Survey of Antimicrobial Resistant Indicator Bacteria and Mechanisms of Resistance from Lake Panorama retention pond and Paremuka Stream in West Auckland

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Abstract

Antibiotic resistant bacteria are becoming an alarming trend throughout the world due to overuse and overprescription of antimicrobial compounds. Death attributed to antimicrobial-resistant micro-organisms was estimated to be 1.27 million deaths in 2019 and the economic cost is projected to reach $1 trillion US dollars in health care related costs by 2050 (World Health Organization, 2025). Treatment of bacterial infections is becoming more challenging as more pathogens develop resistance due to extensive use for both human therapy and in animal husbandry (Nikaido, 2009). Due to their ubiquitous usage, antibiotics and resistant organisms are expelled from people and animals into the waste management system. Most methods of monitoring resistance rely on detection of specific genes (Baba et al., 2022). However, the sewer systems used to transport waste and by extension the resistant organisms are prone to leakage during wet weather and accumulate in artificial reservoirs constructed to prevent flooding of urban areas (Carlisle & Mulamoottil, 1991; Tryland et al., 2011). These artificial basins and reservoirs accumulate stormwater from waste leakages and urban runoff during wet weather and discharge into streams and rivers without treatment (Yang & Lusk, 2018). Most antibiotic resistance surveillance is limited to wastewater or the number of indicator bacteria in water, with little attention given to when potentially harmful bacteria escape into the environment. This study is aimed to survey the presence of any antimicrobial resistance, and the types of mechanisms present in the community of indicator organisms from a local West Auckland stormwater pond and its associated stream.

Five sample sites along the Paremuka stormwater system were selected. Starting from Lake Panorama stormwater retention pond to downstream and then to the outlet at the Waitamatā Harbour. Samples were also taken before and after rainfall events, as well as different seasons for comparison. The number of indicator organisms such as *Enterobacteriaceae* and *Enterococci* spp. was determined using the Most Probable Number (MPN) method and membrane filtration (MF). Presumptive antibiotic resistance was determined using CHROM agar for extended spectrum beta-lactamase (ESBL) *Enterobacteriaceae* and vancomycin-resistant *Enterococci* (VRE). Antibiotic resistant positive isolates was further screened against further antibiotics (e.g. ampicillin, amoxicillin with clavulanic acid, cefalexin and meropenem) using antimicrobial disk assay to determine multidrug resistance (MDR). Of the resistant strains isolated, two MDR isolates was selected to undergo whole genome sequencing to determine the genetic bases that contributed to antibiotic resistance.

The number of indicator organisms during winter, along with heavy rainfall increased the overall count by 200cfu/100ml at upstream sites and downstream sites became too numerous to count (TNTC). Indicator numbers during the summer in resulted in much higher counts ranging from 300cfu/100ml to TNTC.

All isolates were resistant to ampicillin and susceptible to meropenem, with varying degrees of resistance to amoxicillin and cefalexin. Isolates UBr35 and URr35 were the only isolates resistant to ampicillin but showed susceptibility to cefalexin and the combination of amoxicillin and clavulanic acid. Filter isolates, isolate UBd35 and stream isolates showed complete resistance to all tested antibiotics except for meropenem. Estuary isolate BRd42 was the only organism to grow at 42°C that presented resistance to both ampicillin and cefalexin but showed susceptibility to meropenem and amoxicillin.

Genome sequencing was conducted on two isolates UBd35 and SBr35, due to both isolates presenting the greatest resistance to the tested antibiotics. Using MinION and reconstructed using Flye, the assembled genomes were submitted to the Comprehensive Antibiotic Resistance Database (CARD), the Kyoto Encyclopaedia of Genes and Genomes (KEGG), the National Center for Biotechnology Information (NCIB) and the Center for Genomic Epidemiology (CDE) mobile element finder to identify potential resistance genes, mobility of detected genes, and to identify the bacterial species. The resistance mechanisms detected in the UBd35 isolate was presumed to be the bacteria *H. alvei* with the *ACC-3* AmpC beta-lactamase gene. Isolate SBr35 was presumed to be a species of *Citrobacter* with a presumed identity of *C. portcalunesis,* carrying the *CMY-65* class C beta-lactamase gene. The mobile genes detected were insertion sequences and mobile transposons found in SBr35, which was located hundreds of thousands of base pairs away from the *CMY-65* beta-lactamase gene, indicating that the *CMY* gene is not part of the mobile genetic elements.

In summary, the results show that regardless of seasonal conditions, the indicator organisms are present at elevated counts. Among the population of indicator bacteria, antibiotic resistance is present in a local retention pond and its associated stream. Of the two bacteria identified and sequenced, both species have multiple mechanisms capable of resisting common antibiotics. However, of those two bacteria sequenced, only one contained mobile genetic elements relating to insertion sequences and mobile transposons, which do not appear to be related to antibiotic resistance.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor used artificial intelligence tools or generative artificial intelligence tools (unless it is clearly stated, and referenced, along with the purpose of use), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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Chapter 1 Background Information

Introduction

Environmental monitoring of water is typically limited to freshwater water supplies and wastewater surveillance, with stormwater often being overlooked. Stormwater contains a wide range of pollutants that accumulate during heavy rainfall and converge into combined sewer networks or into artificial retention systems. In urban settings, artificial retention ponds are constructed to deal with heavy rain and surface runoff to prevent floodings (Carlisle & Mulamoottil, 1991). Stormwater retention ponds are designed to collect urban runoff during heavy rainfall but also accumulate pollutants ranging from chemical, industrial, and microbial sources (Saifur & Gardner, 2021). Some retention ponds collect surface runoff and discharge into local streams and river systems untreated (Yang & Lusk, 2018). Therefore, microorganisms found in stormwater runoff has been found to contaminate environments downstream and lead to adverse health effects if exposed (Steele et al., 2018; Tseng & Jiang, 2012).

One contaminant that contributes to the composition of stormwater is wastewater. Many urban cities implement combined sewer systems to capture surface water, which is then treated along with municipal sewage (Tibbetts, 2005). The severity of heavy rainfall has become increasingly common and causing the combined sewer infrastructure to overflow and discharge contents into local waterways (Botturi et al., 2021). Wastewater is a prime source of antibiotics being discharged into the environment due to 30 to 90 percent of antibiotics not being metabolised by the body and being discharged into waste unchanged (Watkinson et al., 2007). Due to overprescription, trace amounts of common antibiotics in wastewater have exposed pathogenic strains to non-lethal quantities, leading them to develop resistance (Ferrara, 2017; Karkman et al., 2018). Excessive and inappropriate use of antibiotics discharged into the environment that has resulted in the adaptation of antibiotic-resistant strains of bacteria (Otaigbe & Elikwu, 2023; Serwecińska, 2020). Wastewater undergoes treatment to remove resistant pathogens and waste before being discharged, in contrast to contaminated stormwater that is simply discharged without alteration.

One way to monitor antimicrobial resistant bacteria is to survey wastewater. Wastewater is considered a key environment due to being a collection point for human waste, excreted antibiotics and the resistant organisms (Baba et al., 2022). Of particular concern are the organisms in the family *Enterobacteriaceae* that have been identified as a significant risk to public health due to resistance to common antibiotics such as penicillin, cephalosporins, and monobactams (Pitout & Laupland, 2008; Tiwari et al., 2022). Due to the high concentration of bacteria found in wastewater, the risk of horizontal gene transfer between similar species can result in multidrug resistant bacteria (Karkman et al., 2018). Research in the resistant organisms found in wastewater is well documented, yet research into the resistant organisms found in stormwater, which has been known to contain wastewater remains limited. Stormwater has been known to contain pathogens from wastewater, and without treatment, could establish in local environments could potentially receive or transfer resistance genes between local species.

Pathogenic organisms such as *E. coli* & *Klebsiella pneumoniae* in the family *Enterobacteriaceae* have been identified to be resistant to beta-lactam antibiotics such as penicillin, cefotaxime and aztreonam by producing the enzyme extended spectrum beta-lactamase (ESBL) which deactivate antibiotics (Pitout et al., 2005). According to the Centers for Disease Control (2024a), ESBL-producing Enterobacteriaceae spread via contaminated food and water, although the extent of the spread remains unknown. Organisms like *E. coli* are generally used as an indicator organism for microbial water quality. *E. coli* is found naturally as part of the intestinal microflora of many warm-blooded animals and commonly used as indicator organisms for microbial water quality (Pal, 2014). During heavy rainfall, surface contaminants like faeces are transported with surface water into catchment areas, which increases the risk of pathogenic organisms (Tryland et al., 2011).

While the *Enterobacteriaceae* are of concern, they are not the only bacteria group with resistance. Bacteria in the family *Enterococci* are also used as indicator organisms due to being found in high concentrations in human faecal matter (Boehm & Sassoubre, 2014). *Enterococci* are opportunistic pathogens that have developed resistance to penicillin and are naturally resistant to cephalosporins used in treatments (Byappanahalli et al., 2012; Kristich et al., 2014). Due to these resistances, treatment of *Enterococci* infections require 10 – 100 times the concentration of antimicrobials used to treat similar *Streptococcus* infections (Kristich et al., 2014).

Human activity has a significant impact on the microbial communities of urban waterways. Urbanization in many cities has led to the contamination of waterways with faecal matter from combined sewer overflows (McLellan et al., 2015). Older cities that have combined sewers for both human waste and urban runoff can overflow with even the smallest quantity of rainfall and contaminating local waterways (McLellan et al., 2015). As New Zealand's largest city, the Auckland Council conducts surveillance on the microbial count of the local streams and rivers. Surveillance has found that most streams and rivers in Auckland have an *E. coli* count ranging from 100 – 10, 000 CFU/100ml (Ingley & Groom, 2023). The streams and rivers monitored by the Auckland Council are not associated with urban runoff yet show unsafe levels of contamination. While the Auckland Council monitors the levels of contamination present in its waterways, monitoring of antimicrobial resistance is not part of routine surveillance.

Research Aims

Research Question 1: How prevalent is antibiotic resistance among the indicator bacteria population in the Paremuka stormwater system during winter and summer?

Aim 1: Survey the indicator bacteria population at the Paremuka stormwater system between seasons.

Aim 2: Determine the presence of antibiotic-resistant indicator bacteria from selected sample sites.

Objective 1: Quantify viable indicator bacteria using MPN and MF.

Objective 2: Culture and isolate resistant organisms using CHROM ESBL and VRE agar.

Objective 3: Assess antimicrobial susceptibility profile of individual isolates using disc assays with various antibiotics.

Research Question 2: What are the genetic mechanisms contributing to antibiotic resistance used by organisms isolated from the stormwater system?

Aim 3: Determine the resistance genes in isolates through whole genome sequencing.

Aim 4: Identify potential mobile resistance genes in identified species.

Objective 5: Identify the bacteria species with antibiotic resistance to determine mechanisms using known databases.

Objective 6: Identify resistance genes in relation to mobile genetic elements within sequences.

Chapter 2 Literature review

NZ waterways

New Zealand's water quality has been viewed negatively by the public. Surveys conducted on the population's opinion regarding the overall quality of New Zealand's rivers have ranged from 'somewhat adequate' at best to 'very bad' at worst (Larned et al., 2016). Between the early 1990s and the 2010s, there was a significant increase in total nitrogen and decrease in dissolved oxygen, which was attributed to the expansion of livestock farming (Ballantine & Davies-Colley, 2014). As farming increases, local waterways tend to accumulate more *E. coli* than other waterways unaffected by farming with bacteria levels up to 20 times higher than forested waterways (Foote et al., 2015).

As the rate of farming increases in New Zealand, so too does the use of antimicrobials to treat illnesses among livestock. While New Zealand is among the lowest consumers of antimicrobials in livestock, that number is increasing by 2.5 to 3 percent per year (Pattis et al., 2022). Antimicrobials are generally used in pig, poultry and cattle farming, and as animals are in constant proximity to one another, disease can easily spread from one to another.

As antimicrobial consumption increases among livestock, resistance in the microflora of livestock also develops. Surveillance of a dairy herd over 15 months found that the genes associated with resistance, which were considered a public health risk were low when compared to overseas sources, yet they were present among effluent water discharge and local effluent ponds (Collis et al., 2022). In effluent ponds and the surrounding soils, pathogenic bacteria like *Campylobacter spp., Salmonella spp.,* and *E. coli* have been found to survive for up to 25 days and contaminate waterways (Wang et al., 2004). The number of pathogens present is seasonal dependant with warmer seasons during spring and summer accounting for higher concentrations than cooler seasons of autumn and winter (Selvakumar & Borst, 2006).

While New Zealand's pastoral waterways are of concern for contamination and harbouring resistant organisms, it is the urban waters that pose the greatest risk to public health. Although the number of rivers that flow through urban areas in New Zealand accounts for less than 1 percent of total river length, 85 percent of the population lives in urban areas (Chakravarthy et al., 2019). In lowland pastoral and urban locations, the water quality in New Zealand has consistently elevated levels of nitrogen and *E. coli* associated with the trend of increasing agriculture and urbanisation (Gadd et al., 2020). These changes align with the level in urbanisation observed within the Auckland metropolitan area with the level of urbanisation created multiple impervious surfaces during the 2000s, accounting for 39 percent to 42 percent in 2008 (Meurk et al., 2013).

Auckland Systems

In Auckland, combined sewer systems were implemented to handle surface runoff and integrate into the sewer network to allow for treatment (Chakravarthy et al., 2019). Auckland has experienced multiple heavy rain events that have overwhelmed the combined sewer network and discharging its contents into receiving water ways, with the Auckland Anniversary floods in particular accounting for a total of 265 mm of rain in a single day

(Brook & Nicoll, 2024; Chakravarthy et al., 2019). An alternative method to control stormwater, implemented by the Auckland Council is the use of retention ponds. This approach continues to be, and is expected to remain a part of the Auckland Council strategy (Auckland Regional Council, 2003).

While the Auckland Council maintains regular surveillance on the rivers and streams within its jurisdiction, not all are integrated into the surveillance system. In the report conducted by Ingley & Groom (2023), the faecal coliform and *E. coli* count in the surveyed streams and rivers ranged between 100 – 10,000 CFU/100 ml of water. For *E. coli*, limits for exposure for recreational activities such as swimming cannot exceed an average of 126 CFU/100ml of water, and the maximum limit for *E. coli* exposure from a single exposure event is 235 CFU/100ml of water (Rock et al., 2018). Consistently high numbers of *E. coli* is indicative of waste and faecal contamination; however the report by Auckland Council does not delve in depth into the resistance profile of indicator bacteria or *E. coli.* A study conducted on the resistance profile of bacteria of in a sewer-contaminated river found that bacteria such as *Shigella spp. Citrobacter spp.* and *E. coli* have been found to have resistance to the antimicrobials ampicillin and tetracycline (Young et al., 2013). While the Auckland Council monitors the overall health of the streams and rivers within the Auckland region, no data could be found regarding the number and types of microorganisms found in stormwater retention ponds. Furthermore, it is currently unknown whether the retention ponds are affecting the microbial profiles of the stream environment downstream.

Stormwater

To prevent flooding in developed and urbanised areas, urban planning must consider where surface water drains. In some cities, surface water is integrated into the local sewer system to be treated like municipal sewage (Montserrat et al., 2015). In some instances, the urban environment does not provide any direct route for water to flow naturally through, as structures like roads and pavements are not porous, leading to large pooling of water (Hamer et al., 2012). To prevent large-scale flooding, artificial retention ponds are constructed to divert surface water (Sébastian et al., 2014).

Retention ponds are artificial ponds constructed to manage excess surface water in developed urban areas and allowing its contents to settle before discharging downstream (Wakelin et al., 2003; Yazdi et al., 2021). However, due to the role of retention ponds as collection points for surface water, these artificial ponds accumulate various contaminants ranging from industrial heavy metals to biological contaminants from waste (Hall & Anderson, 1988). Stormwater can contain an amalgamation of various contaminants that are carried by surface water during heavy rain, and allowing stormwater runoff to flow directly into local water sources can have detrimental effects (Greenway, 2010).

Stormwater contains the biological contaminants sourced from waste overflows, farmland, and urban areas, but it also contains heavy metals, polycyclic aromatic hydrocarbons, and inorganic particles (Aryal et al., 2010). While retention ponds are designed to allow contaminants to settle, the resulting water is still discharged into local bodies of water (Yang & Lusk, 2018). Although the contents are slowly discharged into nearby water sources, the resulting discharge can still have a negative effect on the local lakes, streams, or wetlands. Urban

environments produce greater volumes of water during runoff events, which cause adverse effects downstream (Barbosa et al., 2012).

Urban streams are more sensitive to the concentration of pollutants discharged into them, and thus, the methods implemented by retention ponds to reduce contaminant load are generally ineffective (Fletcher et al., 2014). Due to the high correlation between exposure to contaminated water and human illness, water that flows downstream to areas of recreation has a high chance of causing illness (Steele et al., 2018; Tseng & Jiang, 2012). One study analysed the levels of indicator organisms in a river with a wastewater treatment plant discharging into it and found that there was no significant difference in the number of indicator bacteria upstream and downstream of the treated discharge, with authors speculating that the contaminants were transported via storm pipes from animal-derived sources (Gannon & Busse, 1989).

Waste overflow & River contamination

When excreted into the waste system, bacteria with characteristics and resistance unique to an individual are exposed to the collective waste system of the community, exerting ecological stress on multiple organisms (Tiwari et al., 2022). Wastewater exerts unique biological pressures on organisms, which can stress bacteria as wastewater contains an amalgamation of biocides ranging from chlorines, bacteriophages, and antimicrobials from both environmental and individual sources. These pressures can cause bacteria to develop resistances or acquire resistance from other organisms (Tiwari et al., 2022). As such, the removal of pathogens and bactericides is required before discharging treated water.

As urban sewer infrastructure ages and heavy rain events become increasingly common, environmental contamination due to wastewater overflow is becoming increasingly common. Some wastewater systems are integrated to collect surface runoff but become overwhelmed during heavy rain, causing the contents of the sewer to be discharged (known as Combined Sewer Overflow) into urban waterways such as streams and rivers (Muttil et al., 2023). Many cities in both the US and Europe use combined sewers as part of their urban infrastructure as a cost-effective method to manage both urban sewage and surface water runoff, which are responsible for the discharge of micropollutants into nearby water sources (Phillips et al., 2012; Sojobi & Zayed, 2022).

Contamination of waterways with urban sewage and associated faecal bacteria has become more prominent in many cities globally (Kenzaka et al., 2001; Servais et al., 2007). During heavy rain, rivers and waterways can experience an increase in faecal indicator bacteria up to 1.2 log units higher than in dry weather conditions (Nnane et al., 2012). While some contamination does come from wastewater overflows, other sources of faecal contamination can also be derived from farmland, parks and urbanised areas (Kim et al., 2013; Servais et al., 2007).

Sewer overflows are not the only source of faecal contamination in urban waterways. As urban infrastructure ages and the population density increases, damage to wastewater pipelines connecting buildings to the sewer network can cause contents to leak into the surrounding environment (DeSilva et al., 2005). Sewer system failure occurs in two ways: exfiltration and infiltration. Exfiltration results from damage to the sewer network

pipeline, causing contents to leak out and contaminate surrounding ground water and soil (DeSilva et al., 2005; Nguyen et al., 2021). Damage to sewer infrastructure can be caused by many factors such as movement of soil during dry periods, traffic pressure or an overloaded system causing an increase in sewer pressure that leads to rupturing (DeSilva et al., 2005). In contrast, infiltration occurs when sewer systems receive an influx of water either during wet weather or an elevated water table flowing into a system and accelerate failure by straining infrastructure or causing adjacent failure by blocking pipes with backfill (Karpf & Krebs, 2011).

As development of urbanised areas increases and population density increases, the infrastructure originally designed for a lower population struggles to keep up with increased flow rates due to modern consumption behaviour (Perry et al., 2024). As a result, the quantity of untreated waste discharged into the environment contributes to 70 percent of total wastewater discharge (Xia et al., 2024). Bacteria such as *E. coli* and faecal *Enterococci* are used as quality indicators as water contaminated with faecal matter poses a risk to human health due to the diversity of pathogenic organisms present and in combination with the impracticality of testing for every pathogenic bacteria (Baghel et al., 2005). As a result of repeated discharge, the organisms associated with faecal matter mainly *E. coli* integrate with the local bacteria communities called biofilms (Balzer et al., 2010). Due to these indicator organisms integrating into the environmental biofilms, their concentration remains high, independent of free-flowing water, which would normally be contaminated with waste during heavy rain (Mackowiak et al., 2018). Indicator bacteria that contaminate above-ground drinking water sources, like dammed lakes and rivers, have fluctuating concentrations depending on the time of year, with concentrations significantly higher in the summer compared to winter (Reitter et al., 2021).

