs, we observed copper resistance directly downstream of the operon, although in some cases only remnants of the copper resistance were remaining. As such, we combined these resistances and named it the *sil-cop* operon.

Heavy metal resistance was primarily located on plasmids, with two exceptions found on the chromosome. To confirm plasmid localisation, we performed BLAST searches on contigs containing the *sil*-cop operon and identified high sequence coverage against known plasmid sequences from similar or other bacterial species. In certain cases, such as *E. soli*-197 and *E. roggenkampii*-209, we identified an IncFIB-type replicon gene upstream of the *sil-cop* operon, further confirming plasmid association. This combination of the *cop-sil* operon with replicon genes suggests that these plasmids serve as mobile resistance elements capable of spreading heavy metal resistance across bacterial populations.

In two isolates— a novel *Ec*C strain 163 and *E. roggenkampii* strain 214, the *sil-cop* operon was embedded within a 34,276 bp transposon, identified as Tn*7*. BLAST searching of this transposon against GenBank revealed that *Ec*C strain 163 encoded this transposon within the chromosome, whereas it was plasmid-encoded within the *E. roggenkampii* strain. Tn*7* is a class 2 transposon, generating 5 bp DRs at its flanking ends, carrying genes for both copper and silver resistance, organised in the *sil-cop* operon. The transposon carries its own transposition genes (*tnsA-E*) on one end, which facilitate its mobility, and allows for efficient transfer of heavy metal resistance traits between bacterial hosts. This Tn*7* showed high homology to a publicly available Tn*7*, TnECpAPEC (GenBank AN: NC_009838), identified in a pathogenic *E. coli* plasmid in 2009 sourced from an avian bird, having 93% query coverage and 98% identity. The presence of Tn*7*, with its dual metal resistance capabilities, is significant as it suggests a robust adaptation mechanism to environments with high metal concentrations, such as industrial or wastewater settings [109].

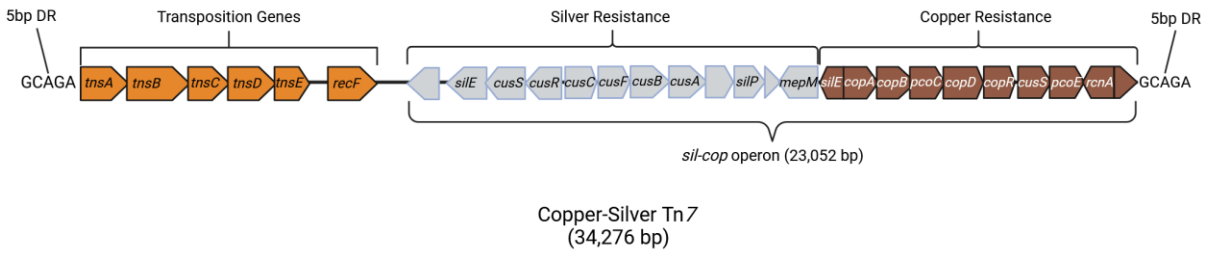



**Figure 29.** Tn*7* encoding heavy metal resistance, copper and silver. Genes in orange are transposition genes. Silver genes represent silver resistance genes. Brown genes represent copper resistance genes. 5 bp direct repeats on each end indicate the transposition of this element into the chromosome and plasmid of strain 163 and 214, respectively.

The *sil-cop* operon itself plays a critical role in heavy metal resistance. The sil operon includes genes such as *silE* and *cus* genes, which confer silver resistance, while the cop operon includes multiple genes for copper resistance (*copA*, *copB*, *pcoC*, etc.). This operon structure allows bacteria to survive in toxic metal environments by sequestering or expelling silver and copper ions.

### 3.9 Virulence within *Enterobacter* spp.

In our *Enterobacter* spp. isolates, we identified several key virulence genes associated with pathogenicity, which was then visualised based on species using the core-genome alignment phylogenetic tree (see figure 30). The *ompA* gene, which plays a significant role in biofilm formation and immune evasion, was present across the entire set of isolates, indicating a ubiquitous capability for biofilm-associated persistence in these bacteria [10]. Additionally, *csgG*, another biofilm-related gene, was detected in all isolates except two *E. roggenkampii* strains, suggesting that biofilm formation is a widespread and potentially conserved virulence trait within this collection [110].

Siderophore-related genes *entA* and *entB*, essential for iron acquisition, were also prevalent among the isolates. These genes facilitate bacterial survival in iron-limited environments, such as within host tissues, by enabling effective iron scavenging—a critical factor for establishing infections [10, 111]. Other virulence genes, including *astA*, *fepD*, and *fliG*, were present less frequently, appearing sporadically across the isolates. The gene *astA* is associated with enterotoxin production, *fepD* contributes to iron uptake, and *fliG* is involved in flagellar motility, which can aid in colonisation and tissue invasion [10, 111].

