Comparison of antibiotic resistance mechanisms in *Enterobacter* and *Citrobacter* spp. bloodstream isolates from the 1980s and 2020 in Bristol

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Abstract

Enterobacter spp. and *Citrobacter* spp. are opportunistic human pathogens that primarily cause bloodstream infections (BSIs) and urinary tract infections (UTIs). Commonly, the β-lactam antibiotics ceftazidime (CAZ) and piperacillin/tazobactam (TZP) are used to treat such infections. However, *Enterobacter* spp. and many *Citrobacter* spp. contain chromosomal *ampC* β-lactamase genes which can become hyper-expressed following mutation, which confers CAZ resistance (CAZ-R). Here, we phenotypically and genotypically characterise antibiotic resistance (ABR) within 99 clinical isolates of *Enterobacter* spp. and *Citrobacter* spp. from the 1980s (19) and 2020 (63) in Bristol and compare ABR mechanisms. We conclude that the predominant mechanism of CAZ-R is AmpC hyper-production due to the inactivation of *ampD,* encoding a protein involved in the *ampC* regulatory pathway. The most common cause of *ampD* inactivation was missense mutations. We characterise AmpD amino acid substitutions in CAZ-R isolates at three previously unreported residues: S73, I78 and S100. A handful of isolates, from 2020 only, contained mobile ABR genes, which accounted for their resistance to a broader range of β-lactam and non-β-lactam antibiotics. Proteomics analysis suggests that cefepime (FEP) resistance, which was seen predominantly in *Enterobacter* spp., and did not differ between the time periods, is occurring through a yet unelucidated mechanism rather than being linked to specific porin changes as reported in other *Enterobacterales* species. We show that there has been a significant increase in TZP resistance from the 1980s to 2020, likely associated with selection pressure from the increased use of TZP. Although likely linked to AmpC hyperproduction, the mechanism for TZP resistance is unclear. This work highlights how external factors, such as antibiotic use, can affect the prevalence of ABR as well as demonstrating the change, or lack thereof, in mechanisms of resistance to different antibiotics over time and between genera.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is my own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: Emily Syvret

DATE: 18 April 2024

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List of Abbreviations

3GC – Third generation cephalosporin

ABR – antibiotic resistance

ACA – aminocephalosporanic acid

AMC – amoxicillin/clavulanate

AMX – amoxicillin

APA – aminopenicillanic acid

AZT – aztreonam

BSI – bloodstream infection

CAZ – ceftazidime

CAZ-R – ceftazidime resistant

CAZ-S – ceftazidime susceptible

CIP – ciprofloxacin

CTX – cefotaxime

ESBL – extended spectrum β-lactamase

ESPAUR – English surveillance programme for antimicrobial utilisation and resistance

EUCAST – European committee on antimicrobial susceptibility testing

FEP – cefepime

IS – insertion sequence

MBL – metallo β-lactamase

NCBI – National Centre for Biotechnology Information

OMP – Outer membrane protein

PBP – penicillin binding protein

PMQR – plasmid mediated quinolone resistance

SBL – serine β-lactamase

SNP – single nucleotide polymorphism

TZP – piperacillin/tazobactam

UTI – urinary tract infection

WGS – whole genome sequencing

WT – wild type

1. Introduction

1.1 Antibiotic resistance (ABR)

ABR – the ability of bacteria to evade antibiotic therapy – is a global threat. The rise of ABR combined with the difficulty in developing new, effective antibiotics has the potential to revert the world to a state in which minor ailments could again be commonly fatal. It is estimated that in 2019, ABR caused 1.27 million deaths (Murray *et al.*, 2022) and some estimates suggest that by 2050 this number could rise to 10 million per year (O'Neill, 2016).

A report by the World Health Organisation (WHO) highlighted 12 pathogens which pose a particular threat to the emergence of ABR and hence should be a priority for research (WHO, 2017). Nine of these 12 species listed were Gram-negative bacteria. These species present a greater problem than Gram-positive bacteria due to their additional outer membrane creating an extra barrier to antibiotics (Cama, Henney and Winterhalter, 2019). The WHO has also classified antibiotics used in clinical settings by their importance to medicine, informing which of these are of upmost importance to preserve (WHO, 2024). Drugs which fall into the highest priority critically important antibiotic category include a subset of β-lactam antibiotics, the third-generation cephalosporins (3GCs). Indeed, 3GC-resistant *Enterobacterales* are some of the highest priority pathogens identified by the WHO for the threat they pose.

1.2 β-lactam antibiotics

Despite first being discovered in 1929 (Fleming, 2001), β-lactam antibiotics are still the most commonly prescribed antibiotic class globally, testifying to their significance (Klein *et al.*, 2018). β-lactams are bactericidal antibiotics which target transpeptidase enzymes (a subset of penicillin binding proteins; PBPs) involved in peptidoglycan crosslinking. Peptidoglycan is the main component of the bacterial cell wall, which is formed by the cross-linking of peptidoglycan chains, catalysed by transpeptidase PBPs, between two pentapeptides, one on each chain. β-lactams covalently bond with the PBP active site by mimicking a key D-Alanine-D-Alanine (D-Ala-D-Ala) moiety within the pentapeptide substrate (Tipper and Strominger, 1965). These irreversible covalent bonds prevent the PBP catalysing peptidoglycan

crosslinking, resulting in an unstable, weak and leaky cell wall which causes the cell to lyse by osmotic pressure.

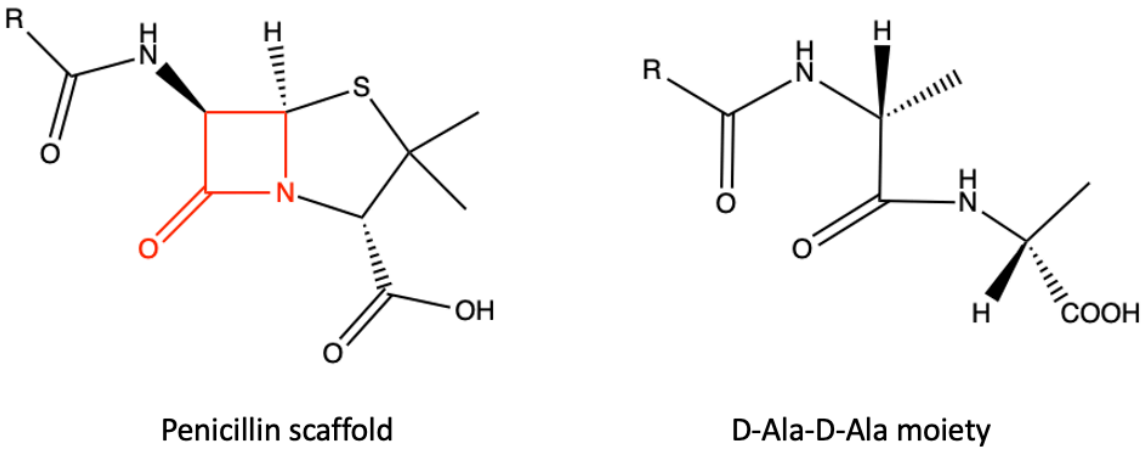

Figure 1 The chemical structure of the core scaffold of the penicillins is similar to that of the natural substrate of the transpeptidase enzyme. The core penicillin structure, with the β-lactam ring highlighted in red (RHS), mimics the D-Ala-D-ala moiety (RHS) of the pentapeptide substrate of the transpeptidase enzyme, inhibiting it from catalysing the final cross-linking step in peptidoglycan synthesis, disrupting the cell wall. Made in ChemDraw 23.0.