Environmental Monitoring Methods

Environmental monitoring tends to be limited to water sources associated with drinking water supplies or in the event of a significant infrastructure failure. Various methods are employed to monitor the types of organisms that are present in different water sources ranging from culture-dependant methods to DNA sequencing methods (Saxena et al., 2015). Depending on which method is employed in surveillance, the various advantages and disadvantages of each must be considered. Cultural methods, like heterotrophic plate count and Most Probable Number (MPN) asses the amount of bacteria in a given volume of water and are typically used as a quality indicator to evaluate the overall water quality (Saxena et al., 2015; Sutton, 2010). While the process to perform the cultural tests is quick, the incubation required for organism growth is time-consuming (Gunda & Mitra, 2016).

As such, contaminants that flow into rivers drastically alter the microbial flora of the affected waterways prompting researchers and public officials to use tests such as MPN to quickly quantify indicator bacteria in a given volume of water and, by extension, potential pathogens (Ahmed et al., 2013). Although commonly used, cultural methods like MPN are not without limitations.

Freshwater conditions such as water temperature, nutrient content or even exposure to sunlight can be bactericidal to faecal indicator organisms. It has been observed that *E. coli* can survive up to thirty days in freshwater (Petersen & Hubbart, 2020; Wcisło & Chrost, 2000). Bacteria when exposed to sublethal

environmental stress become viable but non-culturable, which in turn leads to organisms being undetectable by culturable means, as is the case for some pathogenic strains of *E. coli* O157:H7 (Liu et al., 2008). Outbreaks in relation to sewer overflows have been recorded; one study found that an outbreak of gastroenteritis was the result of underground well water being contaminated with faecal bacteria including *E. coli, Campylobacter jejuni, and Salmonella spp.* (O'Reilly et al., 2007).

AMR Detection

Organisms that contain resistances and remain within a sewer network are generally not an issue as they go further down the treatment. However, conventional wastewater treatments have been found to be somewhat ineffective at removing resistant bacteria genes and associated antimicrobials from both water and organic sludge (Hiller et al., 2019; Pino-Hurtado et al., 2024). Studies conducted on wastewater discharge have found to increase abundancy of resistance genes in the microbial community (Marti et al., 2013; Osińska et al., 2020).

Surveillance methods are already implemented in organisations such as The European Antimicrobial Resistance Network (EARS-Net) and GLASS (Aarestrup & Woolhouse, 2020). Recently, surveillance was conducted to monitor for SARS-CoV-2; however, due to the intrinsic threat such diseases pose to human health, only 59 percent of high-income countries actively monitor wastewater for pathogenic strains and only 13 percent of low-income countries conduct surveillance (Keshaviah et al., 2023). Monitoring in low-and middle-income countries is less prevalent, as resistance genes are in lower concentrations than in high-income countries due to the higher degree of sanitation and public health (Baba et al., 2022). Countries that implement wastewater surveillance, such as Finland, Spain, and Turkey have made it part of national surveillance to track transmission. Other countries, such as the US, UK and Australia, have adopted regional surveillance (Dos Santos et al., 2024).

Surveillance for resistance can be done using two primary methods: HT-qPCR and metagenomics sequencing. HT-qPCR (High Throughput - Quantitative Polymerase Chain Reaction) is a cost-effective method of surveying due to the high sensitivity and low cost per sample, due to the selective nature of the primer sequence targeting (Knight et al., 2024). However, qPCR is not without limitations; due to the highly selective nature of the genetic primers, separate genes related to resistance or completely different novel genes may go undetected (Abramova et al., 2023; Knight et al., 2024). HT-qPCR allows for rapid detection and quantification of select genes from a large volume of samples with high sensitivity, making it the preferred method for large-scale surveying (Srathongneam et al., 2024). Current PCR methods have been implemented in public health responses to monitor for diseases such as SARS-CoV-2 and other pathogenic genes in both waste and environmental monitoring (Dos Santos et al., 2024; Osińska et al., 2020).

Alternatively, metagenomic sequencing allows for a broader scope for DNA analysis at a higher cost, as metagenomic analysis does not require targeted gene sequences. Metagenomic analysis can detect novel genes and provides a taxonomic library. However, metagenomics lacks the sensitivity of PCR, requiring more genetic material for analysis (Knight et al., 2024). While not as cost-effective as HT-qPCR, metagenomic sequencing provides advantages in wastewater surveillance, with 99 percent of all bacteria found in sewage

cannot be cultured and can provide data on bacteria that are not traditionally studied (Garrido-Cardenas et al., 2017).

Traditional metagenomic sequencing tends to be a more expensive method of analysis, with instruments such as HiSeq 2000 costing $6,000 per genome and an upfront cost of $690,000 for the instrument, which requires between 3 to 10 days to sequence (Liu et al., 2012). As advancements in sequencing have developed, more cost-effective methods, such as Nanopore sequencing costs approximately $1000 per sample and provide efficient real time reads of DNA sequences (Lemon et al., 2017). One study conducted whole genome sequencing to identify both the species of carbapenem resistant Gram-negative bacteria and their gene sequence responsible for resistance from wastewater (Cirkovic et al., 2023). The use of technology like Nanopore sequencing has enabled the analysis of resistant bacteria and their mechanisms of resistance, allowing for researchers and health care professionals to apply targeted treatment for infections (Schmidt et al., 2016).

In an effort to find faster methods to detect specific organisms, molecular methods such as Whole Genome Sequencing (WGS) can be used to identify both bacterial species and resistance mechanisms. WGS has been employed against pathogens in food surveillance to track resistance profile, detect outbreaks, and control infection caused by food borne pathogens, including *Salmonella spp., Listeria spp.,* and pathogenic *E. coli* (Oniciuc et al., 2018). Depending on the methodology, WGS employ a wide range of techniques, each with its own limitations in the analysis of bacterial genomes. One such method is Illumina Sequencing, which functions by adding a fluorescent nucleotide to the nucleic acid chain, resulting in a fluorescent signal to be imaged (Hu et al., 2021). Illumina benefits from reduced costs and higher read through puts due to being a next-generation sequencing method, compared to the first-generation methods like Sanger and Maxam-Gilbert, which were time-consuming, expensive, and exposed the user to harmful chemicals (Moti et al., 2022) While effective, Illumina is limited by being able to sequence shorter read lengths and clusters of DNA sequences that interfere with nucleotide reads (Kircher & Kelso, 2010).

Conversely, methods employed by Oxford MinION Nanopore utilise ionic currents to analyse genomic sequence. Oxford Nanopore sequencing is a third-generation sequencing method that employs a portable device with multiple nanopore channels immersed in an ionic solution, with a ionic current passing through the nanopore (Plesivkova et al., 2019). As molecules traverse the nanopore, the ionic current is disrupted, with each nucleotide base pair causing a specific disruption to the ionic current. One of the unique strengths of the MinION is the ability to read large sections of DNA, with an average read length of 1-2 kB, with longer reads ranging up to 98 kB (Laver et al., 2015). However, MinION Nanopore is limited by its accuracy, and despite improvements to computational tools and chemistry, it has a significant error rate ranging from 5 – 15% (Rang et al., 2018). Regardless of the method used, WGS allows for the analysis of the entire bacterial genome, including mobile genetic elements, identify relationships of specific genes and various organisms (Oniciuc et al., 2018). When analysing the genomic profile of unknown or environmental organisms, WGS has advantages over common molecular methods like PCR, as PCR uses primers that are based on the database of cultured bacteria, which diverges from the diverse genetic profile of environmental bacteria (Smith & Osborn, 2009).

Antimicrobial Resistance

Antimicrobial-resistant infections have been increasing and pose a significant burden to treat. Antibiotic resistance is a result of natural selection, with select bacteria species being exposed to antimicrobials and developing mutations to combat effectiveness of antimicrobials (Exner et al., 2017). Medications used to treat infections are becoming less effective, posing a risk to public health in both developed and developing countries (Dadgostar, 2019). Deaths associated with antimicrobial-resistant (AMR) bacteria have documented 33,000 deaths per year in the European Union and costing 1.5 billion euros per year (Aljeldah, 2022). Similar costs occur in the US, where AMR bacteria contribute to an additional 20 billion dollars in health-related costs (Dadgostar, 2019).

An alarming trend of infections resistant to treatments is becoming more common. Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) have been found to show resistance to groups of antimicrobials, such as aminoglycosides, macrolides and tetracyclines (Nikaido, 2009). Such infections are prevalent in healthcare settings, with clinical isolates from the US and Europe showing resistance to 35 percent of cases (Haddadin et al., 2002). While MRSA can be treated with the antimicrobial vancomycin, *Enterococcus spp.* strains have developed resistance to vancomycin (VRE) and have been found to be co-colonize with MRSA. Although rare, there have been instances of shared resistances between species (Furuno et al., 2005; Nikaido, 2009).

MRSA and VRE are not the only resistant organisms that have been isolated from healthcare cases. When comparing infection rates between Gram-positive and Gram-negative infections, Gram-negative organisms contribute to most cases. A study of 191 patients found that 71.6 percent were hospitalized with multidrug resistant Gram-negative infections, contrasting with the remaining 28.4 percent of patients hospitalised with MRSA and VRE. (Lemmen et al., 2004). Prior to the widespread use of antibiotics, infections involving Gram-negative bacteria were rare, with fewer than 100 reported cases in the 1920s (Waterer & Wunderink, 2001). Gram-negative infections have caused many clinical reports such as intra-abdominal infections, urinary tract infections and bacteraemia (Kaye & Pogue, 2015). Treatment of Gram-negative infections relied on antibiotics such as penicillin, cephalosporins and carbapenems (Yusuf et al., 2021). Since the discovery and use of penicillin, the effectiveness of the antimicrobial began to decrease against certain bacteria, and modifications to the to the drug led to the creation of ampicillin to extend and expand the effectiveness against infections (KaushiK et al., 2014). As ampicillin had continued use to treat infections resistant to the original penicillin, strains of bacteria resistant to ampicillin began to emerge.

Antibiotic Usage

Ampicillin and penicillin, in general, function by inhibiting to the protein transpeptidase, thereby preventing the formation of peptidoglycan, a key component in the bacterial cell wall (Ghooi & Thatte, 1995). As ampicillin is used in the treatment of bacterial infections, a variety of bacteria that were previously susceptible to ampicillin have started showing signs of resistance. Of the patients infected with a strain of non-multidrug resistant *Acinetobacter baumannii,* only 4.5% of the isolates were susceptible to ampicillin (Smolyakov et al.,

2003). Resistance to ampicillin was not limited to pathogenic nosocomial isolates. One study found that among the local microbiome of freshwater river sources found that the most common organisms found in the ecosystem resistant to ampicillin were bacteria from the species such as *Acinetobacter spp., Citrobacter spp.,* and *Enterobacter spp.* (Ash et al., 2002). Certain strains of Gram-negative bacteria can to produce enzymes known as extended-spectrum beta-lactamase (ESBL) that hydrolyse and deactivate beta-lactam antibiotics (Falagas & Karageorgopoulos, 2009; Yusuf et al., 2021). ESBL-producing organisms have contributed to infection cases in both community and hospital settings, with rates as high as 25 percent in Western Europe, 30 percent in Latin America, and 15 percent in Australia and New Zealand (Macesic et al., 2025). By the early 1970s, the TEM-1, TEM-2, and SHV-1 ESBL genes responsible for resistance against penicillin in *E. coli* isolates had spread to 50% of other bacteria in the *Enterobacteriaceae* family and later to *Haemophilus influenzae* (Livermore, 2008).

With the increasing resistance of ampicillin spreading through multiple bacterial species, additional compounds are often taken in combination with penicillin to improve bacterial susceptibility. The addition of clavulanic acid to inhibit ESBLs is administered in combination with penicillin to improve the effectiveness of treatment (Falagas & Karageorgopoulos, 2009). Clavulanic acid is a molecule that inhibits specific beta-lactamase enzymes by irreversibly binding to the beta-lactamase protein, thereby preventing the breakdown of the antibiotic (Huttner et al., 2020). The combination of amoxicillin and clavulanic acid has been used in combination to treat a variety of infections caused by *S. aureus, E. coli,* and *H. influenzae* (White et al., 2004). However, resistance to clavulanic acid combination treatments have also been observed. In a study of 9090 *E. coli* isolates taken from patients, 12.5 percent showed nonsusceptibility, and a further 5.1 percent showing resistance (Oteo et al., 2008).

While penicillin is one of the most used medications used to treat infections, another frequently used group of antibiotics is the cephalosporin class. Cephalosporins emerged as an alternative when organisms developed resistance to the first generation of penicillin (Valegård et al., 1998). Cephalosporins function similarly to penicillin by disrupting the stability of the bacterial cell wall during replication, causing the bacterial cell to rupture (Harrison & Bratcher, 2008). Cephalosporin antibiotics such as cefalexin and cefadroxil, are considered the second line of drugs for the treatment of infections of *E. coli, K. pneumoniae* and *Proteus mirabilis* (Nguyen & Graber, 2020). Even with less usage when compared to penicillin, resistance to cephalosporins has been affected by the same mechanisms bacteria used to inhibit penicillin.

Other types of antibiotics used to treat severe infections include carbapenems, such as meropenem. Carbapenems are considered a last-resort antibiotic and function by binding to penicillin-binding proteins, resulting in the deactivation of autolytic inhibitor enzymes that lead to cell lysis (Aslam et al., 2020). Carbapenems are generally used to treat a wide range of bacteria, particularly organisms in the *Enterobacteriaceae* family, which have a 99 – 100 percent susceptibility rate (Breilh et al., 2013). Recently, carbapenem-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa* cases have not only increased but also shown resistance to multiple classes of drugs used in treatment, requiring more novel antibiotics to treat (Bassetti et al., 2019; Waterer & Wunderink, 2001).

Resistance Mechanisms

Resistance to antibiotics is classified into three resistance mechanisms: penetration prevention and efflux pumps, antibiotic target modification, and antibiotic deactivation (Blair et al., 2015). Gram-negative bacteria contain both an inner and outer phospholipid membrane, with the outer membrane containing porin proteins that antibiotics enter the bacterial cell through. By limiting the number of access porins, only limited types of antibiotics penetrate the outer membrane, thereby enhancing bacterial survivability (Blair et al., 2015). Of the antibiotics that manage to penetrate the outer membrane, toxic compounds are then subsequently removed by efflux pumps. Efflux pumps are protein structures that expel waste molecules by the organism and play a role in expelling other compounds toxic to the cell (Piddock, 2006). Efflux pumps utilise multiple mechanisms and have been found in a broad spectrum of bacterial species, targeting a wide range of antimicrobial compounds, often overlapping in their targeted compounds (Lin et al., 2015).

Other organisms employ mechanisms, such as target modification to lower the effectiveness of certain antimicrobials. Antimicrobials like polymyxin function by having both positively charged sections and non-polar sections within the structure, which allows it to attract to the negatively charged outer membrane, disrupting the bacterial cell wall and causing cell lysis (Velkov et al., 2010). Organisms can acquire resistance to cationic antimicrobials by modifying the overall charge to the outer membrane to a neutral charge by adding either phosphoethanolamine (PEtN) or 4-amino-deoxy-4-L-arabinose (L-Ara4N) (Olaitan et al., 2014).

Other organisms produce enzymes that either modify or deactivate the active component in certain antibiotics. The most prominent enzyme that is utilized in the deactivation of antibiotics is Extended Spectrum Beta-Lactamase (ESBL). Penicillin, cephalosporins and carbapenems all fall under beta-lactam antibiotics and, as a result, are susceptible to the wide range of ESBL (ur Rahman et al., 2018). Beta-lactamases operate by disrupting the structure of the antibiotic by hydrolysing the beta-lactam ring, rendering the antibiotic ineffective (Bonomo, 2017). Initially, ESBL genes were grouped into the SHV and TEM gene groups and were the dominant genes in antibiotic resistance found primarily in the bacterium *K. pneumoniae* (Coque et al., 2008). More recently, *E. coli* have been found to harbour the genes responsible for resistance against beta-lactam antibiotics and have expanded to multiple iterations of gene groups such as TEM (TEM-4, TEM-24 & TEM-54), SHV (SHV-5 & SHV-12) and CTX-M (CTX-M9, CTX-M14 & CTX-M15) (Coque et al., 2008; Gniadkowski, 2001).

Many organisms in the *Enterobacteriaceae* family have become resistant to multiple antibiotics used in treatment. *E. coli* has been found to be responsible for severe infections, with certain strains resistant to multiple treatments (Hutinel et al., 2019). Due to *E. coli* having the ability to have multiple resistance to treatments and its presence as part of the biological flora of humans and animals, the organism is used in multiple antimicrobial resistance surveillance organisations such as the World Health Organisation's Global Antimicrobial Resistance and use Surveillance System (GLASS) (Anjum et al., 2021; Hutinel et al., 2019). Due to its prevalence in humans and animals, *E. coli* is also used as an indicator organism in water sources to gauge the level of faecal contamination in drinking, ground, and recreational water (Invik et al., 2017; Ishii & Sadowsky, 2008).

Literature Review Summary

In summary, antibiotic-resistant bacteria is an alarming trend, and surveillance methods can no longer focus solely on wastewater monitoring. Stormwater can contain similar resistant pathogens due to the influx of waste derived from both human and animal sources, posing a risk to public health if overlooked. Due to a combination of extreme weather, ageing infrastructure, and rapid urbanisation, resistant organisms are escaping and contaminating local environments. Local retention systems designed to prevent urban flooding could be a reservoir for pathogens to exchange antibiotic resistance genes with similar organisms, expose organisms trace contaminants to develop resistance, and due to the nature of being untreated, put public health at risk as water is discharged further downstream into recreational areas. Due to the consistently high rates of contamination within Auckland's waterways, coupled with the lack of information regarding the resistance profile of the indicator bacteria highlights the need for research into the resistance profile of the indicator bacteria found in Auckland's waterways.

Chapter 3 Culturing and Antibiotic Resistance Profile

Introduction

New Zealand is subject to multiple natural disasters with extreme flooding becoming more frequent over recent years. It is predicted that the number of severe flooding events, which occur every 50 to 100 years, will become more destructive by 2050 (Wang et al., 2021). As New Zealand's largest metropolitan city, Auckland Council employs Water Sensitive Design, which integrates natural cycles and catchments of the environment to minimise downstream flooding and water degradation (van Roon, 2011; Wong, 2006). One of these methods implemented to reduce flooding is retention ponds, which are designed to catch surface runoff and the associated pollutants, slowly discharging downstream (Baird et al., 2020). Reports from Auckland Council have found that the streams and rivers throughout the region far exceed the safe levels of indicator organisms for recreational activities (Ingley & Groom, 2023; Rock et al., 2018). Auckland Council monitors water quality of multiple streams, both rural and urban, throughout the region, little is known about the retention systems that discharge into local waterways.

Contaminated waterways have the potential to act as hotspots for the acquisition of antibiotic resistance genes, either through exposure or via horizontal gene transfer (Li & Zhang, 2022). Pathogens found in untreated wastewater can share genetic information to acquire resistances to current antibiotics. One known human pathogen, *Vibrio parahaemolyticus* is a pathogenic bacteria found in water that has been shown to have shared resistance and virulence genes with *Enterobacter spp.* and *Shewanella spp.* (Fu et al., 2022). A similar study found strains of *E. coli* susceptible to gentamycin, tetracycline and erythromycin was donated resistance by *Pseudomonas aeruginosa* (Shakibaie et al., 2009). Due the wide range of pathogenic bacteria that are considered a health risk, testing for every individual bacterium is impractical (Baghel et al., 2005). Generally, surveillance of related species acts as an indicator for the overall environmental and resistance profile of a given location (Kojima et al., 2009).

The most common methods used in microbial testing of water is the Most Probable Number (MPN) and membrane filtration (MF). MPN is a microbiological test that is commonly used to estimate the number of a given bacterium in water and milk without directly counting colonies (Cochran, 1950). The other method used in water surveillance is MF. MF is a microbiological test that uses filters of a specific pore diameter to capture cells and determine the number of bacteria present in a given volume of water (Clark et al., 1951).