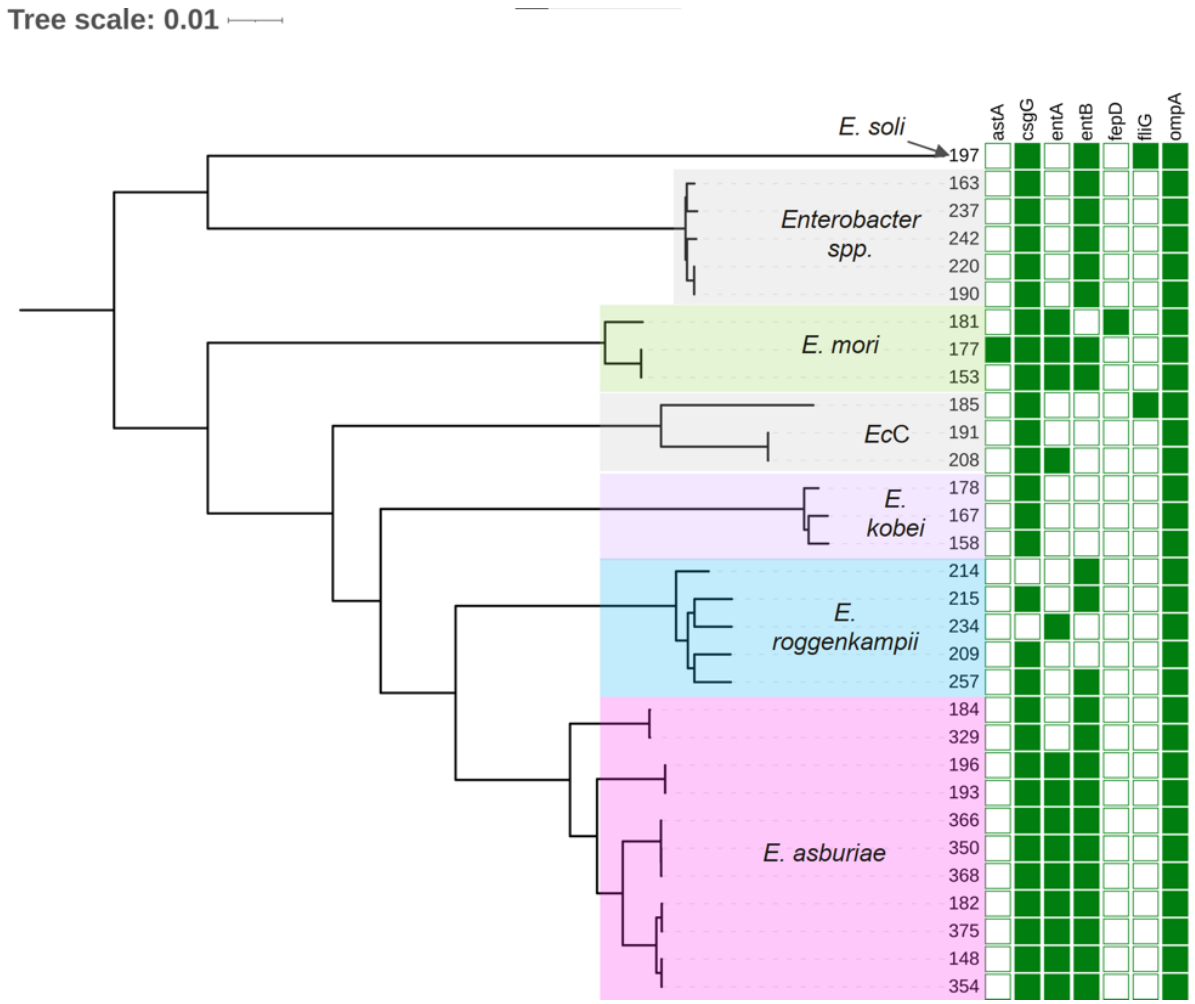



**Figure 30.** Virulence factors profile of our *Enterobacter* spp.

The consistent presence of *ompA*, *csgG*, *entA*, and *entB* in most isolates suggests a core set of virulence factors that may enhance the ability of *Enterobacter* spp. to survive and potentially establish infections. The sporadic occurrence of *astA*, *fepD*, and *fliG* suggests that while these factors may augment pathogenic potential in specific strains, they are not universally necessary across the population. These findings indicate a complex distribution of virulence traits, with core genes likely providing baseline pathogenic potential for *Enterobacter* spp., while others may contribute to specialised adaptations in particular host or environmental conditions.

# Chapter 4: Discussion

## 4.1 *Enterobacter cloacae* complex identification challenges

In this study, the whole genome sequence of 31 environmental *Enterobacter* isolates, sourced from IWW in South Australia, were analysed and contextualised to determine if there was a link between the environment to clinically and globally distributed AMR-significant strains. Identification of species and subspecies within the *Ec*C is often challenging due to the significant number of clonal complexes within the *Ec*C. Therefore, using phenotypic methods to identify subspecies correctly is often irreproducible and unreliable [10, 112]. This issue is amplified by the misreporting of most *Ec*C subspecies as *E. cloacae* on GenBank, often due to misinformation or negligence towards the *Ec*C [113]. This leads to increased complexity in correctly identifying subspecies within the *Ec*C. This issue was prevalent throughout the phylogenetics approach of this study. However, it was mitigated using reference sequences of *Enterobacter* spp. and multiple additions of subspecies within the *Ec*C. As such we determined our collection of isolates to be quite diverse, encompassing mainly *Ec*C species, with nine strains of *E. asburiae*, five strains of *E. roggenkampii*, three novel *E. cloacae* sp., and three strains of *E. kobei and E. mori*. Outside the *Ec*C we determined our set had five novel *Enterobacter* spp. and one *E. soli* strain. Since only 5% of publicly available *Ec*C assemblies are from environmental sources—and none from Australia, our study's focus on environmental isolates is particularly valuable. This emphasis on environmental sampling fills a critical gap in AMR research, as most studies target clinical settings, overlooking the environmental pathways of AMR transmission. Environmental reservoirs, including municipal wastewater and IWW, have been shown to serve as conduits for the spread of AMR genes to human and animal populations, as they often act as a direct snapshot of AMR in a human population [65]. More recently, there have been an increased emphasis on environmental genomic comparative analysis studies, in line with our project, albeit they are still a scarcity in the field.

Our genomic analysis revealed considerable diversity of ARGs across the set, with 89 total ARGs identified. With our environmental isolates carrying significant resistance genes, such as *mcr-9.1*, $bla_{\text{GES-5}}$ and $bla_{\text{SHV-12}}$, which confer resistance to important last-line antibiotics like colistin, carbapenems and third generation cephalosporins, respectively. This aligns with our hypothesis for this project, with environmental strains demonstrating a clear link to clinically significant antibiotic resistance determinants. In particular, two of our isolates, strain 184 *E.*

*asburiae* and 209 *E. roggenkampii*, stood out for their high ARG counts and unique plasmid profiles, and thus it was predicted that these strains were likely to be MDR. Hence, further investigation through our complete hybrid assembly of these strains highlighted a significant genetic link between environmentally derived strains to clinically significant and globally distributed AMR.

### 4.2 Plasmids in *Enterobacter* species drive AMR

Plasmids in our isolates play a key role in carrying and disseminating ARGs, as all of our ARGs were acquired and mobilised via plasmids, with the exception of a few including *qnrE1*, *ampC* and *catA2*. It was found that Inc-type plasmids, specifically IncF, IncHI2 and IncQ types, were considerable vectors for the acquisition and transmission of these ARGs. They had significant IS insertion events and transposons, which introduced ARGs. For instance, the class 1 integron, was found in approximately one-third of our isolates, which was harboured on a transposon that was encoded within a plasmid for all instances. Furthermore, screening of plasmids from our complete genomes (*E. roggenkampii* 209 and *E. asburiae* 184), further elucidated the extensive sharing of plasmids between species. This revealed that these plasmids are common, not only amongst our set, but also to clinical and globally distributed strains from Enterobacterales.