The β-lactam scaffold which mimics the D-Ala-D-Ala moiety consists of a β-lactam ring and its adjoining carboxylate and amide groups (Figure 1). The β-lactam ring is the key pharmacophore – nucleophilic attack of a PBP serine residue on the β-lactam amide opens the β-lactam ring, forming a stable β-lactam-enzyme complex which inhibits PBP activity (Mora-Ochomogo and Lohans, 2021). The bicyclic nature of the β-lactams also contributes to their significant antibacterial activity. The adjoining ring structure confers ring strain on the β-lactam ring, creating a reactive centre which is vulnerable to nucleophilic attack and therefore preferentially reacts with the PBP over the natural D-Ala-D-Ala substrate (Tipper and Strominger, 1965).

There are four main classes of β-lactam antibiotics: penicillins, cephalosporins, carbapenems and monobactams. Each class is derived from natural products, with the core β-lactam ring and adjacent amide and carboxylate groups being retained through the classes. With the exception of the monobactams which are monocyclic systems, β-lactams are bicyclic structures whereby the β-lactam ring is fused to an adjacent ring system.

Penicillins were the first class of β-lactam to be discovered. They are derived from Penicillin G, a molecule isolated from the fungus *Penicillium chrysogenum* (Abraham and Chain, 1940)**.** Elucidation of its structure identified 6-aminopenicillanic acid (6-APA) as the catalytic nucleus of the penicillins (Lima *et al.*, 2020). (Figure 2). This paved the way for development of semi-synthetic penicillin derivatives to optimise pharmacokinetics. In general, the hydrophobic and electron-donating nature of the side chains of Penicillin G and its early analogues resulted in a spectrum of activity limited to Gram-positive species as well as substantial acid-sensitivity limiting administration methods of the antibiotics (Mora-Ochomogo and Lohans, 2021). Furthermore, due to the reactivity of the highly strained β-lactam ring, Penicillin G is highly sensitive to β-lactamase enzymes which hydrolyse the β-lactam ring, rendering the drug ineffective. Notable derivatives of Penicillin G which are still commonly used today include the narrow spectrum antibiotic Penicillin V (phenoxymethylpenicillin) and the broad-spectrum antibiotic, amoxicillin (AMX). AMX has a broader spectrum of activity due to its greater hydrophilicity which enhances permeability through the Gram-negative outer membrane (Lima *et al.*, 2020). Other notable classes of analogues include the carboxypenicillins, (eg. Carbenicillin), the ureidopenicillins (eg. Azlocillin) and the isoxazolyl penicillins (eg. Flucloxacillin) (Figure 2). In 2022, the penicillins were the most prescribed antibiotic in England with the most frequently prescribed being AMX, flucloxacillin and phenoxymethylpenicillin used to treat a variety of common infections such as skin infections, chest infections and Urinary Tract Infections (UTIs) (UK Health Security Agency, 2023) (NHS, 2017).

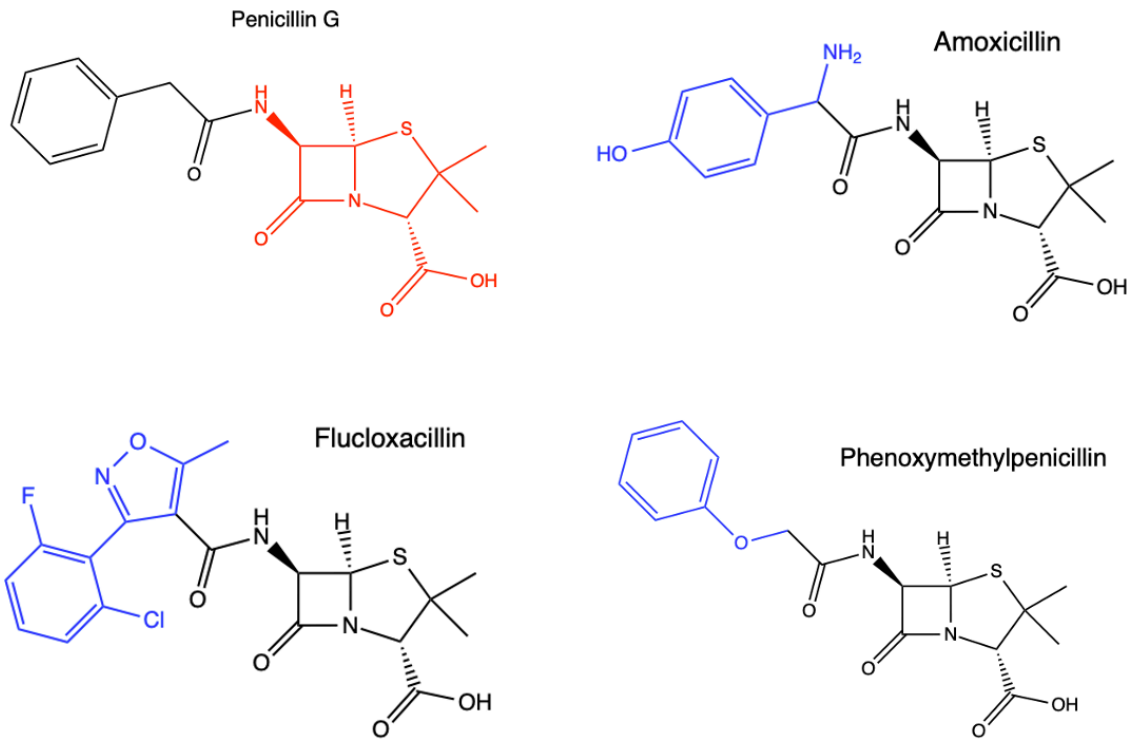

Figure 2 Differences in chemical structure of the first penicillin, Penicillin G, and the three most commonly prescribed penicillin antibiotics in 2022. The 6-aminopenicillanic acid nucleus (red), key to the structure of penicillins, has been retained throughout semi-synthetic derivatives of the penicillins due to its role in catalysis. Varying the R group (shown in blue in the three derivatives) enhances specific pharmacokinetic properties of the drugs.

Cephalosporin C was the first of the cephalosporins to be discovered, isolated from the *Cephalosporium acremonium* fungus (Newton and Abraham, 1955). Structural elucidation of cephalosporin C identified the catalytic nucleus of the molecule as 7-aminocephalosporinic acid (7-ACA) (Loder, Newton and Abraham, 1961). Analogous to 6-APA of penicillins, 7-ACA exhibits a bicyclic structure consisting of a β-lactam ring fused to a six-membered sulphur-containing ring (Figure 3). Compared to penicillins, the β-lactam ring of this cephalosporin scaffold is more stable to nucleophilic attack due to the reduced ring strain conferred by the 6-membered adjoining ring system. This decreases antibacterial activity, but also decreases the vulnerability to hydrolysis by β-lactamases (Lima *et al.*, 2020). Derivatives of cephalosporin C have been synthesised through the optimisation of substituents on the 7-ACA nucleus. This has produced multiple 'generations' of cephalosporins, with marked differences in antibacterial spectrum and activity, the most notable of which is subsequent generations having an increased activity against Gram-negative species (Lima *et al.*, 2020).