MPN and MF provide information regarding bacterial counts and density, but neither method provide information on antibiotic resistance. Due to the numerous bacterial species found within the water, antibiotic-resistant organisms must be selectively cultured and isolated. Selective media works by restricting growth of certain bacteria, either by limiting an essential nutrient or by adding toxic compounds to specific bacterial groups (Bonnet et al., 2020). Multiple species of bacteria can grow in selective media due to their close relation, and they must be isolated before undergoing antibiotic susceptibility testing. Bacterial isolation and cultivation involves growing bacterial colonies on a sterile medium and continuously cultivating by streaking to

isolate individual bacteria to produce pure colonies of a single organism (Hahn et al., 2019). The most common test used to determine antibiotic susceptibility is the disk diffusion test. The disk diffusion test relies on an inoculum of bacterial cells set to 0.5 McFarland standard (Tendencia, 2004). The McFarland standard is a scale that ranges from 0 to 10 on the number of bacterial cells suspended within a solution, and is measured by turbidity (Gayathiri et al., 2018).

Various environmental factors contribute to the antibiotic resistance profile of the bacterial community. The Paremuka stream system including Lake Panorama settlement pond, borders both an agricultural region and the West Auckland suburb of Henderson. This stream system offers unique insight into which environment affects the overall water quality and bacterial community.

Methods

Sampling

Water samples were collected along the Paremuka stream including the Lake Panorama stormwater retention pond. Samples were twice in September, before and after a significant rain event, once in December and once in February. Water samples were collected in 1L sterile plastic bottles and labelled by sample location. Water samples were collected from 3 sites along lake Panorama with the first site being upstream of the lake (36°53’7.597”S, 174°36’7.851”E). The second site was from Panorama Lakeside (36°53’4.837”S, 174°36’9.858”E) and the third was the downstream discharge (36°52’59.522”S, 174°36’18.674”E). The other sample sites were from along the Paremuka Stream (36°52’43.035”S, 147°36’27.024”E) and the Paremuka Estuary (36° 51’ 17.492”S, 174°37’7.711”E). Locations where water was sampled can be seen in Figure 1. Sampling from the Paremuka ponds and the estuary site on the 11/12/24 was omitted due to quarantining for an invasive pond weed and unsafe conditions that prevented sampling.

Figure 1. Sampling Locations of Lake Panorama and Paremuka Stream.

The bottles containing water samples were placed into chiller bags and taken to the AUT laboratory to be analysed within 2 hours of sampling.

Membrane Filtration

A 100ml aliquot from each sample site were vacuum filtered through with a Sartorius 0.45 µm cellulose nitrate filter. This process was done twice for two filtrate samples per location. Each filter was then placed on to CHROM Vancomycin Resistant Enterococci (VRE) and CHROM Extended-Spectrum Beta-Lactamase (ESBL) agar to detect and quantify the number of presumptive VRE and ESBL *Enterobacteriaceae*. Filters on CHROM VRE were incubated for 48 hours at 35°C and 24 hours at 35°C for ESBL agar. After incubation, the colour and number of colonies were identified and enumerated. Due to equipment failure on the 11/12/24, water samples were spread plated on to CHROM VRE and ESBL in placement of vacuum filtration. A 0.1ml aliquot was taken

from each site and pipetted onto respective CHROM VRE and ESBL agar and spread using a flame sterilised glass spreader. CHROM VRE plates were incubated for 48 hours at 35°C and 24 hours at 35°C for CHROM ESBL plates.

Most Probable Number

For the MPN, a three-tube method was used according to the United States Department of Agriculture (2025). Water samples were inoculated into tubes of double strength and single strength Oxiod Lauryl Tryptose Broth (LST). For each location, five tubes of 10ml double strength LST tubes were inoculated with 10ml of sampled water. A further ten tubes containing 10 ml of single strength LST broth was prepared, with five tubes receiving 1 ml of sample water in each tube. The remaining five tubes received 0.1 ml of sampled water in each tube. Tubes were then incubated at 35°C overnight. Tubes were positive if gas was present in the inverted Durham tube and turbidity. Positive tubes were recorded for each location and compared to the MPN chart from the United States Department of Agriculture (2025).

ESBL Isolation from Tubes

Two sets of 10ml of water samples were pre-enriched in double strength LST broth and incubated overnight at 35°C and 42°C respectively. Positive tubes were streaked on CHROM ESBL agar and incubated overnight at their respective temperatures. Growth on CHROM ESBL agar were presumed to have resistance to beta-lactam antibiotics and underwent identification via Gram staining. To create a stock culture of isolates, colonies that had grown on CHROM ESBL agar were then grown in nutrient broth for 24 hours at 37°C and used to make frozen stock culture. Aliquots were store mixed to a final volume of 30% w/v glycerol in cryovials and stored at -80°C until further use.

ESBL and VRE Isolation from filters

Isolates grown from filters on CHROM ESBL and VRE were assumed to have resistance to beta-lactamase antibiotics and vancomycin and underwent identification via Gram staining. Individual coloured colonies from filters grown on CHROM ESBL agar were carefully taken and streaked onto nutrient agar plates and incubated for 24 hours. To ensure a pure isolate was obtained from nutrient agar plates, colonies from the nutrient agar plates was further streaked on respective agars to ensure a single colony type was isolated. Individual colonies were then grown in nutrient broth for 24 hours and used to make a frozen stock culture. Aliquots of cultures were store mixed to a final volume of 30% w/v glycerol in cryovials and stored at -80°C until further use.

Antibiotic Disc Susceptibility Testing

Antibiotic susceptibility test of isolates was performed as per EUCAST (2015) guidelines. Isolates were streaked onto nutrient agar and incubated at 37°C for 16hrs. Once grown, individual colonies were taken and mixed into a vortex tube containing 5ml of 0.85% saline solution and adjusted until a turbidity reading of 0.5 of the McFarland Standard. Once the correct turbidity was achieved, the solution was streaked with a cotton swab over a plate of Muller-Hinton agar, rotating the swab for a total of three spreads to ensure complete coverage over the agar plate. Once streaked, antibiotic discs containing 10µg of ampicillin, 20µg of amoxicillin with 10µg

of clavulanic acid, 30µg of cefalexin, 10µg of meropenem and a blank disk containing 10µL of sterile Muller-Hinton broth as a control blank. Once discs were placed, plates were incubated at 35°C aerobically for 18 h. After incubation, the zone of inhibition was measured and compared to the EUCAST (2015) guidelines for antibiotic susceptibility. According to the EUCAST (2015) guidelines, the minimum diameter for susceptibility for Ampicillin, Amoxicillin with clavulanic acid, Cefalexin and Meropenem were 14mm, 14mm, 14mm and 16mm respectively.

Results

As seen in Table 1, the number of indicator organisms present in the water tended to increase further downstream from the retention pond. Indicator organisms also tended to increase after an increased level of rainfall which caused a large spike in indicator numbers when compared before rainfall. During the dryer months of December and February with minimal levels of rainfall, the indicator numbers far exceeded the levels observed during the winter months with more rainfall. All points along the retention lake and stream had elevated levels of indicator bacteria far exceeding the numbers seen during the winter months.

MPN Results

Table 1. Positive MPN results, presumptive ESBL growth and rainfall data (Auckland Council, 2025)

Location	*Date*	*Rainfall 7 days prior to sampling (mm)*	*MPN/100ml*	*Pres. ESBL count*	*Pre-Enrichment ESBL Growth at 35*	*Pre-Enrichment ESBL growth at 42*
Upstream	10/09/24	47.5	17	23cfu/100ml	Yes	No
	24/09/24	71.5	350	26cfu/100ml	Yes	No
	11/12/24	20	550	15cfu/0.1ml*	Yes	No
	17/03/25	3	1600	4cfu/100ml	Yes	No
Lake	10/09/24	47.5	70	35cfu/100ml	No	No
	24/09/24	71.5	250	66cfu/100ml	No	No
	11/12/24	20	350	3cfu/0.1ml*	No	No
	17/03/25	3	900	14cfu/100ml	No	No
Downstream	10/09/24	47.5	25	45cfu/100ml	No	No
	24/09/24	71.5	350	44cfu/100ml	No	No
	11/12/24	20	900	37cfu/0.1ml*	Yes	No
	17/03/25	3	?	5cfu/100ml	No	No
Stream	10/09/24	47.5	550	240cfu/100ml	Yes	No
	24/09/24	71.5	>1800	TNTC	Yes	No
	11/12/24	20	>1800	7cfu/0.1ml*	Yes	No
	17/03/25	3	1600	59cfu/100ml	Yes	No
Estuary	10/09/24	47.5	900	TNTC	No	Yes
	24/09/24	71.5	900	130cfu/100ml	No	No
	11/12/24	20	XX	XX	x	x
	17/03/25	3	11	TNTC	No	No

* = Samples were analysed using spread plate due to equipment malfunction.

XX = Samples were unable to be collected due to unsafe collection conditions

When observing solely the MPN results, the number of indicator bacteria stay consistently high throughout the year between winter and summer seasons. When observing growth on CHROM ESBL agar plates, a small number do show an increase during the winter and a large decline during the summer months. The stream area showed consistently high indicator counts in both the MPN and on CHROM ESBL agar indicating that the high number of bacteria present were assumed to be somewhat resistant to beta-lactamase antibiotics during the winter. During the summer while numbers were still high, the number of resistant bacteria had fallen. The downstream site showed an inconsistent MPN result during the last sampling period during the summer, leading to no matching value and being unable to confidently quantify the number of indicator bacteria

present. The estuary retained its consistently high number of beta-lactam resistant bacteria, in opposition to the mixed MPN results.

Identification Results

Colonies from each location was selected for further antibiotic susceptibility testing using two primary criteria. The first being able to successfully grow on either CHROM ESBL or VRE agar at the respective incubation temperatures. The second was having visible individual colony growing separate from the main cluster of growth colonies. Each location had the largest individual colony taken from both MF and pre-enrichment plates selected for isolation. As such, all isolates from MF were selected for identification and pre-enriched isolates from the upstream, stream and estuary were chosen due to the only isolates capable of growth on CHROM ESBL agar.

The cultivated organisms that successfully grew from both CHROM VRE and ESBL plates were all found to be Gram-negative rods, regardless of isolation method and location. Gram identification was omitted from colonies from the 17/03/25 as Gram staining was not conducted on isolates from that date.

Zone of Inhibition Results

During the winter period, all pre-enriched isolates showed some degree of susceptibility to all tested antimicrobials except for Ampicillin and can as seen in Table 2. An example of the resistance an isolate has can be seen against a control strain of *E. coli* in Figure 2. At the upstream sampling site, the light rain weather condition had colonies had a similar appearance to the heavy rain weather condition, but the light rain weather condition colonies were found to have resistance to cefalexin and the combination of amoxicillin with clavulanic acid when compared to the similar type of colonies during the heavy rain condition. Further down at the central stream sampling site, all isolates from that site had shown complete resistance to all antimicrobials except for meropenem. The only pre-enriched colonies to grow at 42°C was taken from the estuary site and was found to be only susceptible to amoxicillin and meropenem treatment.

Table 2. Susceptibility of Pre-Enriched Isolate growth from ESBL agar

Location	*Weather Condition*	*Organism Code*	*Blank*	*Ampicillin*	*Amoxicillin + Clavulanic Acid*	*Cefalexin*	*Meropenem*
Control	NA	E. coli 25922	0mm	15mm $_S$	22mm $_S$	16mm $_S$	37mm S
Upstream	Light Rain	UBd35	0mm	0mm $_R$	7mm $_R$	13mm $_R$	34mm $_S$
	Heavy Rain	UBr35	0mm	0mm $_R$	20mm $_S$	22mm $_S$	39mm $_S$
		URr35	0mm	0mm $_R$	19mm $_S$	22mm $_S$	37mm $_S$
Stream	Light Rain	SBd35	0mm	0mm $_R$	6mm $_R$	0mm $_R$	27mm $_S$
	Heavy Rain	SBr35	0mm	0mm $_R$	0mm $_R$	0mm $_R$	29mm $_S$
		SRr35	0mm	0mm $_R$	6mm $_R$	0mm $_R$	24mm $_S$
	Dry	SB-12-35	0mm	0mm $_R$	10mm $_R$	0mm $_R$	33mm $_S$

Estuary	Light Rain	BRd42	0mm	0mm $_{R}$	24mm $_{S}$	8mm $_{R}$	33mm $_{S}$

R = Resistant

S = Susceptible

Figure 2. Comparison between control strain of E. coli and an isolate

When observing the resistance profile of the filter isolates, all showed resistance to tested antibiotics with except for meropenem and can be seen in Table 3 and Figure 3. At the estuary site, filter isolates had shown similar results as the pre-enriched stream isolates.

Table 3. Susceptibility of Filter Isolates

Location	*Weather Condition*	*Organism Code*	*Blank*	*Ampicillin*	*Amoxicillin + Clavulanic Acid*	*Cefalexin*	*Meropenem*
Upstream	Light Rain	UBd-FP	0mm	0mm $_{R}$	0mm $_{R}$	6mm $_{R}$	34mm $_{S}$
	Heavy Rain	URd-FP	0mm	0mm $_{R}$	8mm $_{R}$	0mm $_{R}$	34mm $_{S}$
Lake	Light Rain	LBd-FP	0mm	0mm $_{R}$	0mm $_{R}$	0mm $_{R}$	29mm $_{S}$
	Heavy Rain	LBr-FP	0mm	0mm $_{R}$	6mm $_{R}$	0mm $_{R}$	34mm $_{S}$
Downstream	Light Rain	DBd-FP	0mm	0mm $_{R}$	0mm $_{R}$	0mm $_{R}$	34mm $_{S}$
	Heavy Rain	DBr-FP	0mm	0mm $_{R}$	0mm $_{R}$	0mm $_{R}$	34mm $_{S}$
Stream	Light Rain	SBd-FP	0mm	0mm $_{R}$	0mm $_{R}$	0mm $_{R}$	34mm $_{S}$
	Heavy Rain	SBr-FP	0mm	0mm $_{R}$	15mm $_{R}$	0mm $_{R}$	34mm $_{S}$
Estuary	Light Rain	BBd-FP	0mm	0mm $_{R}$	9mm $_{R}$	0mm $_{R}$	33mm $_{S}$
	Heavy Rain	BBr-FP	0mm	0mm $_{R}$	10mm $_{R}$	0mm $_{R}$	36mm $_{S}$

Figure 3. Filter Isolates from Lake and Downstream site

Discussion

Persistent presence of Enterobacteriaceae and lack of Enterococci

The presence of *Enterobacteriaceae* in both dry and wet conditions in the stormwater system was unsurprising. Between the light and heavy rain conditions in September, initial results indicated that after a period of rain the number of indicator organisms increased. These results were expected and mirror the results of several studies that had found an increase in overall indicator bacteria after rainfall (García-Aljaro et al., 2017; Goto & Yan, 2011; Tornevi et al., 2014). The presence of *Enterobacteriaceae* throughout the sampling period showed a greater increase of *Enterobacteriaceae* in the summer period, even after a rainfall event during the winter. With little to no rainfall over the dry period, it may be possible that the *Enterobacteriaceae* may be proliferating in the retention pond and the associating stream. These results contradict the findings of the papers of Peterson & Hubbart (2020) and Wcisło & Chrost (2000) as it was indicated that bactericidal properties of sunlight, freshwater and other contaminants found within stormwater ponds prevent bacteria like coliforms from surviving. It could be theorised that the higher concentration of bacteria in the summer could be due to proliferation as the increase in water temperature, suspension of particles preventing penetration of sunlight and nutrient abundance could be contributing to higher numbers. A separate paper by Reitter et al., (2021) did find that coliform bacteria were more prevalent in the summer and bacteria were capable of proliferation in the water column is due to the warmer water temperature. A similar paper also theorises that warmer water temperatures that average within the range of 21 to 26°C is the prime factor for survival in water (Abia et al., 2015). During sampling, the water temperature was not recorded at any point and could be an area for future research.

Even with the changes between the winter and summer seasons, the only location that managed to contain faecal coliforms was the winter sampling of the estuary site. Although the estuary site maintained consistently high indicator organism numbers in both rainy and dry conditions, no further growth was observed after the initial sampling. It can be inferred that the resistant faecal coliforms found during the first sampling of the estuary were susceptible to the hostile environment. While a source for the coliforms is up for speculation, a possible source could have been from a wastewater leakage from the nearby residential area or from the local wildlife and waterfowl from the associated park and reserve next to the site. It should be noted that results derived were from water either directly taken from surface standing water or from water that is being discharged in a flowing state. As such, numbers do not account for the potential count or resistance profile of bacteria located in biofilms or sediments.

One observation seen in the results was the lack of culturable *Enterococci* from any sample location. It is possible that *Enterococci* bacteria were present in the water, but the VRE agar used to attempt to culture the bacteria proved to be ineffective due to any *Enterococci* present having no resistance to vancomycin. Another possibility is that any potential *Enterococci* that had resistance to vancomycin were simply outcompeted by Gram-negative bacteria. One paper found that the growth profile of VRE bacteria was significantly slower and could not be grown in the standard incubation time or had grown in such small number and was outgrown by other subspecies (Heß & Gallert, 2016). With the possibility of any present *Enterococci* being out competed by

other environmental bacteria, it would be prudent to try to pre-enrich in a selective medium before streaking on CHROM VRE agar.

A possibility for the lack of VRE could be that the *Enterococci* present were unculturable. Organisms like *E. faecalis* can enter a viable but non-culturable state when exposed to unfavourable or environmental stress (Heim et al., 2002). Due to the complicated nature of VRE, one alternative method to identify the presence of VRE would be to look for the DNA profile of the vancomycin resistance genes. One such method would be to use PCR to detect specific genes species like the *van*A resistance gene (Lemcke & Bülte, 2000). The use of PCR to detect vancomycin resistance genes has been successful in surveying both waste and environmental water, but it should be mentioned that the genes associated with high-level vancomycin resistance come from the common environmental bacteria *Paenibacillus spp.* and can result in false positives (Adegoke et al., 2022; Arias & Murray, 2012).

It should be noted that the organisms that were successfully cultured were from the *Enterobacteriaceae* family or a similar type of fast-growing bacteria that favoured cultivation under laboratory conditions. Cultural methods provided an extremely narrow scope of the types of bacteria present due to the selective nature of the media used. Other organisms present within the water that could harbour similar resistance may have been overlooked due to being either unculturable under laboratory conditions-like the potentially unculturable VRE bacteria-or being grown in incompatible media. Detecting unculturable organisms and their associated genes would require a broader detection method, such as metagenomics, to analyse the entire bacterial community.

Resistance Profile of Isolates

When observing the susceptibility of the bacterial isolates taken from the Paremuka stream against the resistance profile of the control strain of *E. coli,* a wide range of susceptibility against various antibiotics was seen. When observing direct filtrate isolates, all isolates show the exact same resistance to all tested antibiotics with the same susceptibility to meropenem. During the dry conditions, the upstream isolates that underwent pre-enrichment showed a resistance to ampicillin, amoxicillin and cefalexin with a high susceptibility to meropenem as seen in Table 2. As the weather conditions changed, the two-coloured types of bacterial colonies produced isolates that was found still be resistant to ampicillin yet be susceptible to amoxicillin and cefalexin.

The lakeside and downstream sample sites failed to produce any colonies after pre-enrichment, but when grown directly through membrane filtration both sites produced antibiotic resistant colonies. One possible explanation for discrepancy between methods could be that the resistant bacteria that had grown on ESBL agar were not within the *Enterobacteriaceae* bacteria family that was targeted for during the MPN and pre-enrichment process. Indicator bacteria present in the lake and downstream sites that grew in LST broth did not grow on the ESBL agar can be assumed to be susceptible to beta-lactam antibiotics like control strain of *E. coli*.

The stream and estuary sites show the highest levels of indicator bacteria with isolates displaying varying levels of resistance. The stream site showed the highest levels of indicator bacteria with the pre-enriched isolate

showing the same level of resistance as the filter isolate with minimal differences. The estuary site showed the only isolate that produced growth at 42°C, indicating a faecal coliform had some degree of resistance to beta-lactam antibiotics. As seen in Table 2, estuary isolate BRd42 showed susceptibility to meropenem and amoxicillin yet showed resistance to cefalexin and ampicillin. The only antibiotic to be ineffective against all isolates was ampicillin. Resistance to ampicillin among bacterial isolates is unsurprising as resistance to ampicillin has been found in a variety of isolates. One study conducted found that along the Pearl River in China and its tributaries, with one tributary that had water from waste treatment plants discharged into the river had 100% of all isolates resistant to ampicillin. Upstream isolates in a drinking water reservoir with no association with waste had 11 percent of isolates that were resistant to ampicillin (Tao et al., 2010). Among non-pathogenic and opportunistic strains of *E. coli* and *K. pneumoniae* were found to be the prominent hosts of ampicillin resistance (Hammad et al., 2009). While all isolates show resistance to ampicillin, the estuary isolate showed a high level of susceptibility to amoxicillin and clavulanic acid. The estuary isolate showed resistance to ampicillin and cefalexin, yet was highly susceptible to amoxicillin and clavulanic acid, indicating that the mechanisms for resistance lie with the TEM and SHV family of genes (Paterson, 2006).