For instance, the largest plasmid in *E. roggenkampii* 209 (p209-1 – a 300 Kbp IncHI2A type plasmid), was found to play a major role in disseminating several important ARGs, including *mcr-9.1* and $bla_{\text{SHV-12}}$, conferring resistance to last-line antibiotics such as carbapenems and third generation cephalosporins. This plasmid also shared significant coverage to plasmids involved in clinical outbreaks of *E. asburiae* in Denmark (83% coverage/99.88% identity) and animal *E. coli* strains in Alaska (81% coverage/99.98% identity) [114, 115]. Our findings determined that the globally distributed plasmids, from Denmark and Alaska, also encoded clinically important ARGs conferring resistance to carbapenems ($bla_{\text{KPC-2}}$ and $\underline{bla_{\text{OXA-48}}}$), in addition to the colistin resistance already present on these plasmids. This suggests that these plasmid types found in our environment can encode extensive drug resistance, and have the ability to acquire and spread multiple last-line resistance genes. Additionally, a highly similar plasmid to p209-1, was recently resolved from a novel *Ec*C species, *Enterobacter adelaidei* in 2023 from hospital wastewater (83% coverage/99.98% identity) [116]. This aligns with our source for the *Enterobacter* set. Thus, suggesting that this plasmid may have already been involved with clinical distribution within Australia.

This was further highlighted in the case of p184-1, a 120 Kbp IncFIB type plasmid, which extensively encoded resistance to tetracycline, aminoglycosides and phenicols. This plasmid was found to encode a novel transposon, Tn*6928*, which we describe extensively in this study for the first time. Furthermore, this plasmid had significant backbone homology to carbapenem-resistant (containing $bla_{\text{NDM-1}}$ and $bla_{\text{IMP-4}}$) plasmids carried by clinical strains recovered from *E. hormaechei* in Bangladesh (57% coverage/96.67% identity) and *C. freundii* in Hong Kong (77% coverage/99.97% identity) [117, 118]. Thus, highlighting the adaptability of these plasmids of similar nature to not only develop novel MGEs (via the novel transposon, Tn*6928*), but also acquire clinically significant last-line resistances that would impede treatment options and further detriment the AMR crisis.

Our study identified quinolone resistance within nine of our isolates, with resistance located on the chromosome in all three of our *E. mori* strains and on IncQ plasmids in the remaining six. Both chromosomal and plasmid-mediated forms of resistance in these isolates highlight their potential contribution to the AMR crisis. *E. mori*, for instance carried the quinolone resistance gene, *qnrE1*, which from literature has been described as originating from this species via mutation [105]. This quinolone resistance gene has been reported in clinical outbreaks of *K. pneumoniae* in Argentina, Brazil and the US [105, 119]. Previous studies have found that the *qnrE1* gene was mobilised through the IS*EcP1*, which was then captured on a plasmid and disseminated [105, 119]. This highlights the involvement of this species to contribute to the development of AMR via selective pressures which effectively drove mutational resistance within this species.

Furthermore, it was found that plasmid-mediated quinolone resistance was largely acquired via small (6-9 Kbp) mobilisable IncQ-type plasmids. For instance, the IncQ -type plasmid found in strain 214 *E. roggenkampii* had significant links to a plasmid of *Shigella flexneri* sourced clinically from Japan [49]. Thus, reinforcing the linkage of the environment to clinical settings. Furthermore, it was determined that we had an identical IncQ-2 plasmid to an *Aeromonas cavaie* strain sourced from river water in China [48]. This plasmid acquired quinolone resistance via a novel mobile insertion cassette similar to the function of an IS [48]. Although, the plasmid was not clinically sourced, it is still holding clinical relevance as it encoded important resistance genes and entails novel functionality in its capability to acquire resistance. This is further elucidated by the fact that this plasmid had significant coverage of another similar plasmid, pCHE-A, that was involved in carbapenem ($bla_{\text{GES-5}}$) resistance and was

notarised in two of our strains (novel *Enterobacter* strains 190 and 220) [50]. This reinforces the notion that these plasmids, although might not yet involved in clinical outbreaks, they have adaptability and capability to acquire last-line resistances, such as carbapenems, to then be disseminated and detriment health treatments.

### 4.3 Importance of novel mobile genetic elements in genetic acquisition in environmental Australian *Enterobacter* strains

Our study documented the first identification of *dif* modules, previously considered exclusive to *Acinetobacter baumannii*, within *Enterobacter* spp., marking an unprecedented expansion in the known distribution and mobility of these genetic elements. Notably, the *E. asburiae* strain 182 was found to carry macrolide resistance genes *msr-mph*(E) within a XerC/XerD *dif* module site. The presence of these *dif* modules in *Enterobacter* suggests an expanded role for these elements, initially thought restricted to *A. baumannii*, in disseminating AMR traits across multiple genera [52].

GenBank analysis provided additional context, revealing that these *dif* modules were not confined to *Enterobacter* or *A. baumannii* either. They were present across several other species, including *K. pneumoniae, S. enterica*, *E. coli*, *P. mirabilis*, and *M. morganii*, with diverse isolation sources ranging from clinical settings to environmental reservoirs, such as wastewater and animal sources. This widespread presence across genera and environments suggests that *dif* modules act as versatile vectors, facilitating HGT and disseminating AMR traits across ecological and species boundaries. For instance, the *msr-mph*(E) module, discovered in *E. kobei* and *E. hormaechei*, and sourced from environments like coastal waters and human bloodstream infections, further underlines the ability of *dif* modules to bridge environmental reservoirs and clinical sites.

Furthermore, our study reports the GNAT family N-acetyltransferase *dif* module in *Enterobacter* spp., marking its first documented occurrence in this genus. This discovery of p*dif*58 and p*dif*211 sites expands the known understanding of GNAT-based resistance, and highlights their potential role in AMR gene mobility. The GNAT module, containing both a GNAT family N-acetyltransferase and a DUF1778 domain, highlights the capability of *dif* modules as vehicles for resistance dissemination. This plasmid-borne GNAT *dif* module's presence across *Citrobacter*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* signifies a broader relevance, as its mobility enables it to bridge ecological and genus boundaries and adapt to varying selective pressures.

## 4.4 Heavy Metal Resistance widespread throughout *Enterobacter* spp.

In this study, we identified a prevalent mechanism of silver-copper resistance within *Enterobacter* spp. isolates, primarily facilitated by the co-located *sil* and *cop* operons, forming what we termed throughout our study as the "*sil-cop* operon." Heavy metals such as silver and copper have long been used in medical and industrial settings for their antimicrobial properties, with silver commonly incorporated into wound dressings, medical equipment, and various consumer products for its aseptic properties [120, 121]. However, widespread and indiscriminate usage of nanosilver has led to the emergence of resistance, limiting their efficacy in controlling bacterial growth [121, 122]. This poses a growing concern for the field of AMR, as Gram-negative and Gram-positive bacteria have increasingly acquired resistance in both clinical and environmental settings [120-123].