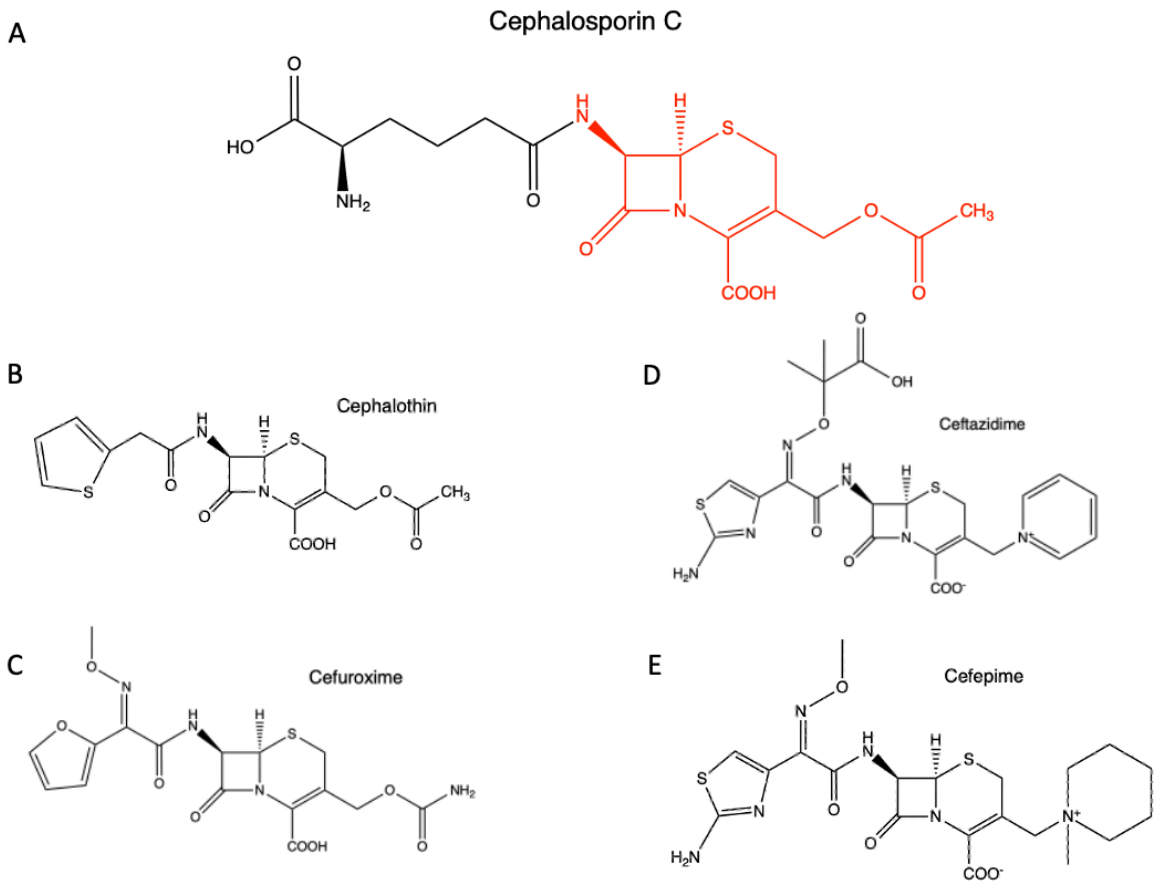

Figure 3 Chemical structures of key antibiotics from each generation of cephalosporin. (A) Cephalosporin C was the first cephalosporin to be isolated and is the structure from which subsequent generations of cephalosporins (B-E) have been derived from. The 7-aminocephalosporinic acid nucleus, key to cephalosporin function, is highlighted in red. Notable derivatives include the third generation oxyiminocephalosporin, ceftazidime, due to its ability to target a broader range of bacteria (D) and the fourth-generation cephalosporin, cefepime (E). Draw in ChemDraw 23.0.

First-generation cephalosporins, such as cephalothin, are mainly active against Gram-positive bacteria due to their low affinity for PBPs of Gram-negative species (Godzeski, Brier and Pavey, 1963). A variety of PBPs have been characterised in both Gram-negative and Gram-positive species, with species containing multiple different types of PBP. There are two main subsets of PBP, high molecular weight and low molecular weight and within these two groups are further subdivisions (Sauvage *et al.*, 2008). As a result, different species contain PBPs with different structures which leave them with a variety of susceptibilities to different β-lactams (Fontana *et al.*, 2000). Second-generation cephalosporins, such as cefuroxime, generally exhibit greater activity against Gram-negative species due to their greater hydrophilicity (Lima *et al.*, 2020). Third-generation cephalosporins (3GCs) exhibit a marked increase in activity against Gram-negative bacteria and increased stability against β-lactamases. Of particular note are the oxyiminocephalosporins (cefotaxime (CTX) and ceftazidime (CAZ)) which have an

oxyimino substituent on the 7-ACA nucleus (Figure 3). This accounts for their increased affinity for PBPs of Gram-negative bacteria and their subsequent increased activity against these species (Klein and Cunha, 1995). As well as improving activity against Gram-negative species, the increase in hydrophilicity in 3GCs reduces their permeability through the highly lipophilic membranes found in the intestinal system (Lima *et al.*, 2020). This increases the spectrum of activity to enteric Gram-negative species such as *Enterobacter* spp. Cefepime (FEP) is a fourth-generation cephalosporin which exhibits similar activity against Gram-negative species as 3GCs but has greater activity against Gram-positive species (Sanders, 1993). It is also stable against most β-lactamases due to its low affinity for the enzymes (Sanders, 1993). 3GCs are mainly used to treat Gram-negative infections, particularly complicated UTIs and bloodstream infections (BSIs). They are classed as highest priority critically important antibiotics used in human medicine (WHO, 2024). Their use should therefore be preserved wherever possible in order to maintain an arsenal of antibiotics which remain effective against such infections.

Carbapenems are a group of β-lactams derived from thienamycin (Figure 4) (Kahan *et al.*, 1979). Although thienamycin was never used clinically, subsequent carbapenems such as meropenem and imipenem exhibit potent antibacterial activity against a broad spectrum of bacterial species (Papp-Wallace *et al.*, 2011). Their structure means they are also highly resistant to β-lactamases rendering them a crucial drug of last resort.

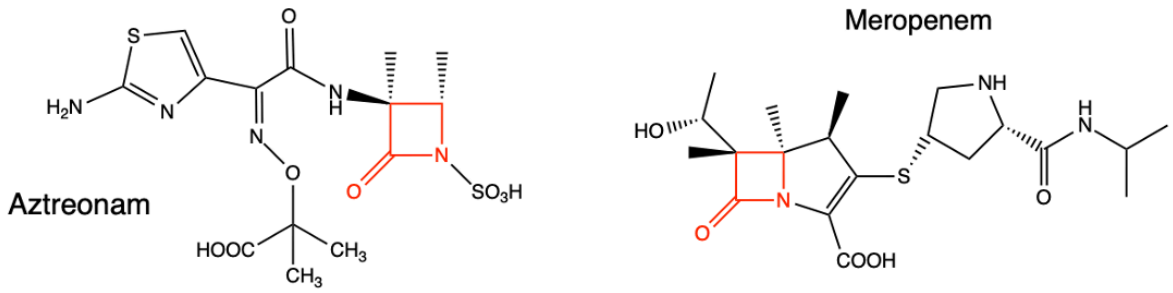

Figure 4 Aztreonam is the only monobactam antibiotic in clinical use and meropenem is a key antibiotic within the carbapenem family. Monobactam and carbapenems are two classes of β-lactam antibiotics in clinical use. The β-lactam ring conserved across these classes is highlighted in red. Meropenem is of particular note as it is a last resort antibiotic.