Isolates from the pre-enriched stream site and all filter isolates showed multiple resistances to the tested antimicrobials except for meropenem. Even with the addition of clavulanic acid with amoxicillin, there was minimal inhibition. One study analysed the resistance profiles of isolated *Enterobacteriaceae* sourced from animals and found varying degrees of resistance to beta-lactam antibiotics, in particular amoxicillin with clavulanic acid. The sources that produced isolates with the highest percentage of isolates that had resistance to amoxicillin were isolated from dairy farms, followed by poultry farms but had full susceptibility to meropenem (Amador et al., 2019). While it is expected that resistance would be found amid livestock, the Paremuka stream site has an urbanised residential area directly to the east and a vineyard directly to the west. While there is farmland near the Paremuka stream sampling site, it is immediately downstream of where the upstream sample was taken. One study analysed the susceptibility of *Enterobacteriaceae* isolated from wastewater against a variety of beta-lactam antibiotics and found that most of the beta-lactam antibiotics tested were ineffective and had varying degrees of susceptibility to meropenem (Zagui et al., 2023). There is a possibility that the organisms that exhibit such a high degree of resistance could have come from a leak in sanitation lines, yet as seen in Table 1, no bacteria from the stream site grew at 42°C that also exhibited any resistance to beta-lactam antibiotics.

One possibility for the high degree of resistance could be due to horizontal gene transfer. The genes associated with resistance to beta-lactam antibiotics such as the *SHV* family of genes are encoded on the transmissible plasmids which can be transferred between the same or similar species of *Enterobacteriaceae* (Doi et al., 2012). A study conducted on amoxicillin resistant *E. coli* and cefotaxime resistant *K. pneumoniae* found that co-incubation with two different species of bacteria with differing resistances under laboratory conditions, both species of bacteria had developed resistances to previously susceptible dosages of the antimicrobials (Vaidya, 2011).

Another possibility for such a high degree of resistance could simply be due to a rapid mutation rate among *Enterobacteriaceae.* Organisms within the family *Enterobacteriaceae* are prone to mutations in a short period of time with one strain of *K. pneumoniae* resistant to quinolone reported in 1998 was originally isolated from a susceptible strain in 1994 (Paterson, 2006). Among strains of *K. pneumoniae* that were isolated from patients 20.6 percent of patients had strains resistant to third-generation cephalosporins. When compared to previous years between 1998 – 2002, strains that were resistant had increased by 47 percent with similar increases in resistance seen in *E. coli* and *Enterobacter spp.* (Paterson, 2006).

The bacteria species *Pseudomonas spp.* exhibit the same physical traits as those found in the group *Enterobacteriaceae.* The bacterium *P. aeruginosa* is commonly found in environmental samples ranging from soils, rivers and wastewater and is naturally resistant to a multitude of antibiotics. *P. aeruginosa* has developed increased resistance to a multitude of antimicrobials like carbapenems, aminoglycosides and fluoroquinolones (Suzuki et al., 2013). Although the filter isolates were identified as Gram-negative via Gram staining, it does not differentiate between similar-looking species like the bacteria in the group *Enterobacteriaceae.* Resistance profile and susceptibility guidelines set by EUCAST indicate that *Pseudomonas spp.* is naturally resistant to specific penicillin and cephalosporins such as the antibiotics amoxicillin and cefalexin tested in this research. Due to the prevalent nature of organisms like *Pseudomonas spp.* being ubiquitous throughout the local environment, observing such a resistance profile throughout the Paremuka stream is likely to be a false positive and would require more in depth biological testing to determine the species.

Furthermore, simply observing the resistance to antibiotics does not explain how each organism is resistant. Depending on the organism, each individual species of bacteria would normally be resistant to a multitude of antimicrobials or have no effect on their susceptibility. A prime example is the use of vancomycin. Vancomycin is the antimicrobial of choice against Gram-positive infections and have little to no effect on Gram-negative bacteria due to antibiotic being unable to pass through the porins of Gram-negative bacteria and bind to the target (Nicolosi et al., 2010). This was seen on the VRE plates that had shown growth of Gram-negative organisms. Even when selectively targeting Gram-negative bacteria, isolates showed some susceptibility to multiple antibiotics, while others displayed complete resistance apart from one antibiotic.

Resistance is due to a multitude of factors to either being naturally resistant due to the other competing organisms within the environment like what is seen in the species *P. aeruginosa* or having the resistance mechanism transferred from a donator species to a receiving species like in the case for horizontal gene transfer seen in strains of *E. coli* (Hasegawa et al., 2018; Sengupta et al., 2013). One of the more effective methods to identify such resistance mechanisms is to identify which genes are present in an organism and to use molecular methods. When attempting to find a specific gene, molecular method such as PCR is a practical method but when attempting to find identify a range of genes, genome sequencing would be the appropriate method.

Chapter 4 Genome Sequencing

Introduction

The prevalence of multidrug-resistant bacteria was initially isolated to infections within hospitals but are now becoming more prevalent in the community (Van Duin & Paterson, 2016). Resistant organisms employ a wide range of mechanisms, including deactivation enzymes, antibiotic pumps and antibiotic target alteration, which target a wide range of antimicrobials to ensure survival (Nikaido, 2009). The presence of micro-organisms that are resistant to commonly used antibiotics can be observed in the previous chapter of this paper. All the isolates displayed some degree of resistance to one or more tested drugs, with all isolates being completely resistant to ampicillin. Due to limited resources, only organisms UBd35 and SBr35 were selected to undergo Whole Genome Sequencing (WGS) due to having the greatest resistance to all tested antibiotics.

Traditional and molecular methods used to identify and characterize microorganisms each present limitations that impact the overall accuracy and efficiency of microbial detection and antibiotic resistance analysis. Traditional methods used to identify organisms involve biochemical tests that rely on culturing organisms in specific mediums to observe metabolization of specific nutrients such as sulphur or urea (Shoaib et al., 2020). Organisms within the family *Enterobacteriaceae* cover a wide range of bacterial species with differing metabolisms and subgroups that individual types of biochemical tests that take time to produce a positive result that ranges from 24 to 48 hours for each individual test (Farmer 3rd et al., 1985). Traditional methods that analyse antibiotic susceptibility, like the disk assay is time-consuming and lack critical explanations of how an organism develops resistance and requiring additional data provided by molecular methods. Molecular methods used in detection and surveillance of antibiotic resistance such as PCR are prone to biases that cause certain resistances to go undetected. The genes that play a role in antibiotic resistance cover a vast range of genes and within the ESBL gene group cover 4 classes of beta-lactamase proteins that are further categorized into subgroups (Bush & Jacoby, 2010). Furthermore, primers have a tendency towards bias, as they are specific genetic sequences that can lead to false conclusions regarding the organisms found in the environment (Sipos et al., 2009).

Implementing both biochemical and selective molecular methods to identify the bacterial species and resistance mechanisms would be impractical from a cost and time perspective. To circumvent the inherent disadvantages of both methods, isolates underwent WGS to identify the bacterial and the mechanisms that potentially contribute to multiple drug resistance. WGS is a comprehensive analysis of an organism's genetic fingerprint that allow officials to observe and identify species, characteristics, and mutations in populations (Centers for Disease Control, 2024b). The current generation WGS has the distinct advantage of being able to identify the sources of outbreaks in less than a day (Reuter et al., 2013).

One of the current generation sequencing methods is Oxford Nanopore MinION sequencing. Oxford Nanopore is a third-generation sequencing technology that sequences genetic material with the use of an electrical current flowing through the nanopores (Plesivkova et al., 2019). MinION technology has emerged as a

competitive and portable method to analyse large quantities of genetic material for analysis without the need for PCR amplification (Jain et al., 2016). One disadvantage of Illumina MiSEQ is the shorter read lengths which has shorter read lengths, which may lead to lower quality assemblies due to repeat regions in the genome (Satam et al., 2023). When compared to PacBio, another next generation sequencing method without a need for PCR amplification, the costs far exceed $525,000 USD, whereas MinION costs a more affordable $1,460 USD for reagents and 12 flow cells (Hu et al., 2021). With the ability to analyse genetic material, insights into the bacterial genome and plasmids can be observed for resistance sequences.

Bacteria can be intrinsically resistant to antibiotics or acquire resistance genes through horizontal gene transfer. Bacterial resistance can be genetically coded into the bacterial chromosome or within the plasmid. The bacterial chromosome is the primary source for an organisms genetic traits and is the source of inherited traits (Krawiec & Riley, 1990). Resistance to antimicrobials bacteria was a natural result of billions of years of evolution, long before the first antibiotics was discovered in the 20th century (Jian et al., 2021). Changes to resistance genes encoded in the bacterial chromosome is the result of natural selection with known pathogenic strains of *K. pneumoniae* and *P. aeruginosa* harbouring resistance and virulence genes encoded in the bacterial genome (Darmancier et al., 2022). Organisms can obtain resistance genes in the bacterial chromosome through the horizontal gene transfer of bacterial transposons. Transposons are a group of mobile genetic elements, often referred to as 'jumping genes' due to their ability to shift position within bacterial genomes. They are defined as discrete DNA segments that are typically integrated in the bacterial chromosome (Choi & Kim, 2009; Whittle et al., 2002). Conjunctive transposons are able to transfer antibiotic resistance in a similar manner to plasmid gene transfer (Whittle et al., 2002). However, resistance genes are not limited to the bacterial chromosome and can also be found in the mobile genetic plasmids that co-exist within the bacterial cell. Bacterial plasmids are self-replicating DNA sequences found within bacterial cells that are considered evolutionary parasites, encoding genes that can be beneficial to the host cell (Rodríguez-Beltrán et al., 2021). Plasmids allow for the rapid adaptation of the bacterial host by exchanging genetic information between species. This exchange benefits the host with advantageous traits, ranging from antibiotic resistance, heavy metal resistance and increased virulence (Pinilla-Redondo et al., 2018). Another method employed by bacteria to obtain resistance genes is the use of bacteriophage. Bacteriophages are viruses that target specific bacteria and closely related organisms, using the bacteria to replicate (Kasman & Porter, 2022). Bacteriophages can transfer resistance to other organisms via transduction, incorporating the previous host's genetic traits that are carried with newly replicated virus particles to recipient cells (Balcazar, 2014).

With multiple unknown isolates resistant to multiple antibiotics, the goal of this section is as follows. The first is to successfully sequence the whole genome of a multidrug-resistant isolate to identify the organism species. Second is to cross reference the assembled genome to detect for any significant matches against known antimicrobial resistance genes to determine potential mechanisms for resistance.

Methods

DNA Extraction

DNA extraction was done using the Qiagen Blood and Tissue kit and followed manufacturers protocol (Qiagen, 2011). Isolates were grown in nutrient broth and incubated at 37°C for 16hrs. 1ml aliquot of the culture was transferred into sterile 1.5ml Lo-bind tubes and centrifuged at 5000 x RCF for 1 minute. The supernatant was then pipetted out while not disturbing the pellet at the bottom of the tube. 180µL of ATL solution was then added and the pellet resuspended in the solution. 20µL of Proteinase K was added and mixed thoroughly and incubated in a well incubator at 56°C for 30 minutes. After incubation, 4µL of RNase A was added, mixed, and left to sit at room temperature. After 2 minutes, 200µL of AL buffer and 100% ethanol was added, mixed thoroughly and transferred into a filter and collection tube. Samples were then centrifuged at 6000 x RCF for 1 minute and the effluent discarded. 500µL of AW1 solution was then added and centrifuged again at 6000 x RCF for 1 minute and the effluent discarded. 500µL of AW2 was then added and centrifuged at 20,000 x RCF for 3 minutes, effluent discarded and allowed to dry in the biosafety cabinet for a minimum of 30 minutes to allow any remaining ethanol to evaporate. Finally, 200µL of buffer AE was added and centrifuged at 6000 x RCF for 1 minute with the final solution kept for DNA purification.

DNA Purification

Extracted DNA was purified using AMPure XP bead protocol by Jackson (2016). 50 µL of extracted DNA was placed into a 1.5ml Lo-bind tube with 90 µL of room temperature AMPure XP bead solution. Solution was mixed by pipetting up and down a minimum of 10 times and allowed to sit at room temperature for 5 minutes. After 5 minutes had elapsed, the tubes were placed in a magnetic stand to allow the solution to clear. Once cleared, the solution was pipetted out and 500µL of 80% ethanol solution was added and allowed to settle for 1 minute. Ethanol was then pipetted out and repeated one more time. After the second removal of the ethanol, tubes were briefly centrifuged and returned to the magnetic stand for 1 minute with the remining trace ethanol removed with a pipette. Bead tubes were then placed into a heat block at 37°C for 3 minutes to evaporate any remaining ethanol. Once evaporated, the bead pellet was mixed with 20µL of autoclaved Millipore water and mixed via pipetting a minimum of 10 times and left to sit for 3 minutes. Once the beads had settled, the tubes were centrifuged briefly and returned to the magnetic stand and allowed to separate. The final solution was then pipetted out and placed into a new tube for gel electrophoresis to check for degradation.

Gel Electrophoresis

Gel electrophoresis is a biochemical test that uses an electrical current to pass through a gel block containing wells and allows for separation of biomolecules by size (Reddy & Raju, 2012). Gel electrophoresis was done by mixing 50ml of 1x Tris/Borate/EDTA (TBE) solution with 0.5g of agarose gel powder. The solution was warmed to allow for the agarose powder to dissolve into the salt solution and allowed to cool to lukewarm temperature before the addition of 5µL of Red Gel indicator. The gel solution was then poured into a gel chamber with a gel comb and allowed to set. Once set, the gel comb was removed, and the gel chamber was transferred into the

appropriate buffer tank and filled with the appropriate volume of 1x TBE solution. Once filled, the first well was loaded with 5µL of 1kb Ladder with the remaining wells filled with a premixed solution of 10µL DNA sample and 2µL of Loading Dye indicator. Once wells have been filled, the gel electrophoresis device was set to run for 35 minutes at 80V/400mA. Once finished, the gel slab was observed under UV light to check for any smearing of the DNA that would indicate degradation.

MinION Sequencing

Sequencing was followed using the Oxford Nanopre (2025) protocol. 5µL of purified DNA was pipetted into a sterile 1.5ml Lo-bind tube with 0.5µL of Fragmentation mix and thoroughly mixed by flicking and spinning down. DNA tubes were then incubated at 30°C for 2 minutes, then at 80°C for 2 minutes then cooled down on a chilled ice block. The rapid adapter buffer was diluted in a separate tube with 1.5µL of Rapid Adapter with 3.5µL of Adapter buffer and 1µL pipetted and mixed with the DNA sample. 5µL of DNA and adapter solution was then mixed with 15µL of sequencing buffer and 10µL of mixed library bead solution and mixed via flicking and spinning to consolidate. Separately, the priming mix for the Nanopore flongle was mixed with 117 µL of Flow Cell Flush and 3 µL of Flow Cell Tether. 200 µL of priming mix was added to the Nanopore flongle along with 75 µL of the prepared DNA library. The Nanopore sequence was then set to read for 24 hours.

Genome Assembly and Annotation

The resulting sequence was assembled using the software package Flye to reconstruct the genomic sequence. The constructed sequence was then submitted to the Comprehensive Antibiotic Resistance Database (CARD), the Rapid Annotation Subsystem Technology (RAST), the National Center for Biotechnology Information – Basic Local Alignment Search Tool (NCBI-BLAST), and Center for Genomic Epidemiology. RAST was used to compare the overall assembly of the genome. NCIB-BLAST was used to match the DNA sequence against known genomes to identify the species. The CARD database was used to compare organisms against a known resistance gene database. Finally, the Center for Genomic Epidemiology (CDE) mobile element finder was used to identify the presence of any mobile genetic elements found in the sequenced micro-organisms.

To manually curate the metabolic and antibiotic resistance pathways, the assembled DNA sequence was annotated into genes and converted into ammino acid protein sequences using Bakta. The amino acid sequence for the reconstructed organisms was then submitted to the Kyoto Encyclopedia of Genes and Genomes (KEGG) tool BlastKOALA to annotate amino acid sequences to assign KEGG Orthology (KO) numbers to reconstruct the pathway of the protein interactions.

Results

Genome Assembly and Identification

To determine the assembly quality, metrics such as N50, L50 and contig count are used to evaluate the differences between the two isolates sequenced, UBd35 and SBr35. When comparing the N50 values of the two isolates, UBd35 had an overall smaller assembly size than SBr35 but maintained the same L50 value and had fewer contigs. This suggests that although UBd35's assembly was smaller, its contigs were more uniform and continuous, indicating more consistent coverage. In contrast, SBr35 displayed a higher N50 value and the same L50 value but had more contigs, indicating more fragmentation and potentially more unresolved genomic regions.

The genomic sequence for both organisms was submitted to NCIB-BLAST to identify the unknown isolates. For isolate UBd35, the organism with the highest match was *Hafnia alvei*. For isolate SBr35, two organisms within the *Citrobacter* species returned a 100 percent identification match: *Citrobacter freundii* and *Citrobacter portucalnesis.* An overview of both isolates can be seen in Table 4 and visual representation of the genomic assembly for both isolates UBd35 and SBr35 can be seen in Figures 4 and 5, respectively.