Our findings revealed that almost half of our *Enterobacter* isolates carry the *sil* operon, typically coupled with copper resistance genes downstream, although, in some cases fragments of the copper operon remained likely due to limitations of short-read sequencing. Previous studies have vocalised the high frequency of silver resistance genes found in Enterobacterales, specifically in *Enterobacter* and *Klebsiella* species [124]. This combination of silver and copper resistance genes was observed primarily on plasmids, but in two instances, we found the operon embedded in the chromosome, a configuration that might enhance stability and persistence within bacterial populations. The operon was often associated with IncFIB-type plasmids, suggesting that these plasmids could act as mobile vectors for transferring heavy metal resistance across different bacterial hosts. In strains such as *E. soli* 197 and *E. roggenkampii* 209, the *sil-cop* operon was embedded within a 34,276 bp Tn*7* transposon, a MGE which facilitates the efficient spread of resistance elements. The acquisition of this resistance via a concerning transposon, such as Tn*7*, highlights the importance of heavy metal resistance to bacteria as they have adaptively acquired this for its survival [109].

## 4.5 Limitations

In this study, certain limitations impacted the breadth of our findings. Primarily, the absence of physical samples of the isolates restricted our ability to conduct phenotypic assays, preventing us from correlating the genotypic resistance profiles with actual resistance phenotypes. This limitation left a gap in understanding the functional expression of resistance genes under real-world conditions. Although resistance genes carried within the assembly is highly-correlated to phenotypic resistance, hence this is not a major limitation of the study.

This is substantiated by the fact that bioinformatics approaches for this field are commonplace [125, 126]. Additionally, while the genomic data allowed us to identify and characterise resistance genes, it limited our ability to examine broader ecological and evolutionary trends that may affect the persistence and dissemination of these genes, such as the role of environmental factors in shaping resistance dynamics. This was heightened by the limited availability of publicly available environmental samples, like GenBank, hindering the maximal capability of our comparative genomic analysis. This would likely be solved by a comprehensive global effort towards the One Health framework, in which environmentally-sourced bacteria is further selected for studies revolving AMR.

### 4.6 Future Directions

This study highlights several pathways for further investigation. Firstly, experimental testing of silver and copper resistance in the isolates would provide validation for the *sil-cop* operon's role in heavy metal resistance. This could be achieved through phenotypic assays to assess the minimum inhibitory concentrations of silver and copper for these isolates, establishing a direct link between genotypic and phenotypic resistance. Additionally, given the complex genetic architecture of plasmids in *Enterobacter* spp., further work should focus on the complete sequencing and structural characterisation of these plasmids, especially those carrying AMR and heavy metal resistance genes. Such work could include hybrid sequencing for all genomes, combining both short and long reads, to produce contiguous assemblies that clarify plasmid origins, structural variations, and mechanisms driving resistance gene acquisition and mobilisation.

Furthermore, the study of novel mobile genetic elements, such as the *dif* modules identified, warrants additional exploration to understand their contribution to AMR dissemination within *Enterobacter* spp. and across bacterial genera. In particular, transformation of the GNAT *dif* module onto a natural plasmid, would serve to characterise the potential resistance of this novel MGE, effectively enriching the field's knowledge towards the significance of these *dif* modules. Moreover, comparative genomic studies with larger environmental and clinical sample sets could help elucidate the trends and environmental pressures that facilitate HGT leading to AMR dissemination in Enterobacterales.

# Chapter 5: Conclusions

Our findings demonstrate that plasmids are central to the dissemination of AMR within environmental *Enterobacter* populations. Key resistance genes conferring resistance to last-line treatment options, such as carbapenem ($bla_{\text{GES-5}}$), colistin (*mcr-9.1*) and third generation cephalosporins ($bla_{\text{SHV-12}}$), were found in genetic contexts mirroring those identified in clinical settings, including plasmids with broad host ranges like IncF, IncHI2, and IncQ. This connectivity highlights the environmental reservoirs' potential role as sources and transit points for clinically relevant resistance genes, confirming the One Health implications of AMR spread across human, environmental, and animal ecosystems.

Additionally, our discovery of *dif* modules within *Enterobacter*—particularly the *msr-mph*(E) *dif* module, previously thought unique to *Acinetobacter*—marks a pivotal finding [52, 53]. This module, and our detection of a GNAT-based *dif* module, suggests that these elements contribute to the genetic versatility of *Enterobacter*, enabling adaptation across ecological boundaries. These results reinforce the critical importance of environmental surveillance and provide foundational insights for future studies on the environmental pathways of AMR transmission.

Overall, this study highlights the importance of environmental surveillance for AMR, demonstrating that influent wastewater acts as a reservoir for ARGs and a breeding ground for genetic exchange. These findings call for ongoing research on environmental AMR, focusing on plasmid dynamics, novel resistance mechanisms, and the evolutionary pressures driving AMR dissemination.

# Appendix

**Appendix A:** Phylogenetic Tree IDs for publicly available RefSeq used in phylogenetic core genome alignment in Figure 12.