The monobactams are a unique group of β-lactams with a monocyclic structure. The only monobactam antibiotic currently in clinical use is aztreonam (Figure 4). Aztreonam exhibits a

very high affinity for PBP3 of Gram-negative bacteria resulting in effective antibacterial activity against these species (Sykes and Bonner, 1985). Its structure around the β-lactam ring also confers a high level of stability to β-lactamase enzymes (Wang *et al.*, 2014).

1.3 β-lactamase mediated antibiotic resistance

There are multiple mechanisms from which resistance to antibiotics can arise including changes in outer membrane permeability leading to reduced influx or increased efflux of drugs, modification of the drug target and production of drug altering enzymes (Avison, 2005). β-lactamases are degradative enzymes which cleave the amide bond of the β-lactam ring and release the product, rendering the β-lactam ineffective (Figure 5) (Tooke *et al.*, 2019). β-lactamases represent the primary resistance mechanism against β-lactam antibiotics in Gram-negative bacteria (Palzkill, 2018) and therefore understanding and finding ways to overcome this resistance is key.

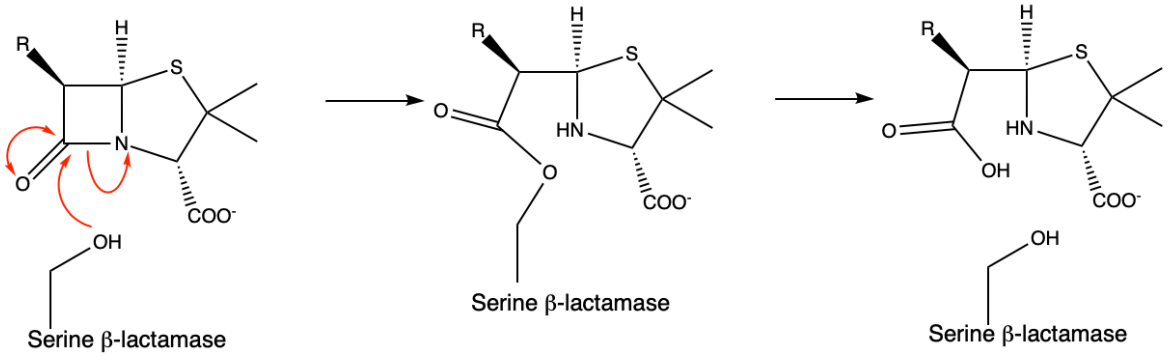

Figure 5 β-lactamase enzymes irreversibly inactivate β-lactam antibiotics. Curly arrow mechanism of the hydrolysis of β-lactams by β-lactamase enzymes. The β-lactamase enzyme cleaves the amide bond within the β-lactam ring and subsequently releases the product, resulting in an ineffective antibiotic and an enzyme which can attack another β-lactam molecule.

There are four classes of β-lactamase enzymes, classes A-D, classified by the Ambler system (Ambler, Baddiley and Abraham, 1997). Original Ambler classification distinguished two unrelated groups of β-lactamases: class A (serine β-lactamases, SBLs) and class B (metallo-β-lactamases, MBLs). The SBLs are thought to have evolved from transpeptidase enzymes (Medeiros, 1997) and have since been divided into three classes of homologous enzymes: class A, C (Jaurin and Grundström, 1981) and D (Ouellette, Bissonnette and Roy, 1987). As

such, the SBLs and MBLs utilise two distinct mechanisms: in SBLs, an active site serine residue undergoes acylation whereas MBLs utilise a zinc-dependent hydrolysis mechanism (Tooke *et al.*, 2019).

Class A β-lactamases are the most widely distributed β-lactamase amongst Gram-negative and Gram-positive species. They mainly comprise enzymes called penicillinases due to their ability to hydrolyse penicillins and little else (Ambler, Baddiley and Abraham, 1997)**.** Class A enzymes TEM-1 and SHV-1, the former plasmid mediated, and the latter chromosomally encoded in *K. pneumoniae,* are the origin from which the earliest known extended-spectrum β-lactamases (ESBLs) have derived from (Bradford, 2001). ESBLs are a subset of class A enzymes which have acquired mutations in their active site enabling them to accommodate, and so hydrolyse oxyimino cephalosporins, including CTX, CAZ and FEP, as well as monobactams. In recent years, the most commonly encountered ESBLs are of the CTX-M type, of which there are many variants. CTX-M enzymes are able to inherently hydrolyse the CTX and FEP, and many variants, particularly CTX-M-15 have good activity against CAZ (Bauernfeind, Schweighart and Grimm, 1990). ESBLs are often mobilised and therefore the current, and potential, spread of resistance through this mechanism is rapid and worrying. Class B MBLs are zinc-dependent metalloenzymes capable of hydrolysing a variety of β-lactams, including carbapenems, but not monobactams. This carbapenem resistance, their increasing global prevalence, and the fact that MBLs are resistant to all β-lactamase inhibitors used in clinic make them an increasing global threat (Mojica *et al.*, 2022)**.** Class C enzymes are the second most abundant class of β-lactamases after those from class A, mainly found in Gram-negative species (Tooke *et al.*, 2019). These are usually chromosomally encoded, inducible enzymes which are common within *Enterobacterales* family, namely *Enterobacter* spp. and *Citrobacter* spp, but also in *Pseudomonas* spp. The inducibility of their production in the presence of β-lactams gives the host intrinsic resistance to penicillins and early generation cephalosporins, giving these enzymes the alternative name of cephalosporinases (Jacoby, 2009). Mutations within genes encoding class C β-lactamase enzymes or their regulatory genes can result in resistance arising to a broader range of β-lactams, discussed in section 1.5. Class D β-lactamases, also termed oxacillinases (OXA) were named due to their ability to hydrolyse oxacillin, a penicillin stable against class-A enzymes (Ouellette, Bissonnette and Roy,

1987). OXA-1 is the most common mobile class D enzyme, and there is a worrying rise of OXA enzymes that have evolved carbapenem hydrolysing activity, of which the OXA-48-like enzymes (Antunes *et al.*, 2014) are the most common OXA carbapenemases in the UK (UK Health Security Agency, 2023). Many OXA enzymes also give resistance to FEP, but generally they are very poor cephalosporinases (Torres *et al.*, 2016). Their lack of sensitivity to β-lactamase inhibitors coupled with their strong penicillinase activity make them important mechanisms of resistance to the β-lactam/β-lactamase inhibitor combination piperacillin/tazobactam (TZP) (Livermore *et al.*, 2019; Dulyayangkul *et al.*, 2023). These substrates are all critically important antibiotics and therefore make OXA enzymes a significant threat.