Table 4. Overview of Organism assembly

CODE	ISOLATE SIZE	N50	L50	NUMBER OF CONTIGS	QUERY COVER	E VALUE	ACCESSION	NUMBER OF RNAS	ORGANISM
UBD35	4,847,414	629,666	2	16	94%	0.0	CP139992.1	117	*Hafnia alvei*
SBR35	5,362,111	1,020,751	2	37	100%	0.0	CP159117.1	112	*Citrobacter spp.*

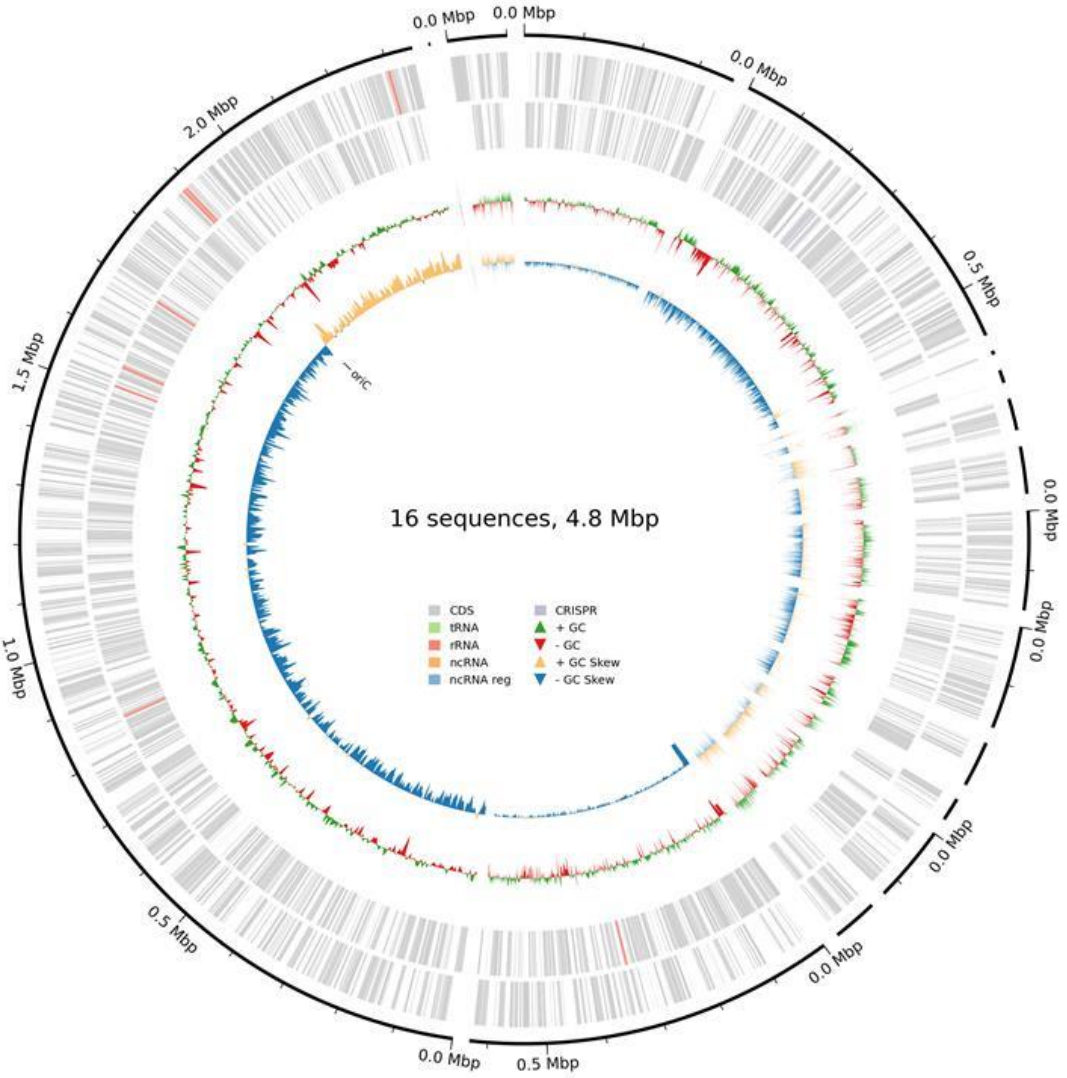

Figure 4. Genome Assembly of UBd35 – H. alvei

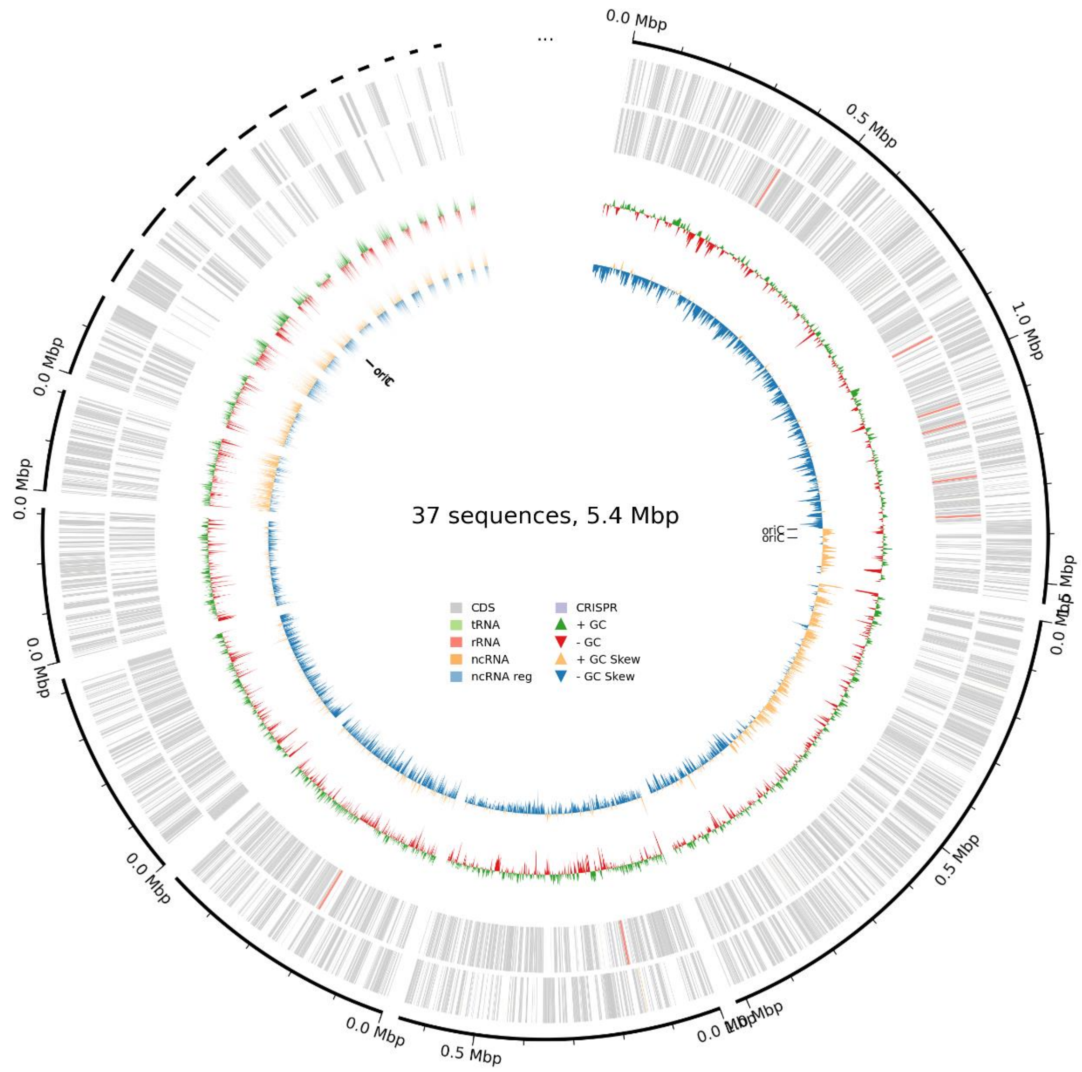

Figure 5. Genome Assembly of SBr35 – Citrobacter spp.

Gene Annotation

Annotation of UBd35 – *H. alvei*

With the DNA sequence of both organisms assembled, both genomes were uploaded to the CARD database to identify matches in the resistance database. Majority of the genes that returned strict matches in isolate UBd35 were majority efflux pump genes. However, one gene was found to be responsible for beta-lactam deactivation: the *ACC-3* gene, a sub-group of the *ampC* beta-lactamases. The presence of the *ACC-3* gene, along with multiple efflux pump genes that also target penicillin and cephalosporins may explain the observed multidrug resistance. Other genes that were found target different groups of antibiotics that were not tested and were not likely to have contributed to resistance to the types tested in this research. The following genes that were found for UBd35 can be seen in Table 5 and the antibiotic protein interactions in Figure 6.

Table 5. Strict CARD matches for UBd35 – H. alvei

CARD Gene	*Gene Family*	*Targeted antimicrobial*	*Resistance Mechanism*
KpnF	Small multidrug resistance (SMR)	Macrolide, Aminoglycoside, Cephalosporin, tetracycline, peptide antibiotics, rifamycin, disinfecting agents and antiseptics	Antibiotic efflux
KpnH	Major facilitator superfamily (MFS)	Fluoroquinolone, macrolide, aminoglycoside, carbapenem, penicillin beta-lactam and peptide antibiotics	Antibiotic efflux
CRP	Resistance-nodulation cell division (RND)	Fluoroquinolone, macrolide and penicillin beta-lactam	Antibiotic efflux
rsmA	Resistance-nodulation cell division (RND)	Fluoroquinolone, diaminopyrimidine and phenicol	Antibiotic efflux
adeF	Resistance-nodulation cell division (RND)	Fluroquinolone and tetracycline	Antibiotic efflux
ACC-3	ACC beta-lactamase	Monobactam, cephalosporin and penicillin beta-lactam	Antibiotic deactivation
ArnT	Phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration

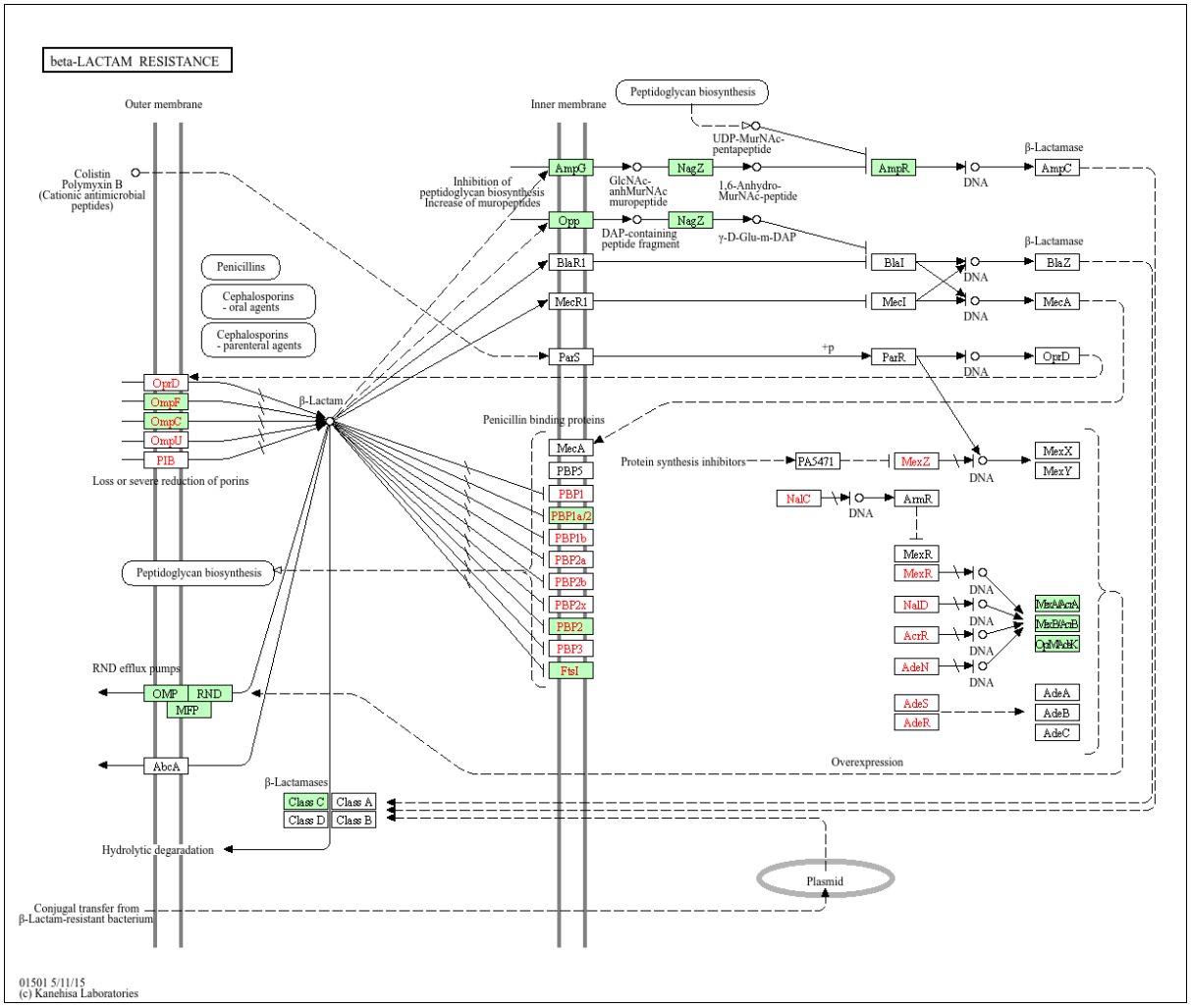

Figure 6. Beta-Lactamase Pathway for isolate UBd35 - H. alvei

Annotation of SBr35 – *Citrobacter spp.*

When referencing the genetic sequence of isolate SBr35, the majority of strict gene matches was greater when compared to Ubd35. Similar to the previous isolate, most of the resistance-associated genes were primarily efflux pump genes. While some genes affected the target site of antibiotics, only one gene was found to be responsible for antibiotic deactivation. The gene found to deactivate antibiotics was the *CMY-65* gene, a class C beta-lactamase that deactivates cephalosporins. The genes found in SBr35 can be seen in Table 6 and interactions in Figure 7.

Table 6. Strict CARD matches for SBr35 – Citrobacter spp.

CARD Gene	*Gene Family*	*Targeted Antimicrobials*	*Resistance Mechanism*
CMY-65	CMY Beta-Lactamase	Cephalosporins	Antibiotic Deactivation
KpnF	Small Multidrug Resistance (SMR)	Macrolide, Aminoglycoside, Cephalosporin, tetracycline, peptide antibiotics, rifamycin, disinfecting agents and antiseptics	Antibiotic Efflux Pump
KpnE	Small Multidrug Resistance (SMR)	Macrolide, Aminoglycoside, Cephalosporin, tetracycline, peptide antibiotics, rifamycin, disinfecting agents and antiseptics	Antibiotic Efflux Pump
marA	Resistance Nodulation Cell Division (RND),Porin with reduced permeability to beta-lactams	Fluoroquinolone antibiotic, monobactam, carbapenem, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic Efflux pump Reduced permeability
CRP	Resistance Nodulation Cell Division (RND)	Macrolide antibiotic, fluoroquinolone antibiotic, penicillin beta-lactam	Antibiotic Efflux Pump
mdtB	Resistance Nodulation Cell Division (RND)	Aminocoumarin antibiotic	Antibiotic Efflux pump
rsmA	Resistance Nodulation Cell Division (RND)	Fluoroquinolone antibiotic, diaminopyrimidine	Antibiotic Efflux pump

		antibiotic, phenicol antibiotic	
baeR	Resistance Nodulation Cell Division (RND)	Aminoglycoside antibiotic, aminocoumarin antibiotic	Antibiotic Efflux pump
Escherichia coli AcrAB-TolC with MarR mutations	Resistance Nodulation Cell Division (RND)	Fluoroquinolone antibiotic, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic Efflux pump
PmrF	pmr phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
ArnT	pmr phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
H-NS	Major facilitator superfamily (MFS) antibiotic efflux pump; resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, cephalosporin, penicillin beta-lactam, tetracycline antibiotic	Antibiotic Efflux pump
E. coli mdfA	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic Efflux pump
mdtG	Major facilitator superfamily (MFS) antibiotic efflux pump	Phosphonic acid antibiotic	Antibiotic Efflux pump
IeuO	Major facilitator superfamily (MFS) antibiotic efflux pump	Nucleoside antibiotic, disinfecting agents and antiseptics	Antibiotic Efflux pump

emrR	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic Efflux pump
emrB	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic Efflux pump
kdpE	kdpDE	Aminoglycoside	Antibiotic Efflux pump
vanG	Glycopeptide resistance gene cluster, Van ligase	Glycopeptide	Antibiotic target alteration
msbA	ATP binding cassette	Nitroimidazole antibiotic	Antibiotic Efflux

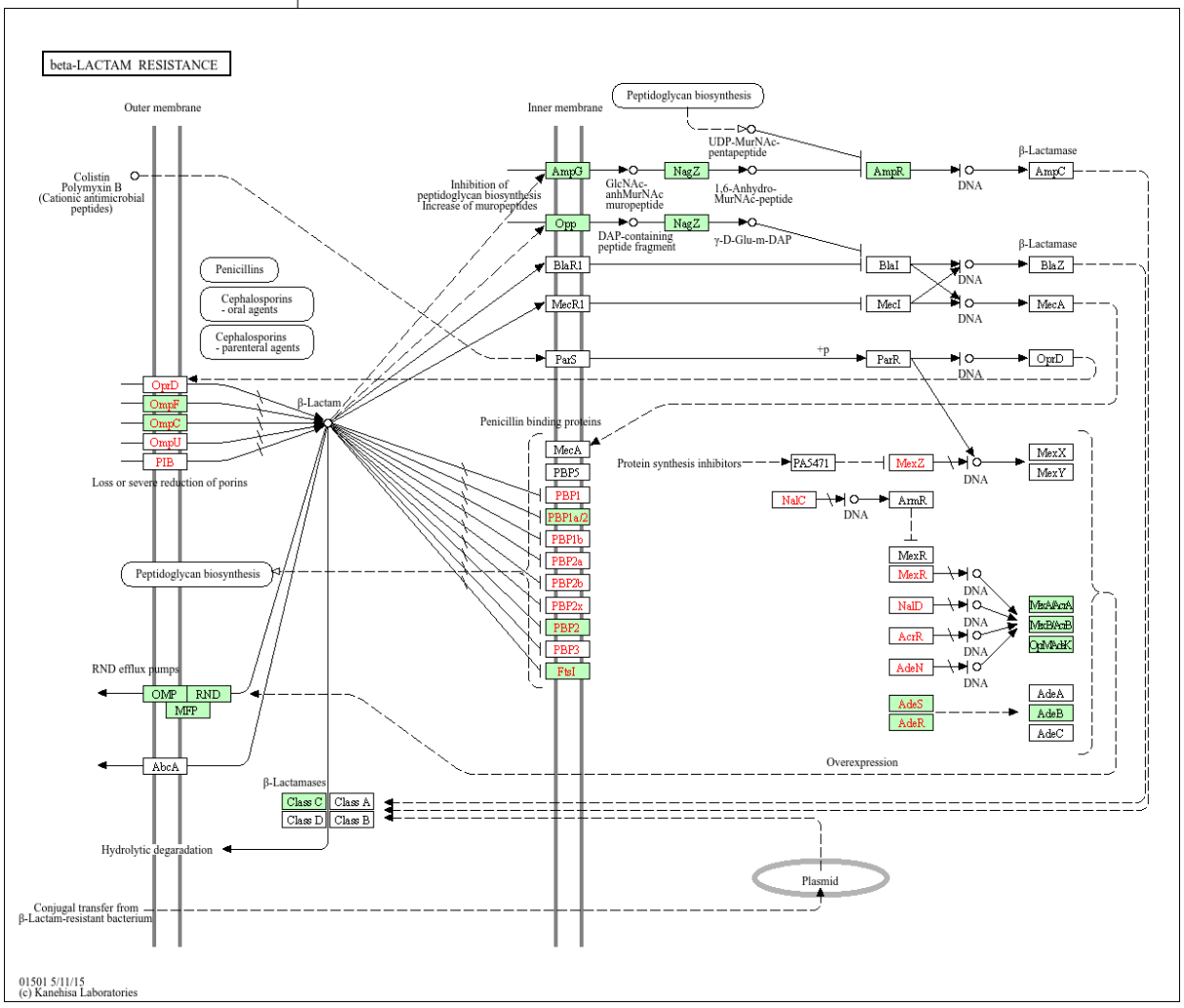

Figure 7. Beta-Lactamase pathway for Isolate SBr35- Citrobacter spp.

NCIB-BLAST identified the isolated organisms as *H. alvei a*nd potentially 2 species of C*itrobacter.* Reference strains of *H. alvei, C. portucalnesis and C. freudii* from the National Center for Biotechnology Information Library (NCIB) were linked via the Kyoto Encyclopaedia of Genes and Genomes (KEGG) to compare resistance genes and resistance mechanism interactions.

Comparison against Reference *H. alvei* strain

The resistance genes found in the reference strain of *H. alvei* showed the exact same resistance genes found in the isolated environment sample with three additional genes relating to antibiotic target alteration. While the CARD databases show the exact same genes and when mapped to the resistance pathway, the reference strain for *H. alvei* shows the production of *ampC* beta-lactamase which encodes the ACC-3 protein. But when compared to the environmental isolate of *H. alvei,* the *ampC* beta-lactamase protein is not present yet is still in a type of class C beta-lactamase. These can be seen in Table 7 and Figure 8 respectively.