| Phylogenetic Tree ID | Accession Number | Species | Strain Name |
|---|---|---|---|
| Cloacae | OW968328.1 | *E. cloacae* | 1782 |
| Cloacae2 | CP077211.1 | *E. cloacae* | FDAARGOS 1431 |
| Cloacae3 | CP056776.1 | *E. cloacae* | DSM30054 |
| Cloacaesubspcloacae | NC_014121.1 | *E. cloacae* subsp. *cloacae* | ATCC 13047 |
| Cloacae5 | NZ_CP073310.1 | *E. cloacae* | CZ862 |
| Hormaechie | CP003678.1 | *E. cloacae* subsp. *dissolvens* | SDM |
| Asburiae | AP019630.1 | *E. asburiae* | 17Nkhm-UP2 |
| Asburiae2 | CP065693.1 | *E. asburiae* | FDAARGOS_892 |
| Asburiae3 | CP011863.1 | *E. asburiae* | ATCC35953 |
| Asburiae4 | CP103751.1 | *E. asburiae* | 2497 |
| Asburiae5 | CP129514.1 | *E. asburiae* | BP16m1 |
| Asburiae6 | CP034336.1 | *E. asburiae* | CAV1043 |
| AsburiaeL1 | AWXI01000001.1 | *E. asburiae* | L1 |
| Dykesii | CP126604.1 | *E. dykesii* | E1 |
| Ludwigii | CP017279.1 | *E. ludwigii* | EN-119 |
| Ludwigii2 | CP077223.1 | *E. ludwigii* | FDAARGOS 1436 |
| Ludwigii3 | CP006580.1 | *E. ludwigii* | P101 |
| Ludwigii4 | CP122442.1 | *E. ludwigii* | I9455333cz |
| Ludwigii5 | CP116347.1 | *E. ludwigii* | CM-TZ4 |
| Roggenkampii | CP017184.1 | *E. roggenkampii* | DSM 16690 |
| Pseudoroggenkampii | CP129026.1 | *E. pseudoroggenkampii* | FY158 |
| Quasiroggenkamppii | RXSJ01000001.1 | *E. quasiroggenkampii* | 090040 1 |
| Chengduensis | CP043318.1 | *E. chengduensis* | WCHEC1-C4 |
| Sichuanensis | CP027986.1 | *E. sichuanensis* | SGAir0282 |
| Vonholyi | JAWNDR010000001.1 | *E. vonholyi* | 211D2 ctg_1 |
| Soli | CP003026.1 | *E. soli* | - |
| Huaxensis | CP043342.1 | *E. huaxensis* | 09008 |
| Nematophilus | JAPKNE010000001.1 | *E. nematophilus* | E-TC7 |
| Oligotrophicus | AP019007.1 | *E. oligotrophicus* | CCA6 |
| Nimipressuralis | DALYVD010000001.1 | *E. nimipressuralis* | CTOTU47227 |
| Bugandensis | CP083403.1 | *E. bugandensis* | XL95 |
| Cancerogenus | CP081105.1 | *E. cancerogenus* | JY65 |
| Chuandensis | CP141063.1 | *E. chuandensis* | AFC |
| Mori | CP091779.1 | *E. mori* | ACYC.E9L |
| Quasimori | NZ_JAHEVU010000001.1 | *E. quasimori* | 120130 |
| Wuhouensis | CP142124.1 | *E. wuhouensis* | AV1 |
| Kobei | KI973153.1 | *E. kobei* | UCI 24 |
| Xiangfangensis 2 | CP017183.1 | *E. hormaechei* subsp. *xiangfangensis* | LMG27195 |
| Hoffmannii | CP017186.1 | *E. hormaechei* subsp. *hoffmannii* | DSM 14563 |
| Hormaechei | CP100388.1 | *E. hormaechei* | VKH10 |
| Quasihormaechei | NZ_SJONO1000001.1 | *E. quasihormaechei* | WCHEQ120003 1 |
| Pasteurii | JADBRO010000001.1 | *E. pasteurii* | P40RS |
| Hormaechei ssp oharae | CP083613.1 | *E. hormaechei* subsp. *oharae* | FDAARGOS 1533 |
| Steigerwaltii | AJ853890 | *E. hormaechei* subsp. *steigerwaltii* | EN-5621 |

# References


1. Learning-Lumen. *Major Types of Mutations*. 2000; Available from: https://courses.lumenlearning.com/wm-nmbiology1/chapter/reading-major-types-ofmutations/.
2. Bönemann, G., et al., *Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, qnrS2, isolated from the bacterial community of a wastewater treatment plant.* Antimicrob Agents Chemother, 2006. **50**(9): p. 3075-80.
3. Lobanovska, M. and G. Pilla, *Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future?* Yale J Biol Med, 2017. **90**(1): p. 135-145.
4. *Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis.* Lancet, 2022. **399**(10325): p. 629-655.
5. OECD, *Stemming the Superbug Tide*. 2018.
6. Aljeldah, M.M., *Antimicrobial Resistance and Its Spread Is a Global Threat.* Antibiotics (Basel), 2022. **11**(8).
7. Glasziou, P., et al., *Antibiotic stewardship.* Australian Journal of General Practice, 2022. **51**(1/2): p. 15-20.
8. IHME, *The burden of antimicrobial resistance (AMR) in Australia.* 2019, University of Washington: healthdata.org.
9. WHO, *WHO bacterial priority pathogens list, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance*. 2024: https://www.who.int/publications/i/item/9789240093461.
10. Davin-Regli, A., J.P. Lavigne, and J.M. Pagès, *Enterobacter spp.: Update on Taxonomy, Clinical Aspects, and Emerging Antimicrobial Resistance.* Clin Microbiol Rev, 2019. **32**(4).
11. Santajit, S. and N. Indrawattana, *Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens.* Biomed Res Int, 2016. **2016**: p. 2475067.
12. Hoffmann, H. and A. Roggenkamp, *Population genetics of the nomenspecies Enterobacter cloacae.* Appl Environ Microbiol, 2003. **69**(9): p. 5306-18.
13. Caliskan-Aydogan, O. and E.C. Alocilja, *A Review of Carbapenem Resistance in Enterobacterales and Its Detection Techniques.* Microorganisms, 2023. **11**(6).
14. Armstrong, T., S.J. Fenn, and K.R. Hardie, *JMM Profile: Carbapenems: a broad-spectrum antibiotic.* J Med Microbiol, 2021. **70**(12).
15. S. Siting, M.D., X. Shiwen, Y. Chenjie, Y. Zhenya, W. HongYu, *Research progress in penicillin binding proteins and their mediated bacterial resistance.* Microbiology China, 2017. **44**(4): p. 902-910.
16. Paudel, R., et al., *Carbapenemase producing Gram negative bacteria: Review of resistance and detection methods.* Diagnostic Microbiology and Infectious Disease, 2024. **110**(1): p. 116370.
17. Vivas, R., et al., *Prevalence of Klebsiella pneumoniae carbapenemase - and New Delhi metallo-beta-lactamase-positive K. pneumoniae in Sergipe, Brazil, and combination therapy as a potential treatment option.* Rev Soc Bras Med Trop, 2020. **53**: p. e20200064.
18. Boyd Sara, E., et al., *OXA-48-Like β-Lactamases: Global Epidemiology, Treatment Options, and Development Pipeline.* Antimicrobial Agents and Chemotherapy, 2022. **66**(8): p. e00216-22.
19. Hirabayashi, A., et al., *Plasmid analysis of NDM metallo-β-lactamase-producing Enterobacterales isolated in Vietnam.* PLoS One, 2021. **16**(7): p. e0231119.