β-lactamase inhibitors are molecules which preferentially bind with the β-lactamase active site, preventing the enzyme from hydrolysing the β-lactam antibiotic and allowing it to remain effective. The first β-lactamase inhibitor was clavulanic acid, a suicide inhibitor of class A β-lactamases used in combination with AMX (Reading and Cole, 1977). Since the discovery of clavulanic acid, multiple β-lactamase inhibitors have been developed although amoxicillin-clavulanate (AMC) remains the most prescribed drug/inhibitor combination in England (UK Health Security Agency, 2023). This is followed by TZP which is now the antibiotic of choice for BSI (Walker *et al.*, 2022) however a rise in emerging resistance has accompanied this increase in use (UK Health Security Agency, 2023). The development of avibactam, a non- β-lactam β-lactamase inhibitor, is used in combination with CAZ and exhibits activity against class-A penicillinases and ESBLs, class-C cephalosporinases and some class-D oxacillinases (Ehmann *et al.*, 2012, 2013).

1.4 *Enterobacter* and *Citrobacter*

Enterobacter spp. and *Citrobacter* spp. are commensal Gram-negative facultative anaerobes belonging to the *Enterobacterales* family. *Citrobacter* spp. are less common than *Enterobacter* spp. and so do not pose as much of a burden on human healthcare (figure 6) (UK Health Security Agency, 2023) however they are both a common cause of nosocomial BSIs, UTIs and respiratory tract infections and exhibit similar biochemical properties, mechanisms of

antibiotic resistance and resistance trends (Gajdács and Urbán, 2019). As a result, they are often discussed together, along with *Serratia* spp. The opportunistic nature of these pathogens means they are often associated with complicated infections such as those in immunocompromised patients, or catheter associated UTIs, and outbreaks have been reported in neonatal and adult intensive care units (Gajdács and Urbán, 2019; Ferry *et al.*, 2020). Since 2012, the incidence rates of *Enterobacter* spp. and *Citrobacter* spp. BSI in England has increased from 2.64% to 4.46% and 1.24% to 2.41% per 100,000 population respectively (Figure 6) (UK Health Security Agency, 2023).

The proportion of these BSIs which are CAZ-resistant (CAZ-R) has reduced slightly over the past decade (Figure 6). This is likely due to a greater appreciation of intrinsic CAZ resistance in AmpC producing species and hence a reduction in its use as a treatment. In contrast to this, incidences of TZP resistance have been increasing since 2017, likely due to the increase in prescribing of TZP (Figure 6) (UK Health Security Agency, 2023). This is also apparent in other high-priority Gram-negative species such as *E. coli* and *K. pneumoniae* (UK Health Security Agency, 2023). This trend is worrying as TZP-resistance will result in the increased use of last-resort carbapenems.

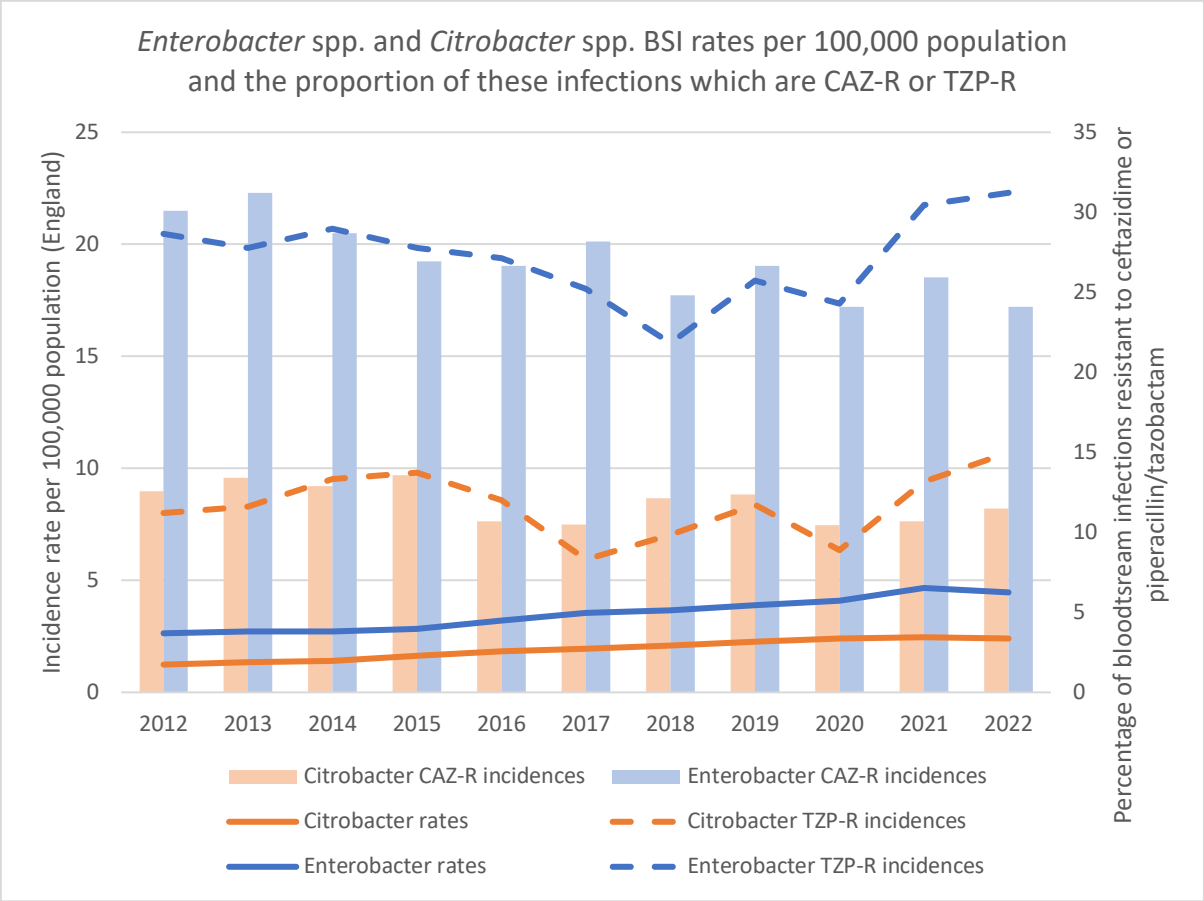

Figure 6 The frequency of infections caused by *Enterobacter* and *Citrobacter* spp in England is increasing. Incidence rates (line graph) of *Enterobacter* spp. (blue) and *Citrobacter* spp. (orange) infections per 100,000 population within England (obtained from ESPAUR) show that the number of infections caused by these species has increased from 2012-2022. The percentage of these infections which are CAZ-R (bar graph) is relatively stable but the percentage of TZP resistant (TZP-R) is rising (dashed line graph).

1.5 AmpC mediated antibiotic resistance in *Enterobacter* spp and *Citrobacter* spp.

Enterobacter spp. and some *Citrobacter* spp. have a chromosomal β-lactamase gene, *ampC,* which encodes class C β-lactamase enzymes, termed AmpC (Jacoby, 2009). The *ampC* β-lactamase gene is transcriptionally upregulated (induced) in the presence of many β-lactam antibiotics. Induction occurs through the AmpR transcriptional regulator and results in increased levels of AmpC in the cell, conferring intrinsic resistance to those β-lactams that induce its production. As is characteristic for Class C β-lactamases, AmpC enzymes always confer resistance to AMX and AMC, but only give resistance to 3GC when AmpC becomes hyper-produced, as discussed below.