Table 7. Strict CARD Genes for Reference Strain of H. alvei

CARD Gene	*Gene Family*	*Targeted Antimicrobials*	*Resistance Mechanism*
rsmA	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic	Antibiotic efflux
Klebsiella pneumoniae KpnH	Major facilitator superfamily (MFS) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, aminoglycoside antibiotic, carbapenem, cephalosporin, penicillin beta-lactam, peptide antibiotic	Antibiotic efflux
qacG	Small multidrug resistance (SMR) antibiotic efflux pump	Disinfecting agents and antiseptics	Antibiotic efflux
Klebsiella pneumoniae KpnF	Small multidrug resistance (SMR) antibiotic efflux pump	Macrolide, Aminoglycoside, Cephalosporin, tetracycline, peptide antibiotics, rifamycin, disinfecting agents and antiseptics	Antibiotic efflux
ArnT	pmr phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration

adeF	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, tetracycline antibiotic	Antibiotic efflux
CRP	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, penicillin beta-lactam	Antibiotic efflux
ACC-3	ACC beta-lactamase	Monobactam, cephalosporin, penicillin beta-lactam	Antibiotic inactivation
Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin	Elfamycin resistant EF-Tu	Elfamycin antibiotic	Antibiotic target alteration
Haemophilus influenzae PBP3 conferring resistance to beta-lactam antibiotics	Penicillin-binding protein mutations conferring resistance to beta-lactam antibiotics	Cephalosporin, penicillin beta-lactam	Antibiotic target alteration

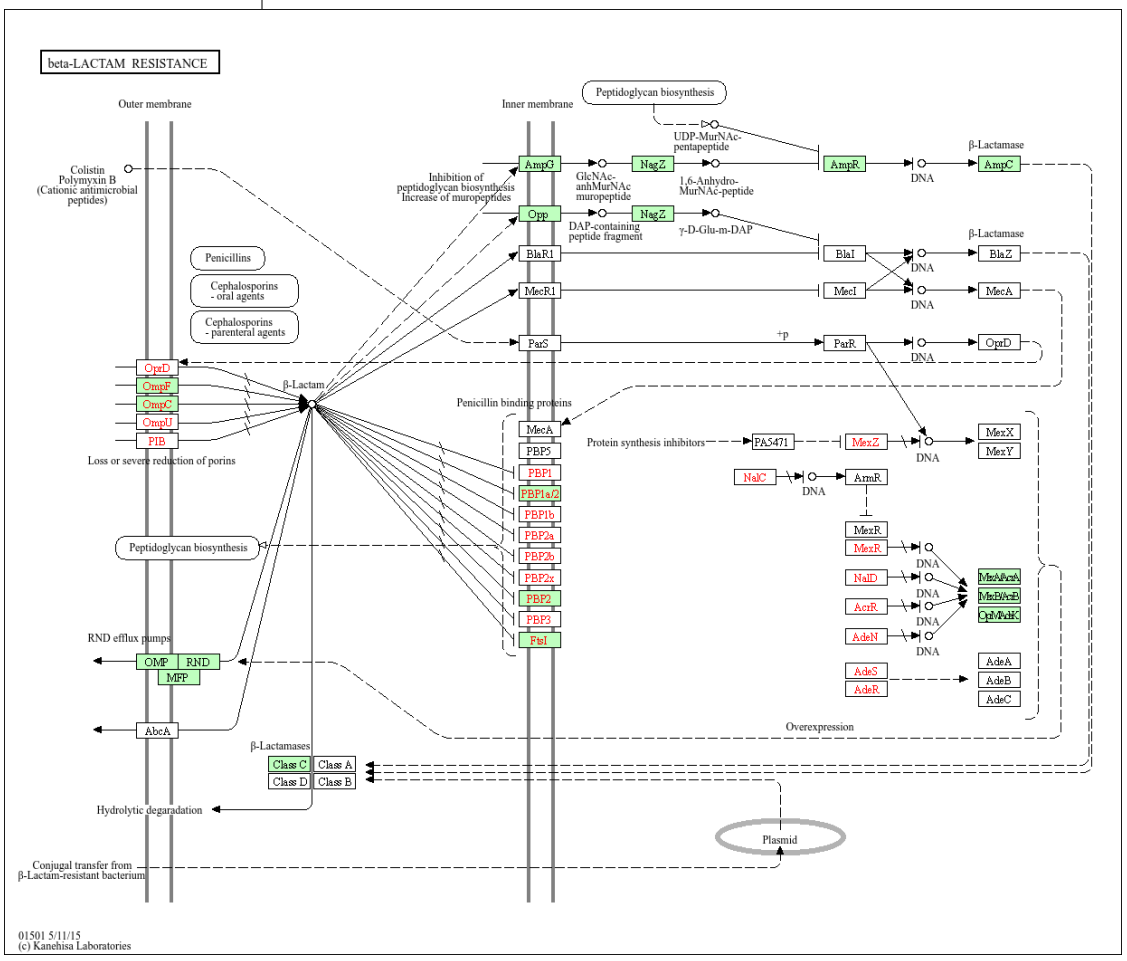

Figure 8. Kyoto Encyclopaedia of Genes and Genomes (KEGG) reference for H. alvei Beta-Lactamase Pathway

Comparison against reference strains of *Citrobacter spp.*

When observing the resistance genes found in the reference strain of *C. portucalnesis,* many of the efflux pump genes were present in both the reference strain and the environmental isolate. The key differentiating feature is the beta-lactamase gene found in the reference strain. The reference strain contains the beta-lactamase gene *CMY-86,* which differs from the *CMY-65* gene found in the environmental isolate. Additionally, the pathway for the *Citrobacter* isolate indicates there is presence of the Adeb protein and the precursor signalling proteins. Yet no genes for the specified proteins are found in either of the CARD gene databases or the KEGG pathway for the reference strain of *C. portucalnesis* and can be seen in Tables 6, 8 and Figure 9 respectively.

Table 8. Strict CARD Genes for Reference strain C. portucalnesis FDAARGOS_617

CARD Gene	*Gene Family*	*Targeted Antimicrobials*	*Resistance Mechanism*
CMY-86	CMY Beta-Lactamase	Cephalosporins	Antibiotic Deactivation
QnrB30	Quinolone resistance protein (qnr)	Fluoroquinolone antibiotic	Antibiotic target protection
marA	Resistance-nodulation-cell division (RND) antibiotic efflux pump, reduced permeability to beta-lactams	Fluoroquinolone antibiotic, monobactam, carbapenem, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic efflux, reduced permeability to antibiotic
Klebsiella pneumoniae KpnF	Small multidrug resistance (SMR) antibiotic efflux pump	Macrolide antibiotic, aminoglycoside antibiotic, cephalosporin, tetracycline antibiotic, peptide antibiotic, rifamycin antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
Klebsiella pneumoniae KpnE	Small multidrug resistance (SMR) antibiotic efflux pump	Macrolide antibiotic, aminoglycoside antibiotic, cephalosporin, tetracycline antibiotic,	Antibiotic efflux

		peptide antibiotic, rifamycin antibiotic, disinfecting agents and antiseptics	
mdtG	Major facilitator superfamily (MFS) antibiotic efflux pump	Phosphonic acid antibiotic	Antibiotic efflux
msbA	ATP-binding cassette antibiotic efflux pump	Nitroimidazole antibiotic	Antibiotic efflux
Escherichia coli mdfA	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
Escherichia coli acrA	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
vanG	Glycopeptide resistance gene cluster, Van ligase	Glycopeptide antibiotic	Antibiotic target alteration
leuO	Major facilitator superfamily (MFS) antibiotic efflux pump	Nucleoside antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
CRP	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, penicillin beta-lactam	Antibiotic efflux
rsmA	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic	Antibiotic efflux

emrB	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic efflux
emrR	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic efflux
ArnT	pmr phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
PmrF	pmr phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
mdtC	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminocoumarin antibiotic	Antibiotic efflux
H-NS	Major facilitator superfamily (MFS) antibiotic efflux pump; resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, cephalosporin, penicillin beta-lactam, tetracycline antibiotic	Antibiotic efflux
Escherichia coli EF-Tu	Antibiotic-resistant EF-Tu	Elfamycin antibiotic	Antibiotic target alteration
Escherichia coli UhpT	Antibiotic-resistant UhpT	Phosphonic acid antibiotic	Antibiotic target alteration
Escherichia coli AcrAB-TolC with MarR	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic target alteration, antibiotic efflux
Escherichia coli soxS	ATP-binding cassette, major facilitator superfamily, resistance-nodulation-cell division,	Fluoroquinolone antibiotic, monobactam, carbapenem, cephalosporin,	Antibiotic target alteration, antibiotic efflux, reduced

	Bacterial Porin with reduced permeability	glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	permeability to antibiotic
baeR	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminoglycoside antibiotic, aminocoumarin antibiotic	Antibiotic efflux

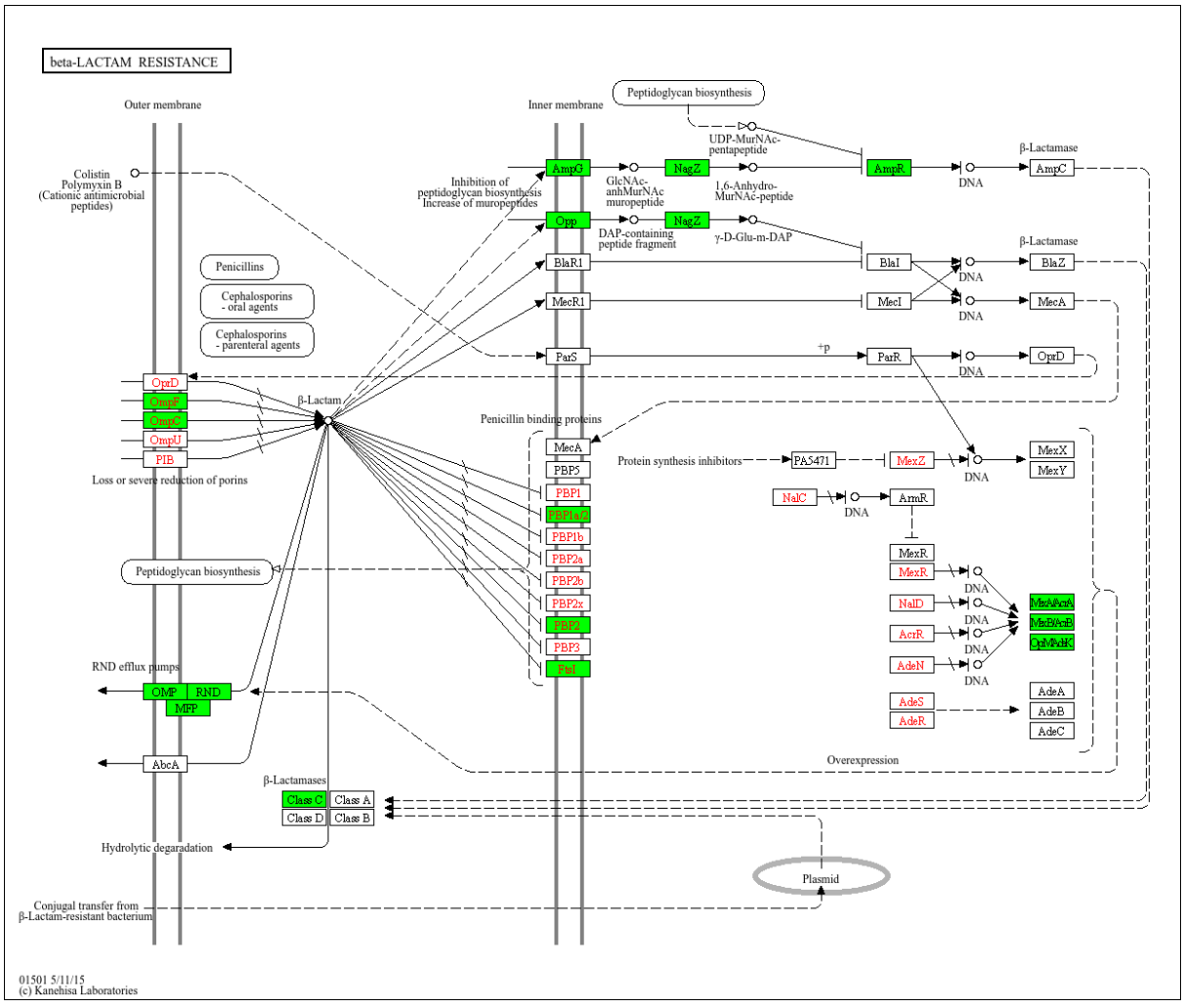

Figure 9. Kyoto Encyclopaedia of Genes and Genomes reference strain C. portucalnesis FDAARGOS_617 Beta-Lactamase Pathway

In contrast to the genes found in the reference strain of *C. portucalensis,* the antibiotic resistance genes found in the reference strain of *C. freundii* includes multiple genes in relation to beta-lactam deactivation. Several beta-lactamase genes from different beta-lactamase group classes, primarily from class A and C, are present.

Notably, the beta-lactamase genes *FOX-5, SHV-5 and CMY-159* were identified, along with the multiple target alteration, efflux pump, and other antibiotic deactivation genes. These genes and resistance protein interactions can be seen in Table 9 and Figure 10.

Table 9. Strict CARD Genes for reference strain C. freundii CFNI1H1

CARD Gene	*Gene Family*	*Targeted Antimicrobials*	*Resistance Mechanism*
FOX-5	FOX beta-lactamase	Cephalosporin	Antibiotic inactivation
KPC-2	KPC beta-lactamase	Monobactam, carbapenem, cephalosporin, penicillin beta-lactam	Antibiotic inactivation
SHV-5	SHV beta-lactamase	Cephalosporin, penicillin beta-lactam	Antibiotic inactivation
CMY-159	CMY beta-lactamase	Cephalosporin	Antibiotic inactivation
catA1	Chloramphenicol acetyltransferase (CAT)	Phenicol antibiotic	Antibiotic inactivation
catB3	Chloramphenicol acetyltransferase (CAT)	Phenicol antibiotic	Antibiotic inactivation
AAC(6')-Ib10	AAC(6',)	Aminoglycoside antibiotic	Antibiotic inactivation
AAC(3)-Ia	AAC(3)	Aminoglycoside antibiotic	Antibiotic inactivation
sul1	Sulphonamide resistant sul	Sulphonamide antibiotic	Antibiotic target replacement
mphE	Macrolide phosphotransferase (MPH)	Macrolide antibiotic	Antibiotic inactivation
emrR	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic efflux
emrB	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic efflux
rsmA	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic	Antibiotic efflux

CRP	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, penicillin beta-lactam	Antibiotic efflux
leuO	Major facilitator superfamily (MFS) antibiotic efflux pump	Nucleoside antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
vanG	Glycopeptide resistance gene cluster, Van ligase	Glycopeptide antibiotic	Antibiotic target alteration
acrB	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
Escherichia coli acrA	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
kdpE	kdpDE	Aminoglycoside antibiotic	Antibiotic efflux
msbA	ATP-binding cassette (ABC) antibiotic efflux pump	Nitroimidazole antibiotic	Antibiotic efflux
mdtG	Major facilitator superfamily (MFS) antibiotic efflux pump	Phosphonic acid antibiotic	Antibiotic efflux
Klebsiella pneumoniae KpnE	Small multidrug resistance (SMR) antibiotic efflux pump	Macrolide antibiotic, aminoglycoside antibiotic, cephalosporin, tetracycline antibiotic, peptide antibiotic, rifamycin antibiotic, disinfecting agents and antiseptics	Antibiotic efflux

Klebsiella pneumoniae KpnF	Small multidrug resistance (SMR) antibiotic efflux pump	Macrolide antibiotic, aminoglycoside antibiotic, cephalosporin, tetracycline antibiotic, peptide antibiotic, rifamycin antibiotic, disinfecting agents and antiseptics	Antibiotic efflux
marA	Resistance-nodulation-cell division (RND) antibiotic efflux pump, reduced permeability to beta-lactams	Fluoroquinolone antibiotic, monobactam, carbapenem, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic efflux, reduced permeability
H-NS	Major facilitator superfamily (MFS) antibiotic, resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, cephalosporin, penicillin beta-lactam, tetracycline antibiotic	Antibiotic efflux
mdtB	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminocoumarin antibiotic	Antibiotic efflux
PmrF	pmr phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
ArnT	pmr phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration
Escherichia coli parC	Fluoroquinolone resistant parC	Fluoroquinolone antibiotic	Antibiotic target alteration
Escherichia coli EF-Tu	Elfamycin resistant EF-Tu	Elfamycin antibiotic	Antibiotic target alteration
Escherichia coli UhpT	Antibiotic-resistant UhpT	Phosphonic acid antibiotic	Antibiotic target alteration

Haemophilus influenzae PBP3	Penicillin-binding protein mutations to beta-lactam antibiotics	Cephalosporin, penicillin beta-lactam	Antibiotic target alteration
Escherichia coli gyrA	Fluoroquinolone resistant gyrA	Fluoroquinolone antibiotic	Fluoroquinolone antibiotic
Escherichia coli soxS	ATP-binding cassette, major facilitator superfamily, resistance-nodulation-cell division, Bacterial Porin with reduced permeability	Fluoroquinolone antibiotic, monobactam, carbapenem, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic target alteration, antibiotic efflux, reduced permeability to antibiotic
Escherichia coli AcrAB-TolC with MarR mutations	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, cephalosporin, glycylcycline, penicillin beta-lactam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics	Antibiotic target alteration, antibiotic efflux
baeR	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminoglycoside antibiotic, aminocoumarin antibiotic	Antibiotic efflux

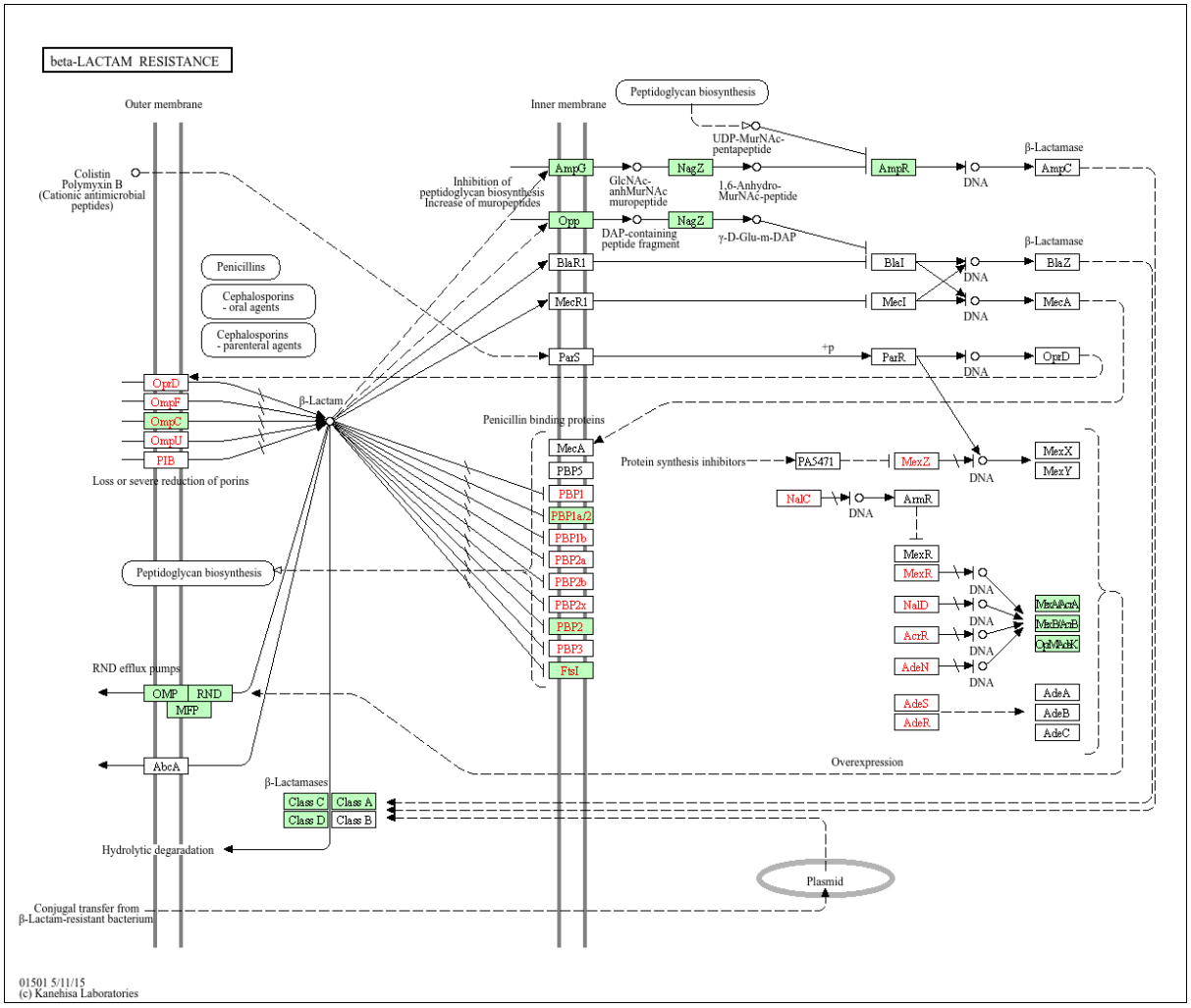

Figure 8. Kyoto Encyclopedia of Genes and Genomes (KEGG) reference strain C. freundii CFNIH1 Beta-Lactamase Pathway

When examining the beta-lactamase pathway of isolate SBr35 and resistance genes present, it shows a similar pattern to both *C. freundii* and *C. portucalensis,* with multiple overlapping resistance genes. *C. freundii* can produce class A, C and D beta-lactamases when exposed to antimicrobials, whereas *C. portucalensis* produces beta-lactamases the only class made is the class C beta-lactamases. Based on the pathway and gene profile of SBr35, the closest match from the KEGG and CARD databases is C*. portucalensis,* suggesting that SBr35 is potentially a strain of *C. portucalenesis.*

Gene Mobility

Both reconstructed sequences were submitted to CDE mobile element finder to identify any potential mobility within the genetic sequences. For *H. alvei,* the results showed that the *ACC-3* beta-lactamase gene matched the same gene in the CDE database with 95 percent identity. Of the 56 potential mobile elements for *H. alvei,* none were confirmed as mobile by the CDE database, suggesting that antimicrobial resistance in this strain is unlikely to be the result of horizontal gene transfer. The mobility results for *C. portucalnesis* showed a similar trend. Although the strain was associated with 128 potential mobile gene elements, only 47 were confirmed to be mobile.