20. Meletis, G., *Carbapenem resistance: overview of the problem and future perspectives.* Ther Adv Infect Dis, 2016. **3**(1): p. 15-21.
21. Annavajhala, M.K., A. Gomez-Simmonds, and A.-C. Uhlemann, *Multidrug-Resistant Enterobacter cloacae Complex Emerging as a Global, Diversifying Threat.* Frontiers in Microbiology, 2019. **10**.
22. Strikas, R.A., et al., *6 - Active Immunization*, in *Principles and Practice of Pediatric Infectious Diseases (Fifth Edition)*, S.S. Long, C.G. Prober, and M. Fischer, Editors. 2018, Elsevier. p. 43-71.e4.
23. Andrade, F.F., et al., *Colistin Update on Its Mechanism of Action and Resistance, Present and Future Challenges.* Microorganisms, 2020. **8**(11).
24. Ledger, E.V.K., A. Sabnis, and A.M. Edwards, *Polymyxin and lipopeptide antibiotics: membrane-targeting drugs of last resort.* Microbiology (Reading), 2022. **168**(2).
25. Doijad, S.P., et al., *Resolving colistin resistance and heteroresistance in Enterobacter species.* Nature Communications, 2023. **14**(1): p. 140.
26. Xu, T., et al., *Frequent convergence of mcr-9 and carbapenemase genes in Enterobacter cloacae complex driven by epidemic plasmids and host incompatibility.* Emerg Microbes Infect, 2022. **11**(1): p. 1959-1972.
27. Kieffer, N., et al., *mcr-9, an Inducible Gene Encoding an Acquired Phosphoethanolamine Transferase in Escherichia coli, and Its Origin.* Antimicrobial Agents and Chemotherapy, 2019. **63**(9): p. 10.1128/aac.00965-19.
28. Dong, X., et al., *Metallo-β-lactamase SMB-1 evolves into a more efficient hydrolase under the selective pressure of meropenem.* J Inorg Biochem, 2023. **247**: p. 112323.
29. Gibson, J.S., et al., *Identification of Qnr and AAC(6')-1b-cr plasmid-mediated fluoroquinolone resistance determinants in multidrug-resistant Enterobacter spp. isolated from extraintestinal infections in companion animals.* Veterinary Microbiology, 2010. **143**(2): p. 329-336.
30. Nouri, R., et al., *The role of gyrA and parC mutations in fluoroquinolones-resistant Pseudomonas aeruginosa isolates from Iran.* Braz J Microbiol, 2016. **47**(4): p. 925-930.
31. Mahillon, J. and M. Chandler, *Insertion sequences.* Microbiol Mol Biol Rev, 1998. **62**(3): p. 725-74.
32. Shropshire, W.C., et al., *IS26-mediated amplification of blaOXA-1 and blaCTX-M-15 with concurrent outer membrane porin disruption associated with de novo carbapenem resistance in a recurrent bacteraemia cohort.* J Antimicrob Chemother, 2021. **76**(2): p. 385-395.
33. Pray, L., *Transposons: The Jumping Genes.* Nature Education, 2008. **1**: p. 204.
34. Bourque, G., et al., *Ten things you should know about transposable elements.* Genome Biology, 2018. **19**(1): p. 199.
35. Ghaly, T.M., et al., *The Natural History of Integrons.* Microorganisms, 2021. **9**(11).
36. Domingues, S., G.J. da Silva, and K.M. Nielsen, *Integrons: Vehicles and pathways for horizontal dissemination in bacteria.* Mob Genet Elements, 2012. **2**(5): p. 211-223.
37. Smillie, C., et al., *Mobility of plasmids.* Microbiol Mol Biol Rev, 2010. **74**(3): p. 434-52.
38. Schröder, G. and E. Lanka, *The mating pair formation system of conjugative plasmids—A versatile secretion machinery for transfer of proteins and DNA.* Plasmid, 2005. **54**(1): p. 1-25.
39. Che, Y., et al., *Conjugative plasmids interact with insertion sequences to shape the horizontal transfer of antimicrobial resistance genes.* Proc Natl Acad Sci U S A, 2021. **118**(6).

40. Johnson, T.J. and L.K. Nolan, *Plasmid Replicon Typing*, in *Molecular Epidemiology of Microorganisms: Methods and Protocols*, D.A. Caugant, Editor. 2009, Humana Press: Totowa, NJ. p. 27-35.
41. Agyekum, A., et al., *blaCTX-M-15 carried by IncF-type plasmids is the dominant ESBL gene in Escherichia coli and Klebsiella pneumoniae at a hospital in Ghana.* Diagn Microbiol Infect Dis, 2016. **84**(4): p. 328-33.
42. Alcántar-Curiel, M.D., et al., *Emergence of IncFIA Plasmid-Carrying bla(NDM-1) Among Klebsiella pneumoniae and Enterobacter cloacae Isolates in a Tertiary Referral Hospital in Mexico.* Microb Drug Resist, 2019. **25**(6): p. 830-838.
43. Kim, J., et al., *Characterization of IncF plasmids carrying the blaCTX-M-14 gene in clinical isolates of Escherichia coli from Korea.* Journal of Antimicrobial Chemotherapy, 2011. **66**(6): p. 1263-1268.
44. Feng, Y., et al., *Coexistence of Two bla(NDM-5) Genes on an IncF Plasmid as Revealed by Nanopore Sequencing.* Antimicrob Agents Chemother, 2018. **62**(5).
45. Lima, T., et al., *Occurrence and Biological Cost of mcr-1-Carrying Plasmids Co-harbouring Beta-Lactamase Resistance Genes in Zoonotic Pathogens from Intensive Animal Production.* Antibiotics (Basel), 2022. **11**(10).
46. Forde, B.M., et al., *Discovery of mcr-1-Mediated Colistin Resistance in a Highly Virulent Escherichia coli Lineage.* mSphere, 2018. **3**(5).
47. Hendrickx, A.P.A., et al., *Plasmid diversity among genetically related Klebsiella pneumoniae blaKPC-2 and blaKPC-3 isolates collected in the Dutch national surveillance.* Scientific Reports, 2020. **10**(1): p. 16778.
48. Wen, Y., et al., *High prevalence of plasmid-mediated quinolone resistance and IncQ plasmids carrying qnrS2 gene in bacteria from rivers near hospitals and aquaculture in China.* PLoS One, 2016. **11**(7): p. e0159418.
49. Rodríguez-Martínez, J.M., et al., *Plasmid-mediated quinolone resistance: an update.* J Infect Chemother, 2011. **17**(2): p. 149-82.
50. Poirel, L., et al., *Integron mobilization unit as a source of mobility of antibiotic resistance genes.* Antimicrob Agents Chemother, 2009. **53**(6): p. 2492-8.
51. Carattoli, A., et al., *Evolution of IncA/C blaCMY-₂-carrying plasmids by acquisition of the blaNDM-₁ carbapenemase gene.* Antimicrob Agents Chemother, 2012. **56**(2): p. 783-6.
52. Blackwell, G.A. and R.M. Hall, *The tet39 Determinant and the msrE-mphE Genes in Acinetobacter Plasmids Are Each Part of Discrete Modules Flanked by Inversely Oriented pdif (XerC-XerD) Sites.* Antimicrob Agents Chemother, 2017. **61**(8).
53. Balalovski, P. and I. Grainge, *Mobilization of pdif modules in Acinetobacter: A novel mechanism for antibiotic resistance gene shuffling?* Mol Microbiol, 2020. **114**(5): p. 699-709.
54. Samreen, et al., *Environmental antimicrobial resistance and its drivers: a potential threat to public health.* J Glob Antimicrob Resist, 2021. **27**: p. 101-111.
55. Cooper, L., et al., *Supporting global antimicrobial stewardship: antibiotic prophylaxis for the prevention of surgical site infection in low- and middle-income countries (LMICs): a scoping review and meta-analysis.* JAC Antimicrob Resist, 2020. **2**(3): p. dlaa070.
56. Sijbom, M., et al., *Trends in antibiotic selection pressure generated in primary care and their association with sentinel antimicrobial resistance patterns in Europe.* J Antimicrob Chemother, 2023. **78**(5): p. 1245-1252.
57. ReActGroup. *Mutations and selection*. 2023; Available from: https://www.reactgroup.org/toolbox/understand/antibiotic-resistance/mutation-and-selection/.