β-Lactams induce the transcription of *ampC* through a regulatory pathway linked to peptidoglycan recycling (Jacobs *et al.*, 1994) (Figure 7). In a wild-type cell, peptidoglycan in the cell wall is constantly broken-down and re-synthesised to accommodate cell growth. The peptidoglycan molecules are broken down into 1,6-anyhdro-muropeptides which are transported into the cell by the inner membrane permease, AmpG (Dietz and Wiedemann, 1996). Here, the cytoplasmic amidase, AmpD, cleaves the muropeptide amide bond, and the products are then further broken down and resynthesized to produce UDP-MurNac-pentapeptides. These pentapeptides are re-incorporated into the peptidoglycan synthesis pathway (Tipper and Strominger, 1965) but also act as the repressor substrate of the AmpR transcriptional regulator, resulting in repression of *ampC* transcription (Lindberg, Westman and Normark, 1985).

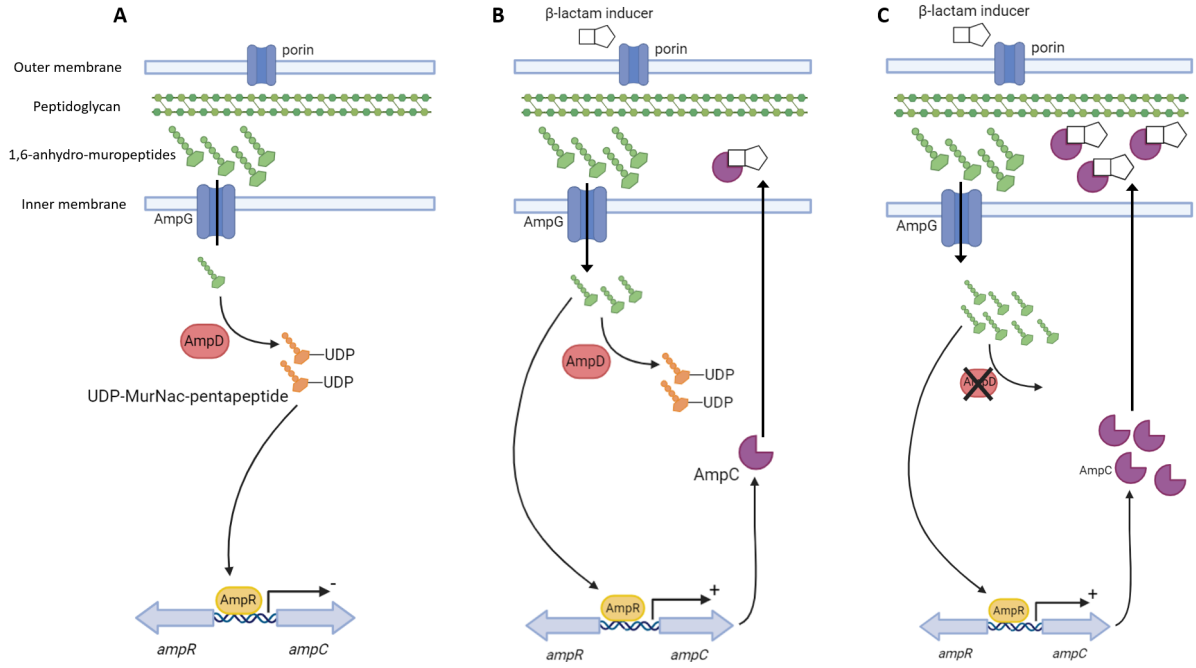

Figure 7 Transcription of *ampC* encoding the AmpC β-lactamase enzyme is inducible by β-lactam antibiotics and can be disrupted by mutations within regulatory genes, particularly *ampD*. Schematic of the peptidoglycan pathway linked to *ampC* transcription. (A) Wild-type (absence of β-lactams) conditions in which the peptidoglycan cell wall is broken down into 1,6-anhydro-muropeptides that enter the cytoplasm through membrane permease AmpG. AmpD breaks down these muropeptides into the repressor substrate of AmpR, UDP-MurNac-pentapeptides, repressing the transcription of *ampC*. (B) In the presence of β-lactams, peptidoglycan breakdown increases, increasing the concentration of 1,6-anhydro-muropeptides. These act as the activatory substrate for AmpR, upregulating transcription of *ampC*. (C) The presence of β-lactams and an inactivated AmpD leads to increased 1,6-anhydro-muropeptide concentration leading to constitutive expression of *ampC* and a high concentration of AmpC β-lactamase able to hydrolyse β-lactams.

β-Lactams affect the peptidoglycan recycling pathway in two ways: they induce peptidoglycan breakdown and inhibit its synthesis. This causes saturation of the AmpD enzyme which cannot break down 1,6-anyhdro-muropeptides at a fast enough rate, causing them to build up inside the cell. These muropeptides act as the activatory ligand of AmpR and preferentially bind over

the UDP-pentapeptide repressor substrate (Dietz, Pfeifle and Wiedemann, 1997). Hence, when muropeptide concentration increases, they cause an AmpR conformational change triggering upregulation of *ampC* transcription and increased AmpC production (Figure 7) (Lindquist, Lindberg and Normark, 1989). Different β-lactams affect *ampC* expression to different extents, depending on their inducing ability. For example, penicillins, AMC and early generation cephalosporins are strong *ampC* inducers (Jacoby, 2009). They cause a greater increase of anhydro-muropeptide concentration, particularly of the most activatory anhydro-muro-pentapeptides resulting in high levels of AmpC which will confer resistance to these drugs. On the other hand, weak inducers such as CAZ and other 3GCs result in AmpC levels low enough for the drug to remain effective (Jacoby, 2009). This can be overcome by mutations that cause *ampC* to be constitutively overexpressed.

Overexpression of *ampC* occurs due to mutations in genes involved in this peptidoglycan recycling pathway. The most frequent cause of this is *ampD* loss of function mutations (Schmidtke and Hanson, 2006). AmpD loss of function means the 1,6-anhydro-muropeptides are not broken down (Jacobs *et al.*, 1995). This permanent increase in anhydro-muropeptide concentration means AmpR is in a constant activatory state, resulting in constitutive expression of *ampC* (Lindberg, Lindquist and Normark, 1987). This occurs regardless of the inducing strength of the β-lactam, meaning resistance occurs even to weak inducers like CAZ. Activatory mutations in AmpR which cause it to lose its repression can also cause constitutive *ampC* expression (Lindberg, Westman and Normark, 1985). Although less common, mutations in other regulatory genes encoding enzymes involved in the peptidoglycan pathway have been implicated in *ampC* overexpression. These include AmpE which senses β-lactams (Honoré, Nicolas and Cole, 1989; Juan *et al.*, 2005), Mpl involved in production of muropeptides (Calvopiña and Avison, 2018) and DacD, a DD-carboxypeptidase PBP whose loss increases the number of anhydro-muropeptides with pentapeptide side chains (Sarkar *et al.*, 2011).

1.6 Aims and Objectives

Since the characterisation of the *ampC* induction system in the 1980s and 1990s, there have been few studies investigating mechanisms of AmpC hyper-production among clinical isolates. The work that has occurred has primarily focused on *Enterobacter cloacae, Enterobacter*

hormaechei and *Citrobacter freundii* and no studies have compared CAZ-R strains isolated from the time of the introduction of CAZ into clinical practice (1980s) with isolates from the present day. Given our rare position of having access to bacterial strains isolated from the 1980s and 90s, and also more recently in 2020, all from patients in Bristol, our aim was to investigate differences and similarities between these sets of isolates. This study demonstrates three main findings: (i) CAZ-R in *Enterobacter* spp. and *Citrobacter* spp. is sporadic and does not cluster within certain species, (ii) there is no striking difference between genetic causes of *ampC* overexpression over the 40 years, but there is more horizontally acquired resistance in the 2020 isolates, and more TZP resistance, a combination not used at the time of the isolation of the 1980s/90s isolates but one of the most common antibiotics in use today and (iii) we characterise three novel AmpD substitutions causing this *ampC* overexpression.