Table 10. Mobile Elements of SBr35 in relation to CMY-72

Mobile Gene Element	*Family*	*Type*	*Sequence Identity*	*Contig Location*	*Contig Position*	*Accession*
CMY-72	CMY beta-lactamase	Antibiotic Deactivation	99.56%	30	901,303 – 902,448	JX440352
ISEcl1	IS2	Insertion Sequence	97.9%	30	206,615 - 207,950	AF342826
IS30	IS30	Insertion Sequence	99.84%	30	325,490 – 326,710	X00792
ISSen4	IS3	Insertion Sequence	99.75%	30	1,113,134-1,114,354	EU219534
IS903	IS5	Insertion Sequence	96.69%	11	30,731 – 31,787	J01839
IS102	IS5	Insertion Sequence	92.24%	11	45,981 – 47,037	J01728
Cn 31787 I903	IS5	Composite transposon	96.69%	11	30,730 – 47,037	J01839
IS5	IS5	Insertion Sequence	98.09%	6	7,881 – 9,084	J01735
ISCfr26	IS110	Insertion Sequence	99.78%	13	4,485 – 5,849	MG3871191
Cn 3873 IS26	IS6	Composite Transposon	100%	14	964 – 4,837	X00011
Cn 6857 ISEcl1	IS3	Composite Transposon	97.9%	37	647,530 – 654,487	AF342826

Similar to the *H. alvei* isolate, the gene that matched the CDE database was the *CMY-72* gene with a 99.5 percent identity match and differing from the CARD. However, of the mobile genetic elements returned, matches in the CDE database indicated that the matched *CMY-72* gene was not found to be near a mobile genetic element. The only mobile genetic material found in the strain of *C. portucalnesis* were insertion sequences and mobile transposons. The *CMY-72* beta-lactamase gene was detected in contig 30, while genes relating to composite transposon was a part of contigs 11, 14 and 37. Nearby genes surrounding the *CMY* beta-lactamase gene can on contig 30 be seen in Figure 9.

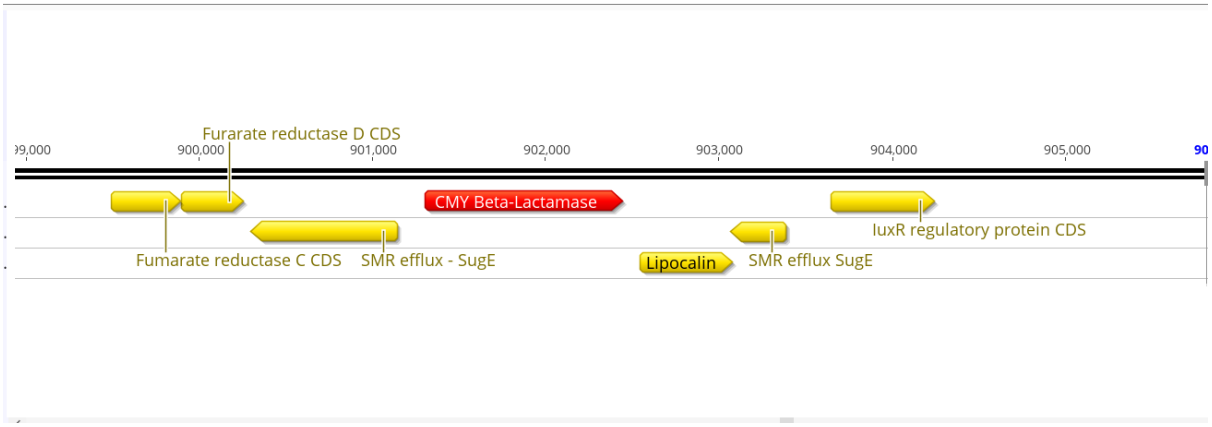

Figure 9. Location of CMY Beta-Lactamase Gene on Contig 30 in Isolate SBr35 – C. portucalnesis.

Discussion

Hafnia alvei

H. alvei is a rod-shaped, opportunistic Gram-negative pathogen in the family *Enterobacteriaceae,* commonly found in soils, food and the intestinal tract of animals (Yin et al., 2019). As a part of the natural flora of various animals, including fish, birds, and mammals - *H. alvei* can be found in a wide range of environments including sewage, soil and freshwater (Stock et al., 2005). *H. alvei* has been detected in multiple aquatic environments, such as rainwater, well water or tap water (Okuwaki et al., 1982). The presence of the *H. alvei* strain in this study was unsurprising, due to the sampling location being directly downstream from local farmland. The strain may have originated from livestock waste and was washed into the stream or sourced from nearby aquatic birds that frequent the area.

H. alvei is considered an opportunistic pathogen and shares at least one virulence gene with pathogenic strains of *E. coli.* Infections caused by *H. alvei* typically occur in patients who have undergone separate treatment and have supressed immune systems, leading to conditions such as septicaemia, pneumonia and surgical wound infections (Ramos & Dámaso, 2000). More commonly, *H. alvei* is associated with gastroenteritis in humans, with outbreaks in countries like Japan, Russia, and Canada (Janda & Abbott, 2006).

The genome assembly of UBd35, H. *alvei* showed an N50 size of 629,666 with an L50 value of 2. The N50 value indicated that the smallest contig covering 50 percent of the genome is 629,666 base pairs long. The L50 value represents the number of contigs required to cover 50 percent of the assembled genome, which in this case was 2 contigs. With a total of 16 contigs, the overall quality of the DNA used in assembly was relatively intact.

Previous works conducted to test the susceptibility of *H. alvei* against a multitude of antibiotics found that aminoglycosides, carbapenems, and combined treatment penicillin (Ticarcillin-clavulanic acid) were found to be effective against all tested isolates (Abbott et al., 2011). The same study also tested the same isolates against cephalosporins, single-agent penicillin treatments, and tetracycline, and found that varying degrees of susceptibility. Fewer than 10 percent of isolates resistant antimicrobials such as cefoxitin, while over half of isolates being resistant to tetracycline. A similar study evaluated the susceptibility of eight *H. alvei* strains to several antibiotic groups, including aminoglycosides, penicillin, and cephalosporins. The authors reported that against aminoglycosides such as gentamycin and tobramycin successfully inhibited all strains of *H. alvei.* Penicillin showed limited effectiveness, with only 2 to 3 strains being susceptible to ampicillin and amoxicillin. In contrast, cephalosporins displayed variable efficacy; some strains were inhibited by cefotaxime and only 2 strains susceptible to cefazolin (Ramos & Dámaso, 2000).

The works of Abbot et al. (2011) and Ramos & Dámaso (2000) reflect similar findings in this study. Resistance to penicillin and cephalosporins against the strain of *H. alvei* is most likely due to the *ACC-3* gene, which was the only matched gene in the CARD database responsible for antibiotic deactivation. The *ACC-3* gene is an antibiotic resistance gene found naturally in *H. alvei* and is a part of the *ampC* family of extended spectrum beta-lactamase antibiotic resistance genes (Zhou et al., 2022). In addition to the *ACC-3* gene, *H. alvei* is the

primary reservoir for the *ACC* group of beta-lactamase genes and has been known to harbour variants such as the *ACC-1* and *ACC-4* beta-lactamase genes (Zhou et al., 2022). As such, the presence of the *ACC-3* gene found in the environmental *H. alvei* isolate is a common occurrence.

The *ACC-3* gene is a beta-lactamase encoding gene in the *ampC* family, responsible for the deactivation of penicillin and effective in targeting cephalosporins (Jacoby, 2009). This is reflected in the observed resistance to ampicillin and cefalexin, which are penicillin and cephalosporin antibiotics, respectively. The isolate also showed resistance against amoxicillin combined with clavulanic acid, a beta-lactamase inhibitor. This result is expected, as enzymes produced by *ampC* beta-lactamases are not inhibited by clavulanic acid, which is primarily effective against theTEM-1 group of extended spectrum beta-lactamases (Peter-Getzlaff et al., 2011).

A notable point of contention arose between the two databases used to identify resistance mechanisms in both isolates.

While the primary role for using the CARD and KEGG databases was to identify matching genes and pathways within their respective libraries, discrepancy between the datasets were apparent. The main discrepancy involved the KEGG database, which indicated that the environmental isolate of *H. alvei* lacks the AmpC protein responsible for producing class C beta-lactamases, despite being present in the reference strain of *H. alvei.* In contrast, the CARD database identified the *ACC-3* gene, which belongs to the *ampC* family of beta-lactamases. While this inconsistency could be a case of mistaken identity, as a related bacteria *Hafnia paralvei* lacks a variant of the *ampC* gene, it would contradict the NCIB-BLAST results, which showed over 94 percent coverage and an associated E value of 0.0, indicating that the alignment is not due to chance.

The presence of the *ampC ACC-3* beta-lactamase gene warrants further investigation, as the resistance to penicillin and cephalosporins used in disk diffusion testing could be a result of active production of the beta-lactamase enzyme, multiple efflux pump genes being expressed, or a combination of both mechanisms. The discrepancy may also stem from the specific type of *ACC* gene typically found in *H. alvei s*trains. While *H. alvei* is known to produce AmpC enzymes, they are typically of the A*CC-1 v*ariety (Ruppé et al., 2006). The difference between the CARD and KEGG databases could simply be due to the *ACC-3 g*ene being absent from the KEGG database, which may only include *H. alvei s*trains carrying the *ACC-1* gene, and not variants with mutations.

In addition to the *ampC ACC-3* gene found in *H. alvei*, several other genes - *kpnF, kpnH*, CRP, *rsmA* and *adeF* were identified, all of which encodes for efflux pumps that remove antibiotics form the bacterial cell. The *kpnF* gene is a gene responsible for the efflux pump for removal of antimicrobials from the bacterial cell (Srinivasan & Rajamohan, 2013). Efflux pumps play a critical role in microbe survival by actively transporting bactericidal compounds out of the cell. The *kpnF* gene is found in the bacteria *K. pneumoniae* and belongs to the *kpnEF* gene subgroup, which is associated with small multidrug resistance (SMR) systems (Maurya et al., 2019; Srinivasan & Rajamohan, 2013; Webber & Piddock, 2003).

Another efflux pump gene identified in the isolate was *kpnH*, which is a part of the major facilitator superfamily (MFS) - a key physiological trait that allows for organisms such as UBd35 and *K. pneumoniae* to survive

exposure to a multitude of antibiotics, including ciprofloxacin and piperacillin, as well as disinfectants like benzalkonium chloride and triclosan (Srinivasan et al., 2014).

The remaining efflux genes – *CRP*, *rsmA* and *adeF* – belong to the Resistance-nodulation-cell division (RND) family, that much like the previous *KpnF* and *KpnH* genes, function to remove toxic compounds from the bacterial cell (Fernando & Kumar, 2013). RND type efflux pumps are protein complexes embedded in bacterial membrane. In Gram-negative bacteria, these pumps consist of three components: the inner membrane protein (IMP), the outer membrane protein (OMP) and the periplasmic membrane fusion protein that connects the IMP and OMP (Venter et al., 2015). RND efflux systems are considered a major contributor to antimicrobial resistance in Gram-negative bacteria, with similar genes responsible for antimicrobial resistance in pathogenic strains of *E. coli* and *Campylobacter jejuni* (Routh et al., 2011).

The overall resistance genes found in the strain of *H. alvei* was inferred to be intrinsic to the bacteria, and was unrelated to horizontally acquired through mobile elements. Although the isolated strain of H. alvei did not contain any mobile genetic elements, *H. alvei* is capable of obtaining a variety of genetic traits through horizontal gene transfer. One paper had found that strains of *H. alvei* and *H. paralvei* contained multiple genes within the plasmids that contributed to metabolic pathways, virulence and antibiotic resistance, with three strains of *H. alvei* acquiring Type 3 secretion system (T3SS) genes from *Salmonella enterica* (Yin et al., 2019). While the strain of *H. alvei* isolated from the stream did not contain mobile genetic elements, the organism itself does have the ability to exchange genes via horizontal gene transfer and potentially acquire more resistances than what was currently found.

Citrobacter portucalnesis

Organisms in the genus *Citrobacter* are generally found in the soil, water, food and the intestinal tract of humans and animals (Samonis et al., 2009). Due to the widespread nature of the *Citrobacter* species, pathogenic strains have been found in surface water and the associated waterways (Kuczynski, 2016). Organisms from the *Citrobacter spp.* have been known to cause infection in patients ranging from pneumonia, urinary tract infections and meningitis (Hart, 2006). While the strains of *C. freundii* and *Citrobacter koseri* are known to be opportunistic pathogens primarily known to affect patients, other species within the *Citrobacter* group have been found to also be just as opportunistic (Jabeen et al., 2023). While the rate of infections caused by species of *Citrobacter spp.* are lower than other bacteria from the *Enterobacteriaceae* family, infection rates from patients have been increasing with the species having multiple resistances, producing AmpC beta-lactamase and carbapenemase that can be easily spread to other organisms within the *Enterobacteriaceae* group (Fonton et al., 2024).

The overall assembly for SBr35 presented similar N50 and L50 results similar to UBd35. The sequence for SBr35 resulted in a N50 value of 1,020,751 base pairs, providing 50 percent coverage, and an L50 value of 2, indicating that only 2 contigs were required to achieve 50 percent coverage. However, where similarities deviate from UBd35 was in the number of contigs present. UBd35 consisted of 16 smaller uniform contigs, while SBr35 consisted of 37 total contigs. While the large N50 value and identical L50 value indicates that there

are larger contigs capable of providing 50 percent coverage, the remaining contigs are smaller and are likely more fragmented. This fragmentation is most likely due to the timing of the sequencing after the purification process. The first sequence, UBd35 was sequenced within 48 hours after the purification and was held in chilled storage prior. The second sequence was delayed due to unavailability of personnel, resulting in the DNA to be left in storage for an extended period. This delay likely resulted in the fragmentation, degradation, and missing sections between contigs.

Multiple *Citrobacter spp.* have been known to have resistance against penicillin, cephalosporins and fluroquinolones (Metri et al., 2013). One study isolated a strain of *C. portucalensis* from a patient contained resistance genes against four antibiotic groups ranging from beta-lactams, aminoglycosides, quinolones and sulphonamides (Luo et al., 2022). A separate study had found similar results in a strain of *C. portucalenesis* isolated from two separate community trading hubs and found that 80 and 55 percent were resistant to second and third generation cephalosporins respectively (Okafor et al., 2024).

These results match what was found in the susceptibility testing of the *C. portucalnesis* isolate with additional data regarding antibiotics that were not tested in this study. Although other species of *Citrobacter* from other studies are likely to have similar and differing classes of beta-lactamase similar to the reference strain of C*. freundii,* the presence of one beta-lactamase gene could contribute significant resistance. The degree of resistance of the isolated strain of *C. portucalnesis* against tested antibiotics, like the previous strain of *H. alvei,* could be due to the presence of the beta-lactamase gene *CMY-65.* This could possibly be a reason for the resistance exhibited to the cefalexin disk as the CMY beta-lactamase enzyme would have deactivated the antibiotic. Penicillin's ineffectiveness on the isolate would likely be due to the plethora of efflux pump genes that all overlap in targeting penicillin-type antibiotics, with multiple efflux pumps capable of removing penicillin, and thus limiting the overall effectiveness of the antibiotic

The *CMY-65* gene was the only gene found in the CARD database that returned a perfect match against the reference sequence in the CARD database. However, the CDE database returned a similar match with the same sequence returning the beta-lactamase gene *CMY-72.* The C*MY* gene is a sub-group of the beta-lactamase that target penicillin, cephalosporins, and beta-lactam antibiotics with beta-lactamase inhibitors (Kim et al., 2006). The *CMY* gene is found in Gram-negative bacteria and is typically associated with nosocomial infections, but has been increasing in community acquired infections (Doi et al., 2010). When observing the prevalence of the *CMY* family of genes, it is predominantly found in the chromosome of multiple species of *Citrobacter* including, but not limited to, *C. portucalnesis, Citrobacter youngae,* and C*. freundii* (Cao et al., 2021; Cheng et al., 2021; Liu et al., 2025). Although predominantly found within the species of *Citrobacter,* the genetic sequence for *CMY* genes have been found in other species related to *Citrobacter.* One research paper found that the *CMY* gene in a strain of *E. coli* matched the chromosomal fragment located in the bacteria *C. freundii,* indicating that the genetic sequence has the ability to be mobile between species (Manageiro et al., 2015). A similar paper had identified novel *CMY* genes from *P. mirabilis* originating from *C. freundii* via horizontal transfer (Literacka et al., 2004).

The transfer of the *CMY* beta-lactamase gene to other bacteria could be due to the transmission of insertion sequences carrying the resistance gene. Insertion sequence genes are short sections of DNA segments that encode only enzymes that range in length from 0.7 to 2.5 kb (Siguier et al., 2015). Individual insertion sequences are capable carrying nearby genes as passengers (Razavi et al., 2020). Two flanking insertion sequences can combine and transfer the genetic material between them to form composite transposons and transfer larger genetic sequences (Galiot et al., 2023).

When observing the location of the insertion sequences and composite transposons in relation to the *CMY* beta-lactamase gene in SBr35, the likelihood of the beta-lactamase gene being mobile is low. The *CMY* beta-lactamase gene is located on contig 30, spanning base pairs 901,303 to 902,448. The closest insertion sequence on the same contig is located base pairs 1,013,490 to 1,014,825. The number of base pairs between the two gene sequences is over 100,000 base pairs. Most individual insertion sequences average between 500 to 2,500 base pairs (Siguier et al., 2006). The hypothesis that the composite transposons being the mobility vector for the CMY gene is also unlikely, due to the location of the composite transposons. The only known composite transposons found in SBr35 are on contigs 11, 14 and 37. In order for the *CMY* gene to be considered mobile due to the composite transposons, it would need to be flanked by the two insertion sequences that form the transposon. While it is unlikely for this specific strain to transfer resistance due to the location of both the resistance gene and composite transposons, the potential for similar organisms to obtain mobile insertion sequences and mobile transposons near resistance genes to transfer resistance in the future must be considered.

Like the reference strains of *Citrobacter,* multiple mechanisms were found to play a role in increased survivability against antibiotics not limited to beta-lactamase. The *C. portucalnesis* isolate was found to have the *acrAB-TolC* gene, which is a stress induced efflux pump gene found in *E. coli* (Ma et al., 1995). The *acrA g*ene is a part of the AcrAB-TolC efflux structure consisting of the AcrB inner membrane protein, the AcrA membrane fusion protein and the TolC outer membrane factor (Jang, 2023). Expression of the acrAB-TolC protein is regulated by the AcrR protein, which in turn is regulated by the *MarRAB* gene, of which the *marR* gene is a part of (Praski Alzrigat et al., 2017). It has been theorised that mutations to the *marR* gene prevent the repression of the AcrAB-TolC protein pump allows for greater survivability of cytotoxic compounds (Watanabe & Doukyu, 2012). Such mechanisms and mutations play a key role in the multidrug resistance of bacteria such as *Citrobacter spp. and Enterobacter aerogenes* (Chenhaka et al., 2023; Pradel & Pagès, 2002)

The *mdfa g*ene was another gene detected in the suspected strain of *C. portucalnesis* that is derived from *E. coli.* The MdfA protein is a multidrug efflux pump a part of the MFS family that targets antimicrobials, such as tetracycline, erythromycin and chloramphenicol (Wang et al., 2014). *E. coli s*trains that overexpress the *mdfA* gene have been found to have sixfold increase in resistance to fluoroquinolone (Abdelhamid & Abozahra, 2017). Although the types of antibiotics that the *MdfA ge*ne targets were not tested, it could be an area of future research to observe if the efflux pump plays a role in the survivability of the particular strain of *C. portucalnesis.*

The final efflux pump gene of interest is the *msbA* ATP binding cassette gene (ABC) found in the *Citrobacter* isolate. ABC proteins, and by extension the MsbA protein, are found in the cytoplasmic membrane that functions by using ATP hydrolysis to export harmful compounds across the cell membrane (Chang & Roth, 2001; Westfahl et al., 2008). ABC proteins typically consist of four modules comprising two membrane-spanning regions and two nucleotide-binding domains (Chang & Roth, 2001). Such mechanisms play a critical role in the survival of *C. freundii* against aminoglycosides, fluoroquinolones and macrolides (Chenhaka et al., 2023).