58. Larsen, M.V., et al., *Multilocus sequence typing of total-genome-sequenced bacteria.* J Clin Microbiol, 2012. **50**(4): p. 1355-61.
59. Lumbreras-Iglesias, P., et al., *High-risk international clones ST66, ST171 and ST78 of Enterobacter cloacae complex causing blood stream infections in Spain and carrying bla(OXA-48) with or without mcr-9.* J Infect Public Health, 2023. **16**(2): p. 272-279.
60. Chen, C.-M., et al., *Emergence and transmission of the high-risk ST78 clone of OXA-48-producing Enterobacter hormaechei in a single hospital in Taiwan.* Emerging Microbes & Infections, 2024. **13**(1): p. 2404165.
61. Tymensen, L., et al., *Clonal expansion of environmentally-adapted Escherichia coli contributes to propagation of antibiotic resistance genes in beef cattle feedlots.* Science of The Total Environment, 2018. **637-638**: p. 657-664.
62. Larsson, D.G.J. and C.-F. Flach, *Antibiotic resistance in the environment.* Nature Reviews Microbiology, 2022. **20**(5): p. 257-269.
63. Honda, R., et al., *Transition of antimicrobial resistome in wastewater treatment plants: impact of process configuration, geographical location and season.* npj Clean Water, 2023. **6**(1): p. 46.
64. Fouz, N., et al., *The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings.* Trop Med Infect Dis, 2020. **5**(1).
65. Chau, K.K., et al., *Systematic review of wastewater surveillance of antimicrobial resistance in human populations.* Environ Int, 2022. **162**: p. 107171.
66. Djordjevic, S.P., et al., *Genomic surveillance for antimicrobial resistance - a One Health perspective.* Nat Rev Genet, 2024. **25**(2): p. 142-157.
67. Tello, A., B. Austin, and T.C. Telfer, *Selective pressure of antibiotic pollution on bacteria of importance to public health.* Environ Health Perspect, 2012. **120**(8): p. 1100-6.
68. Hazards, E.P.o.B., et al., *Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain.* EFSA Journal, 2021. **19**(6): p. e06651.
69. Wattam, A.R., et al., *Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center.* Nucleic Acids Res, 2017. **45**(D1): p. D535-d542.
70. Clark, A.E., et al., *Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology.* Clin Microbiol Rev, 2013. **26**(3): p. 547-603.
71. Quail, M.A., et al., *A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers.* BMC Genomics, 2012. **13**(1): p. 341.
72. Jain, M., et al., *The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community.* Genome Biology, 2016. **17**(1): p. 239.
73. Andrews, S., *FastQC: A quality control tool for high throughput sequence data*. 2010.
74. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-2120.
75. Seemann, T., *Shovill*. 2019.
76. Bankevich, A., et al., *SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing.* Journal of computational biology, 2012. **19**(5): p. 455-477.
77. Parks, D.H., et al., *CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes.* Genome Res, 2015. **25**(7): p. 1043-55.
78. Wick, R.R., et al., *Trycycler: consensus long-read assemblies for bacterial genomes.* Genome Biology, 2021. **22**(1): p. 266.

79. Li, H., *Minimap and miniasm: fast mapping and de novo assembly for noisy long sequences.* Bioinformatics, 2016. **32**(14): p. 2103-10.
80. Wick, R.R. and K.E. Holt, *Benchmarking of long-read assemblers for prokaryote whole genome sequencing.* F1000Res, 2019. **8**: p. 2138.
81. Kolmogorov, M., et al., *Assembly of long, error-prone reads using repeat graphs.* Nature Biotechnology, 2019. **37**(5): p. 540-546.
82. Tonkin-Hill, G., et al., *Producing polished prokaryotic pangenomes with the Panaroo pipeline.* Genome Biology, 2020. **21**(1): p. 180.
83. Croucher, N.J., et al., *Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins.* Nucleic Acids Research, 2014. **43**(3): p. e15-e15.
84. Nguyen, L.-T., et al., *IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies.* Molecular Biology and Evolution, 2014. **32**(1): p. 268-274.
85. Letunic, I. and P. Bork, *Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation.* Nucleic Acids Research, 2021. **49**(W1): p. W293-W296.
86. Seemann, T., *Prokka: rapid prokaryotic genome annotation.* Bioinformatics, 2014. **30**(14): p. 2068-2069.
87. Seemann., T., *ABRicate*. 2020: github.
88. Maiden, M.C., *Multilocus sequence typing of bacteria.* Annu Rev Microbiol, 2006. **60**: p. 561-88.
89. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.
90. Ross, K., et al., *TnCentral: a Prokaryotic Transposable Element Database and Web Portal for Transposon Analysis.* mBio, 2021. **12**(5): p. 10.1128/mbio.02060-21.
91. Doster, E., et al., *MEGARes 2.0: a database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data.* Nucleic Acids Res, 2020. **48**(D1): p. D561-d569.
92. Siguier, P., et al., *ISfinder: the reference centre for bacterial insertion sequences.* Nucleic Acids Res, 2006. **34**(Database issue): p. D32-6.
93. Alcock, B.P., et al., *CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database.* Nucleic Acids Research, 2020. **48**(D1): p. D517-D525.
94. Chen, L., et al., *VFDB: a reference database for bacterial virulence factors.* Nucleic Acids Res, 2005. **33**(Database issue): p. D325-8.
95. Florensa, A.F., et al., *ResFinder - an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes.* Microb Genom, 2022. **8**(1).
96. Carattoli, A., et al., *In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing.* Antimicrob Agents Chemother, 2014. **58**(7): p. 3895-903.
97. Jolley, K.A., J.E. Bray, and M.C.J. Maiden, *Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications.* Wellcome Open Res, 2018. **3**: p. 124.
98. NCBI. *Bethesda (MD)*. National Center for Biotechnology information. 2024; Available from: https://www.ncbi.nlm.nih.gov/.
99. Benslama, O. and A. Boulahrouf, *High-quality draft genome sequence of Enterobacter sp. Bisph2, a glyphosate-degrading bacterium isolated from a sandy soil of Biskra, Algeria.* Genomics Data, 2016. **8**: p. 61-66.