2. Materials and methods

2.1. Bacterial isolates

A total of 99 non-duplicated (by patient) clinical isolates were used in this study; 80 bloodstream isolates collected during 2020 from samples referred to the Severn Pathology laboratory from hospitals in Bristol and Bath and 19 isolates collected from 1983-1990 from patients being treated at the former Bristol Childrens' Hospital. All isolates were grown on cation adjusted Mueller-Hinton (MH) agar and incubated at 37 °C unless stated otherwise.

2.2. Antibiotic susceptibility testing

Two methods were used to obtain antibiotic susceptibility data for all isolates for the following antibiotics: β-lactams (AMX; CAZ; CTX; FEP; meropenem, aztreonam), β-lactam/inhibitor combinations (AMC; TZP), aminoglycosides (gentamicin, tobramycin, amikacin), ciprofloxacin and cotrimoxazole. The Severn Pathology laboratory at the Bristol Royal Infirmary provided antibiograms for the clinical isolates from 2020 as determined using the Vitek system. In addition, disc diffusion testing (performed myself), in accordance with EUCAST guidelines (http://eucast.org) was used to obtain susceptibility data of the isolates from the 1980s. Disc diffusion testing was also used to confirm the phenotypes of isolates from 2020 determined as CAZ-R by Severn Pathology, and to test for any antibiotics where there were missing data points.

2.3. Whole genome sequencing and genomic analysis

Whole genome sequencing (WGS) was performed by MicrobesNG (https://www.microbesng.com/) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2x250 bp paired-end reads. Reads were assembled into contigs using SPAdes (http://cab.spbu.ru/software/spades/) (Bolger, Lohse and Usadel, 2014) and contigs were annotated using Prokka (https://vicbioinformatics.com/software.prokka.shtml) (Seemann, 2014). ABR genes were identified using ResFinder 4.1 (Bortolaia *et al.*, 2020), running the programme with 'other' as a species parameter and using 90% similarity threshold for ABR genes except for when there were none identified, in which case the threshold was lowered

to 80%. Amino acid sequences were aligned against sequences from the reference genome of the relevant species (Table 1) using the CLUSTAL Omega service from the European Bioinformatics Institute (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) (Madeira *et al.*, 2022) Sequence alignments were visualised with Jalview (Waterhouse *et al.*, 2009). Insertion sequences were identified using IS Finder (Siguier, 2006).

2.4. Phylogenetic analysis

Phylogenetic analysis was carried out using the computational facilities of the Advanced Computing Research Centre, University of Bristol *(*http://www.bristol.ac.uk/acrc/*).* Genome sequence alignment against reference sequences (Table 1) was performed using Snippy and Snippy-core software (https://github.com/tseemann/snippy) and single nucleotide polymorphism (SNP) distances were calculated using SNP-dists software (https://github.com/tseemann/snp-dists). Maximum likelihood phylogenetic trees were produced using the RAxML program (Stamatakis, 2014) with the GTRGAMMA model rate of heterogeneity. Phylogenetic trees were visualised and illustrated using Microreact (https://microreact.org/) (Argimón *et al.*, 2016).

Species	Strain	GenBank assembly number
E. cloacae	ATCC 13047	GCA_000025565
E. hormaechei	FDAARGOS 1433	CP077308
E. xiangfangensis	LMG 27195	CP017183
E. hormaechei subsp. hoffmannii	DSM 14563	CP017186
E. hormaechei subsp. steigerwaltii	DSM 16991	NZ_CP017179
E. hormaechei subsp. oharae	DSM 16687	CP017180
E. roggenkampii	DSM 16690	CP017184
E. bugandensis	DSM 29888	CP077206
E. kobei	DSM 13645	GCA_001729765
E. ludwigii	EN 119	GCA_001750725
C. freundii	ATCC 8090	CP049015
C. youngae	ATCC 29220	NZ_LR134485
C. murliniae	ATCC 51118	GCA_004801125
C. koseri	ATCC 27028	NC_009792
C. amalonaticus	ATCC 25405	NZ_CP014070
C. werkmanii	ATCC 51114	NZ_CP044101
C. pasteurii	FDAARGOS 1424	CP077262
C. portucalensis	DSM 104542	CP044098

Table 1 Type strain and GenBank assembly numbers of reference genomes used for phylogenetic and genomic analysis.

2.5. Amino acid mutability analysis

The substitution rates of amino acid residues within AmpD sequences were determined using the blastp database (Altschul *et al.,* 1990). AmpD query sequences were submitted under search parameters defining the relevant organism (Table 2) with maximum target sequences set at 5000. Only results with a coverage over 99% were used.

Species	AmpD accession number
C. freundii	QIH67718
E. hormaechei	QXB33216.1
K. pneumoniae	WP_002888692
S. enterica	AAL19110.1
M. morgannii	QCY20502
E. coli	AIL17344
S. marcescens	QDL84602

Table 2 Accession numbers from the NCBI database of the AmpD proteins used for genomic analysis from the given species. Species with inducible *ampC* are *C. freundii, E. hormaechei, S. marcescens and M. morgannii.* Species with non-inducible *ampC* are *K. pneumoniae, S. enterica and E. coli.*

2.6. Proteomics analysis

Bacterial strains were grown overnight on MH agar (susceptible control isolates) or CAZ-infused MH agar (4 mg/L) for CAZ-R strains (E1, E38, E33, E50, E54). A single colony was subcultured overnight in 5 ml Ca^{2+} adjusted MH broth. For each strain, this was repeated three times to obtain biological replicates. OD_{600} of overnight broth culture was measured. Culture was added to 50 ml Ca^{2+} adjusted MH broth to reach a starting OD_{600} of 0.1 and further incubated until an OD_{600} of 0.8 ± 0.2 was reached (to ensure strains are in logarithmic phase). Cells were pelleted by centrifugation (4°C, 4000 x *g*, 10 min), resuspended in 25 ml Tris-HCl (30 mM, pH 8.0) and lysed, on ice, by sonication (63%, pulsing one second on-one second off for three minutes). The lysis product was centrifuged (4°C, 7600 x *g,* 15 min; Sorval RC5B PLUS using an SS-34 rotor) and the supernatant discarded. Protein concentration was measured using 10 μl of supernatant in a working concentration Bradford assay at OD_{595}. Protein (3 μg) was separated by SDS-PAGE gels (Table 3) which were run at 180 V until the dye front had travelled 1 cm into the separating gel. This separated out any impurities from the crude protein extract. The gels were stained, whilst shaking, using Quick Coomassie stain (Protein Ark) for 20 min, and de-stained in deionised water.

	Volume	
	Separating gel	**Stacking gel**
30% acrylamide/bis solution 37.5:1	5 ml	3 ml
15% SEPARATING GEL	3 ml	-
1% STACKING GEL	-	2.5 ml
Deionised water	2.16 ml	4.5 ml
TEMED	10 µl	10 µl
Ammonium persulfate (APS)	100 µl	100 µl

Table 3 Contents of separating and stacking gels used to make SDS page gels for proteomics.