The remaining antimicrobial gene of interest was the *arnT* gene. The *arnT* gene is responsible for the creation of the 4-amino-4-deoxy-L-arabinose transferase enzyme, which affects positively charged antimicrobials like polymyxin by removing the negative charge on the bacterial outer membrane that prevents binding of the antimicrobial (Deng et al., 2013). Polymyxins are a class of antimicrobials that function by destabilizing the lipopolysaccharide outer membrane causing cellular permeability and leading to cell death (Trimble et al., 2016). *Salmonella typhimurium* strains have been found to harbour *arnT* genes that allow resistances to polymyxins and strains of *E. coli* having the similar *pmrA* gene that allow for survival when treated with polymyxin (Yan et al., 2007). *K. pneumoniae* have also been found with the *arnT* gene along with similar *mcr-1* and *eptA* genes that resulted in initial success in treatment, but had later regrown with an increased expression of *ernT* and *eptA* genes (Nang et al., 2019).

Many pathogenic Gram-negative bacteria have multiple genes encoding different types of RND efflux pumps that either target a large group of antimicrobials or selectively remove specific compounds. The *CRP* and *rsmA* genes have been found to be the resistance mechanisms in strains of the bacteria *Morganella morganii* isolated from clinical patients (Behera et al., 2023). Other similar mechanisms have been found in other pathogenic strains of Gram-negative bacteria. Organisms like *E. coli, Enterobacter spp.* and *P. mirabilus* have been found to have the RND grouped genes *AcrAB-TolC,* which target fluoroquinolones, glycylcylines and various biocidal chemicals (Poole, 2007).

Multiple resistance mechanisms can be present in a singular bacterial species that target similar or a wide range of antimicrobials, allowing the bacteria to survive. The bacteria species *Salmonella spp.* have a multitude of efflux pumps from the RND, Major Facilitator (MF), Multidrug and Toxic compound Extrusion (MATE) and ATP-binding cassette (ABC) family group of genes that allow the organism to achieve multidrug resistance (Nishino et al., 2021). Similar mechanisms can be seen in the bacteria *K. pneumoniae* employ three types of efflux pumps in the RND, SMR and MATE groups in combination with *CTX-M15* ESBL gene that attributes greater resistance against beta-lactam antimicrobials (Maurya et al., 2019).

With the resistance genes that may or may not have contributed to the organisms' degree of resistance, there were discrepancies between the two databases and reference strains. The first discrepancy was between the reference strain of *C. portucalnesis a*nd the isolate. The only difference between the database pathway of *C. portucalensis* and the stream isolate is the presence of the precursor combined sensor/regulator AdeS and AdeR protein group and the presence of the AdeB multidrug efflux pump.

While there were multiple mechanisms shown in the supposed isolate of *C. portucalnesis,* the *adeB* efflux protein gene is a part of the AdeABC efflux protein pump, similar to the AcrAB-TolC efflux pump, as it consists of three components used to remove cytotoxic compounds from the bacterial cell (Wieczorek et al., 2008). The AdeB efflux pump is a part of the AdeABC group of multidrug efflux pumps that is found in the bacteria *Acinetobacter baumannii* that plays a critical role in the resistance to multiple antibiotics in the aminoglycoside group (Leus et al., 2024). The AdeABC protein is a multidrug efflux protein in the RND family group consisting of the outer membrane protein (AdeC), the multidrug transporter (AdeB) and the attaching fusion protein (AdeA) (Xu et al., 2019). Although it is possible to exchange the genetic sequence between organisms the possibility that the *C. portucalnesis* isolate contains the *acrAB-TolC* genes, it could be plausible to assume that only a partial transfer of the genetic material was transferred. However, when comparing to CARD and the CGE database, the *adeB* gene is absent among the listed genes present or a part of the mobile genetic elements.

If the KEGG pathway is to be believed, it would indicate that the isolate of *C. portucalensis* isolated from the stream site contains the precursor genes for sensory and regulation of the *adeABC* RND efflux pump genes but only the multidrug transporter portion of the three protein structure. When the DNA sequence was placed into CARD, multiple genes relating to RND type efflux pumps were present in the bacteria. However, no strict matches were found relating to the *adeABC* genes were matched with the CARD database. This difference could simply be due to the degraded nature of the DNA, causing a false positive due to having multiple contigs that cause multiple gaps between the DNA sequence.

Similarly, the CDE database indicated that the antibiotic deactivation gene was the *CMY-72* gene, with the only mobile elements being related to insertion sequences and composite transposons. The difference in databases for the type of *CMY* gene could simply be due to the commonality between strains of *C. portucalnesis* to have multiple variations of the *CMY* gene (Chenhaka et al., 2023; Sellera et al., 2023). When considering the mobile elements shown by the CDE database, the only mobile elements found were unrelated to resistance or virulence. Separate studies have found that strains of *C. portucalenesis* are capable of transferring diverse groups of resistance genes between species ranging from NDM-1, CTX-M14 and SFO-1 (Guo et al., 2024; Wang et al., 2022). While the CDE database indicated that no resistance mechanisms were mobile, the potential threat for environmental organisms to acquire more resistance mechanisms on top of what is currently present is a concern.

Comparison between *H. alvei* and *C. portucalnesis*

The primary objective of sequencing the presumed isolates of *H. alvei* and *C. portucalnesis* was to determine the degree of resistance presented during the antibiotic disk assay. Initial tests were conducted to determine the spectrum of resistance against beta-lactam antibiotics produced by each organism. Initial findings indicated that the type of beta-lactamase produced by the organisms were the result of being the original source of the beta-lactamase gene. Although both *H. alvei* and *C. portucalnesis* isolates returned positive matches for ESBL genes, the resulting resistance was due to naturally occurring resistance rather than acquired resistance from a separate organism. However, while the beta-lactamase genes were naturally a part of the respective genomes,

both organisms showed strict matches for multidrug efflux genes associated from different species of bacteria. The first being the *kpnF*, *kpnE* and *kpnH* group of genes that are present in *K. pneumoniae.* While the potential for horizontal gene transfer is possible, it is unknown whether it was acquired from an environmental contamination with *K. pneumoniae* and wastewater before passing the genetic material before all viable cells succumbed to the hostile environment. The CDE database would indicate that none of the resistance genes found in the *H. alvei s*train contained any mobile genetic elements, but such results could simply be due to gaps within the databases. It should be noted that the *H. alvei* isolate was taken during a period of increased rainfall, and the potential for faecal indicator organisms to be a part of the environment when sampled is a possibility. Due to the *C. portucalnesis i*solate location consistently having an MPN count far exceeding 1,600 CFU/100ml, it would be possible for strains of *E. coli* or similar bacteria to transfer the genetic sequence for the acrAB-TolC protein to reinforce the resistance to beta-lactam antibiotics.

It should be noted that all databases used provided contradicting information in regard to what types of genes were present. Both the CARD and CDE databases matched the A*CC-3* gene found within the *H. alvei* strain, while the KEGG database detected the presence of a type C beta-lactamase, but failed to associate it with the *ampC* subgroup the gene was a part of. Both the CARD and CDE databases resulted in contradicting data with CARD displaying the efflux pump genes found in both *H. alvei* and *C. portucanesis* originated from *K. pneumoniae,* while the CDE database indicated that no mobile genes were related to resistance mechanisms, suggesting that the resistance genes were intrinsic to the respective bacteria. This discrepancy may simply be due to limitations between both databases. The CARD database reported strict, not perfect matches, with missing regions between contigs, may have contributed to inconsistent results between databases.

One point of comparison is the number of efflux pump resistance genes found in each isolate. While still expressing resistance to the same antibiotics, the *H. alvei* isolate, excluding the AmpC beta-lactamase *ACC-3* gene had 6 strict matches within the CARD database. Conversely, the *C. portucalnesis* isolate returned 19 strict matches excluding the *CMY-65* beta-lactamase gene. While the *H. alvei s*train did produce a small inhibition zone for 2 antibiotics, the size of the inhibition zone still classified the diameter to be resistant. When observing *C. portucalnesis* for comparison, the inhibition zone is only present for meropenem, which the organism is susceptible. While there is overlap in some genes that are potentially shared between both *H. alvei a*nd *C. portucalnesis* isolates, the greater number of efflux pump genes would indicate that multiple efflux pumps are enabling greater resistance to the *C. portucalnesis* isolate. Although the number of efflux pump genes in the *H. alvei* isolate is lower when compared to *C. portucalnesis,* the genes that are present would still allow the isolate to be considered resistant.

One similarity noticed between the two isolates was the high susceptibility to meropenem. Of all detected genes found in both isolates, only one gene was found to target carbapenems. This limited presence of carbapenem-targeting genes, along with the absence of overlapping resistance mechanisms for carbapenems, may explain the high susceptibility to meropenem observed in both organisms.

Sequencing Limitation

It should be noted that the Oxford MinION Nanopore technology used for sequencing the genomes of both isolates was prone lower base-calling accuracy. Early iterations of Oxford MinION were capable of producing long reads of genetic material but were hindered by the low base-calling accuracy, with the initial release of the technology reporting an estimated a base-calling accuracy of 38.2 percent (Laver et al., 2015; Mikheyev & Tin, 2014). As the technology developed, Oxford Nanopore technology sequencing had improved in accuracy, with the first modified kits achieving a median accuracy of 87 to 92 percent, and the most recent kits reaching 94 percent accuracy (Liu-Wei et al., 2024).

Despite initial lower base-calling accuracy, improvements to the technology have significantly increased the overall accuracy of Oxford Nanopore over recent years. The longer reads allow for better assembly as overlapping regions can be connected into larger contigs that allow for better assembly (Kerkhof, 2021; Lu et al., 2016). In contrast, Illumina sequencing is limited by shorter read lengths ranging from 300bp to 550bp. However, it could be used in tandem with Oxford MinION Sequencing as a polishing tool to remove the lower base-calling and improve overall accuracy (Cook et al., 2024).

However, when it comes to identifying specific resistance genes, such as the beta-lactamase family of genes, alternative sequencing methods should be considered. As mentioned, the lower base-calling accuracy of Oxford MinION Nanopore could lead to false negatives or misidentification, potentially allowing resistance genes to go undetected. Beta-lactamase genes, including CTX, CMY, and TEM groups, are known to have multiple variants within the same gene family.

Given the need for higher accuracy when sequencing gene families with multiple variants, platforms such as Illumina may be more suitable. Illumina sequencing is known to have a significantly lower error rate, estimated to be around 1 percent (Moti et al., 2022).

Resistance genes found in New Zealand waters

Previous studies conducted in New Zealand have found a variety of resistance genes in local waterways. One study found that during the spring season, CTX beta-lactamase genes were detected in the Selwyn and Rangatata rivers in the Canterbury region, but was absent in the same rivers during the autumn season (Davis et al., 2021). A separate study found strains of *E. coli* containing the CTX-15 beta-lactamase gene downstream of a treatment site along the Manawatu river and in a stormwater drain in Palmerston North (Gray et al., 2025).

Despite being in different geographical locations, both studies were able to detect the same group of resistance genes from their respective sources. In comparison, the results from this study detected two types of beta-lactamase genes. However, while these genes were identified in the initial isolates, it remains unknown how prevalent they are throughout the year or across the country. Although the *ACC-3* and *CMY-65 g*enes were detected in one Auckland stormwater system, future studies could investigate how prevalent the distribution of these genes are throughout the Auckland region and New Zealand's waterways in general.

Chapter 5 Final discussion

Bacteria becoming resistant to current treatments is an alarming trend for public health officials and requires intervention to prevent further preventable deaths (Tang et al., 2023). Surveillance methods are being implemented in hospitals and waste treatment plants at a global scale as a precautionary measure to track changes in infections to allow health professionals to prescribe effective treatment (Chau et al., 2022; Moolchandani et al., 2017; Simonsen et al., 2004). However, the infrastructure in which surveillance relies on is prone to gaps that allow for escape into local environments that go undetected. This study has found that a local stormwater retention basin and the associated stream does contain AMR bacteria.

The resistance mechanisms employed by the AMR bacterial strains found in the local waterways and two bacterial species utilise these mechanisms to be resistant to multiple antibiotics. Understanding the resistance mechanisms is an important requirement to effectively treat any potential spread when they arise. Most studies investigate the prevalence of the indicator bacteria present in waterways, and their associated sources or the resistance profile of bacteria affected by wastewater, but few studies have observed the resistance profile of bacteria from water sources designed to contain contaminated water. The goal of this study was to first observe the number of indicator bacteria between seasonal and weather change, then survey for the presence of antimicrobial resistant organisms. Of the resistant bacteria that were cultivated, both the species and resistance mechanisms were identified.

During the winter months, the indicator numbers before and after heavy rain were found to slightly increase in upstream sites with the increased rainfall, but counts in downstream locations remained at high numbers regardless of the weather conditions. In the summer months, despite having no significant levels of rainfall, the indicator organism counts found in the same sites exceeded the count found during the rainy conditions. The number of indicator bacteria from the MPN results approximated the genera of bacteria present. It should be noted that the MPN results represent were only from one set of winter and summer sampling and could have been an outlier and not the expectation. Further research in the fluctuations between seasons over time is required before conclusions regarding the indicator bacteria population can be made.

Only one positive MPN tube produced growth at 42°C, but it was never cultured again in later sampling. This indicated that the initial sampling contained faecal coliform bacteria, but further sampling no longer contained faecal bacteria. With the lack of detection of faecal bacteria from the estuary site indicated that the environment provided a hostile environment to prevent growth and sources of contamination were limited to the initial period. The organisms that were cultured throughout the duration of this study can be assumed to be a part of the natural flora of their given location.

All environmental filters produced some small quantity of ESBL bacteria and the upstream, stream and estuary sites had produced indicator bacteria with resistance to ESBL antibiotics. While resistance profile of each isolate varies from site to site, all isolates whether taken as an enrichment or environmental isolate showed full susceptibility to meropenem regardless of rainfall condition or season. Of the isolates that showed the highest level of resistance, all environmental filters displayed the highest degree of resistance. As for the enrichment

isolates, the stream location displayed similar resistance to the environmental isolates regardless of weather conditions or seasonal shift. The other isolate that displayed similar resistance to the enriched stream site was the upstream isolate taken during light rainfall. Interestingly, when taken after heavy rain, two different isolates from the same location showed reduced resistance and susceptibility to previously resistant antibiotics.

The sites that contained ESBL producing bacteria, the upstream site was found to have the environmental bacteria *H. alvei* with the *ACC-3* gene group that is part of the *ampC* family of ESBL genes that deactivate beta-lactam and cephalosporin antibiotics. The *ACC-3* gene detected during sequencing is a potential reason for the isolate being resistant to two penicillin drugs and cefalexin during initial antibiotic disk susceptibility testing. Of the other resistance genes found, 3 were a part of the RND family of efflux pumps genes with the final resistance mechanism involving antibiotic alteration. Although RND and *ampC* genes were found in the isolated bacteria, it is unknown whether those genes played an active role in the resistance of the tested antibiotics.

In regards to the mobility of the genes, the isolated strain of *H. alvei* did not contain any mobile gene elements. However, while one isolated strain of *H. alvei* did not contain any mobile gene elements it is unknown if other strains from the same location have mobile elements. The one isolate of *H. alvei* represented a single point in time and various environmental factors could have altered other *H. alvei s*trains in the original sampling location to develop mobile resistance elements.

Similar results came from the stream isolate that was found to have the bacteria *C. portucalnesis* with a similar C class beta-lactamase gene *CMY-65.* The presence of the *CMY* beta-lactamase group of genes that target cephalosporins, which could be the reason for the resistance against cefalexin seen during antibiotic disk susceptibility testing. As for the resistance against both types of penicillin*,* the strain of *C. portucalnesis* was found to have a multitude of efflux pump genes that had overlapping targeting of the penicillin group that could have resulted in resistance.

The strain of *C. portucalnesis,* while containing beta-lactamase resistance genes, was not observed to have any mobile genetic elements that attributed to resistance. Mobile genetic elements found in the isolate of *C. portucalnesis* were related to insertion sequences and mobile transposons, far away from the beta-lactamase gene.

Future Directions

Although the results produced gave insight into the resistance profile of the bacteria present, many changes to improve the quality of research can be done. First was the failure to enumerate *Enterococci* bacteria. Due to the failure to enumerate any *Enterococci*, it can neither be confirmed nor denied that the bacteria were present or contained any significant resistance. While the number of indicator bacteria given from the MPN result and streaking would indicate that the *Enterobacteriaceae* present were from environmental sources rather than faecal sources, the lack of any evidence of *Enterococci* would be beneficial for local health bodies to confirm if the stormwater ponds had been previously infected with faecal sources.

The second improvement would be to analyse the resistance genes of all isolates. Currently, only two isolates had their genome sequenced to identify bacteria species and resistance genes. At present, the only susceptibility testing conducted was done with antibiotic disks with concentrations set by the EUCAST guidelines. Looking into the Minimum inhibitory Content (MIC) for isolates would be recommended for future studies as some isolates, while resistant did have a small zone of inhibition. An increase in the concentration of selected antimicrobials could help identify the effective inhibitory concentration. With a longer time frame and budget for multiple MinION sequence flongles would have been ideal to investigate the full profile of the environment. It should be noted that the difference in databases used to identify the same resistance type of genes in a single organism were present in the CARD database but not in the KEGG database. Changes to a genetic sequence could potentially lead to organisms with resistance genes to slip past databases undetected. The same principle can be applied to the types of PCR primers used in wastewater and environmental surveillance. Due to the large variation in resistance genes in the ESBL group, multiple variations of the same gene have the potential to be undetectable if the specific set of primers are not used in the surveillance rotation.

Another improvement would be the use of barcoding to sequence multiple isolates using a single Oxford MinION Nanopore flongle. Although only 5 locations were sampled, some locations produced multiple resistant isolates. In practice, this would require multiple flonges to sequence each isolate individually, increasing the overall costs. By incorporating genomic barcoding, multiple isolates could be combined into a single sequencing run, generating more data on the resistant bacteria present, while reducing costs.

Another improvement would be to survey for the potential for resistant pathogenic anaerobic bacteria within the sediment and silt. Results derived from this study were taken from the organisms distributed from the surface and free-flowing water. Organisms that were cultivated from each water source were done under aerobic conditions, and as a result produced organisms that were either aerobic or facultative anaerobic. Further work into other potentially resistant organisms either require anaerobic conditions for growth or the bacterial biofilm community within the sediment.

Finally, the last improvement would be to examine which genes play a role in the resistance to beta-lactam antibiotics. The simple presence of resistance genes does not indicate that the genes are active in the organism found. Removing the specific section of genomic sequence and implementing the exact same genetic sequence

in an organism that is known to have susceptibility to the same antibiotics like *E. coli* 25922 or testing the organism against the same antimicrobial again to observe for susceptibility. Additional research into other combined antimicrobials like penicillin with boric acid to test if it is the specific gene is effective in deactivating the beta-lactamase supposedly found in the isolates.

The data from this paper is a result of the initial screening of the antimicrobial profile of organisms isolated from a West Auckland stormwater system. The number of indicator organisms fluctuated between both weather and season, but remained high regardless of when sampling occurred. Throughout the stormwater system, organisms were found to be resistant to be resistant to at least one or more antibiotic. Of the resistant organisms isolated, WGS revealed the resistance mechanisms of two isolates. Although resistant to multiple beta-lactam antibiotics, these isolates did not contain resistance genes near any mobile genetic elements that would enable transfer. While results indicated that the two isolates were unable to transfer resistance elements, it is important to note that these findings represent the resistance profile of only two organisms, which are part of a larger microbial community. Other bacterial species found in the same or different stormwater systems are still unaccounted for and warrant further research.

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