100. Chavda Kalyan, D., et al., *Comprehensive Genome Analysis of Carbapenemase-Producing Enterobacter spp.: New Insights into Phylogeny, Population Structure, and Resistance Mechanisms.* mBio, 2016. **7**(6): p. 10.1128/mbio.02093-16.
101. Rafaï, C., et al., *Dissemination of IncF-type plasmids in multiresistant CTX-M-15-producing Enterobacteriaceae isolates from surgical-site infections in Bangui, Central African Republic.* BMC Microbiol, 2015. **15**(1): p. 15.
102. Potter, R.F., A.W. D'Souza, and G. Dantas, *The rapid spread of carbapenem-resistant Enterobacteriaceae.* Drug Resist Updat, 2016. **29**: p. 30-46.
103. Jacoby, G.A., *AmpC beta-lactamases.* Clin Microbiol Rev, 2009. **22**(1): p. 161-82, Table of Contents.
104. Tamma, P.D., et al., *A Primer on AmpC β-Lactamases: Necessary Knowledge for an Increasingly Multidrug-resistant World.* Clin Infect Dis, 2019. **69**(8): p. 1446-1455.
105. Albornoz, E., et al., *qnrE1, a Member of a New Family of Plasmid-Located Quinolone Resistance Genes, Originated from the Chromosome of Enterobacter Species.* Antimicrob Agents Chemother, 2017. **61**(5).
106. Souza, R.C.d., et al., *Dissemination of blaKPC-2 in an NTEKPC by an IncX5 plasmid.* Plasmid, 2019. **106**: p. 102446.
107. Grant, J.R., et al., *Proksee: in-depth characterization and visualization of bacterial genomes.* Nucleic Acids Research, 2023. **51**(W1): p. W484-W492.
108. Piotrowska, M., et al., *Molecular Characterization and Comparative Genomics of IncQ-3 Plasmids Conferring Resistance to Various Antibiotics Isolated from a Wastewater Treatment Plant in Warsaw (Poland).* Antibiotics (Basel), 2020. **9**(9).
109. Parks, A.R. and J.E. Peters, *Tn7 elements: engendering diversity from chromosomes to episomes.* Plasmid, 2009. **61**(1): p. 1-14.
110. Benomar, S., G. Di Venanzio, and F. Feldman Mario, *Plasmid-Encoded H-NS Controls Extracellular Matrix Composition in a Modern Acinetobacter baumannii Urinary Isolate.* Journal of Bacteriology, 2021. **203**(21): p. 10.1128/jb.00277-21.
111. Kathi, S., *Enterobacter spp. Virulence Factors and Biofilm Components: Synthesis, Structure, Function, and Inhibitors*, in *ESKAPE Pathogens: Detection, Mechanisms and Treatment Strategies*, S. Busi and R. Prasad, Editors. 2024, Springer Nature Singapore: Singapore. p. 349-365.
112. Mezzatesta, M.L., F. Gona, and S. Stefani, *Enterobacter cloacae Complex: Clinical Impact and Emerging Antibiotic Resistance.* Future Microbiology, 2012. **7**(7): p. 887-902.
113. Godmer, A., et al., *Revisiting Species Identification within the Enterobacter cloacae Complex by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry.* Microbiol Spectr, 2021. **9**(1): p. e0066121.
114. Samuelsen, O., Hansen,F., Aasnaes,B., Hasman,H., Lund,B.A.,, et al., *Dissemination and characteristics of a novel Enterobacteriaceae-encoded carbapenem-hydrolyzing class D beta-lactamase OXA-436 from four patients involving six different hospitals in Denmark.* 2017, Detection of Antimicrobial Resistance, University Hospital of North Norway, Department of Microbiology and Infection Control, Tromso: Norwegian National Advisory.
115. Ahlstrom Christina, A., et al., *Repeated Detection of Carbapenemase-Producing Escherichia coli in Gulls Inhabiting Alaska.* Antimicrobial Agents and Chemotherapy, 2019. **63**(8): p. 10.1128/aac.00758-19.
116. Siderius, N.L., et al., *Enterobacter adelaidei sp. nov. Isolation of an extensively drug resistant strain from hospital wastewater in Australia and the global distribution of the species.* Microbiol Res, 2024. **288**: p. 127867.

117. Farzana, R.a.W., T.R., *Determining the burden of carbapenem-resistant Enterobacterales from a tertiary public heath setting in Bangladesh: a clinical, epidemiological, and molecular study*. 2022: Medical Microbiology.
118. Hawkey, P.M., et al., *Occurrence of a new metallo-beta-lactamase IMP-4 carried on a conjugative plasmid in Citrobacter youngae from the People's Republic of China.* FEMS Microbiol Lett, 2001. **194**(1): p. 53-7.
119. Monte, D.F., et al., *Early Dissemination of qnrE1 in Salmonella enterica Serovar Typhimurium from Livestock in South America.* Antimicrob Agents Chemother, 2019. **63**(9).
120. Gupta, A., et al., *Molecular basis for resistance to silver cations in Salmonella.* Nature Medicine, 1999. **5**(2): p. 183-188.
121. McNeilly, O., et al., *Emerging Concern for Silver Nanoparticle Resistance in Acinetobacter baumannii and Other Bacteria.* Front Microbiol, 2021. **12**: p. 652863.
122. Gunawan, C., et al., *Widespread and Indiscriminate Nanosilver Use: Genuine Potential for Microbial Resistance.* ACS Nano, 2017. **11**(4): p. 3438-3445.
123. Graves, J.L., et al., *Rapid evolution of silver nanoparticle resistance in Escherichia coli.* Frontiers in Genetics, 2015. **6**.
124. Sütterlin, S., et al., *High frequency of silver resistance genes in invasive isolates of Enterobacter and Klebsiella species.* Journal of Hospital Infection, 2017. **96**(3): p. 256-261.
125. Vanstokstraeten, R., et al., *Genotypic resistance determined by whole genome sequencing versus phenotypic resistance in 234 Escherichia coli isolates.* Scientific Reports, 2023. **13**(1): p. 449.
126. Van Camp, P.J., D.B. Haslam, and A. Porollo, *Bioinformatics Approaches to the Understanding of Molecular Mechanisms in Antimicrobial Resistance.* Int J Mol Sci, 2020. **21**(4).