For each sample, the 1 cm of gel containing protein was sent for LC-MS/MS analysis at Bristol Proteomics Facility, as previously described (Dulyayangkul *et al.*, 2020). The raw data files were processed and quantified as previously described (Dulyayangkul *et al.*, 2020) and searched against the UniProt database for the relevant reference strain (Table 1). Proteomics analysis was repeated in triplicate for each strain, from a separate batch of cells. Protein abundance was normalised based on the average abundance of the 50S and 30S ribosomal proteins in each sample. Comparisons of normalised protein abundance were compared between samples using an unpaired t-test, and significance was defined with $p < 0.05$. Fold change in abundance between samples was calculated by finding the ratio between the average normalised abundance value of each sample.

3. Results Chapter 1: Characterisation of clinical bloodstream *Enterobacter* and *Citrobacter* spp. isolates from 2020

3.1 Whole genome sequencing

Whole genome sequencing (WGS) of 80 deduplicated clinical bloodstream isolates from 2020, 55 *Enterobacter* spp. and 25 *Citrobacter* spp, was conducted. ResFinder (Zankari *et al.*, 2012) was used to identify antibiotic resistance (ABR) genes present in the genomes of all clinical isolates. ResFinder identifies acquired (mobile) ABR genes and their homologues. Some mobile β-lactamase genes in the ResFinder database have been mobilised from the chromosomes of *Enterobacter* or *Citrobacter* or related genera. Therefore, the β-lactamase gene variant that ResFinder identifies as a match to the chromosomal β-lactamase gene present in the sequenced bloodstream isolates is the closest mobile variant found in its database.

In all 55 *Enterobacter* spp. isolates, chromosomal *ampC* β-lactamase genes were identified. These genes were most similar to the following mobile variants: bla_{MIR} in *E. roggenkampii* and bla_{ACT} in all other species (Table 4). Within the *Citrobacter* spp. isolates, 12 isolates were *C. koseri* and one isolate was *C. amalonaticus.* These species contain class-A β-lactamase genes identified by ResFinder as bla_{MAL}, bla_{SED} and bla_{CKO} (Table 4). bla_{MAL} and bla_{CKO} are non-inducible genes whereas bla_{SED} is inducible (Petrella *et al.*, 2001). The remaining 12 *Citrobacter* spp. isolates were species containing inducible *ampC* genes*: C. freundii; C. pasteurii; C. braakii; C. werkmanii* and *C. portucalensis.* ResFinder identified the β-lactamase genes in all isolates from these species as being most similar to the bla_{CMY} variant, originally isolated from *C. freundii* (Table 4).

Mobile ABR genes were found in only four of 80 isolates; two *E. hormaechei* and two *C. freundii* isolates. Five mobile β-lactamase genes and genes associated with resistance to non-β-lactam antibiotics were identified and are discussed in section 3.2.2.

Mobile β-lactamase gene families	β-lactamase class of chromosomal homologue	*ampR* present	Genus	Species
ACT	C	Yes	*Enterobacter*	*hormaechei* *kobei* *ludwigii* *bugandensis*
MIR	C	Yes	*Enterobacter*	*roggenkampii*
CMY	C	Yes	*Citrobacter*	*freundii* *pasteurii* *werkmanii* *portucalensis* *braakii*
MAL	A	No	*Citrobacter*	*koseri*
CKO	A	No	*Citrobacter*	*koseri*
SED	A	No	*Citrobacter*	*amalonaticus*

Table 4 A variety of *Enterobacter* and *Citrobacter* spp. contain inducible class C β-lactamase genes, the mobile homologues of which are identified by ResFinder. The mobile β-lactamase gene families identified in different species of clinical isolates and the class of β-lactamase enzyme that the chromosomal homologue belongs to are shown. The presence of *ampR* indicates that the chromosomal *ampC* is inducible.

3.2 ABR phenotype

A combination of hospital VITEK data (provided by Severn Pathology) and antibiotic disc testing (performed myself) were used to define the antibiotic susceptibility profiles of the clinical isolates. Disc testing was performed when there was no result from the VITEK, and to confirm the phenotypes of ceftazidime (CAZ), cefepime (FEP) and piperacillin/tazobactam (TZP) for CAZ-resistant (CAZ-R) isolates as defined by Vitek. On five occasions, there were discrepancies between the VITEK data and disc testing results. All five were when testing against FEP; three isolates were reported from VITEK as intermediate when they were resistant by disc tests. Two isolates were reported susceptible from VITEK when they were intermediate by disc tests. On these occasions, the disc testing result was taken over the VITEK results, because this would allow more equal comparison with isolates discussed later in the

thesis, for which disc testing data were all that was available. Table 5 shows a breakdown of the percentage (and number) of isolates from each genus exhibiting resistance to the different antibiotics tested.

	***Enterobacter* spp.**	***Citrobacter* spp.**		**Total inducible *ampC***	**Total in all isolates**
		***ampC* species**	**non-*ampC* species**		
Amoxicillin (AMX)	100% (55)	100% (12)	100% (13)	100% (67)	100% (80)
Amoxicillin/ clavulanate (AMC)	100% (55)	100% (12)	0% (0)	100% (67)	84% (67)
Ceftazidime (CAZ)	20% (11)	8% (1)	0% (0)	18% (12)	15% (12)
Cefotaxime (CTX)	20% (11)	8% (1)	0% (0)	18% (12)	15% (12)
Cefepime (FEP)	15% (8)	8% (1)	0% (0)	13% (9)	11% (9)
Piperacillin/ tazobactam (TZP)	20% (11)	17% (2)	0% (0)	19% (13)	16% (13)
Aztreonam	20% (11)	8% (1)	0% (0)	18% (12)	15% (12)
Meropenem	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
Ciprofloxacin (CIP)	2% (1)	17% (2)	0% (0)	4% (3)	4% (3)
Cotrimoxazole	4% (2)	17% (2)	0% (0)	6% (4)	5% (4)
Gentamicin	4% (2)	17% (2)	0% (0)	6% (4)	5% (4)
Amikacin	2% (1)	8% (1)	0% (0)	3% (2)	3% (2)
Tobramycin	4% (2)	8% (1)	0% (0)	4% (3)	4% (3)

Table 5 Antibiotic susceptibility data from clinical isolates shows highest levels of resistance to β-lactam antibiotics, and resistance to 3GC solely in species with inducible ampC. The percentage (and number) of isolates from each genus that show resistance to the listed antibiotics. All *Enterobacter* spp. isolates contain inducible ampC. The *Citrobacter* spp. are broken down into species containing inducible ampC (with the presence of ampR) and species containing a different class of β-lactamase enzyme. (non-ampC species).

3.2.1 β-lactam resistance

The antibiotics to which there is the highest level of resistance across all isolates is amoxicillin (AMX) (100%) and amoxicillin/clavulanate (AMC) (84%). The only isolates susceptible to AMC were those that do not contain *ampC* β-lactamase genes (Table 5). Resistance to the third-generation cephalosporins (3GCs) CAZ and cefotaxime (CTX) only occurred in *ampC* positive isolates and was more frequent in *ampC* positive *Enterobacter* spp. (20%) than *Citrobacter* spp. (8%).

Nine isolates (75%) were resistant to FEP. One of these was a *C. freundii* (*ampC* positive) isolate, and the other eight were *Enterobacter* spp. (Table 5). Resistance to TZP occurred in 16.25% (13) of isolates. 12 of these 13 isolates were CAZ-R, and the outstanding isolate contained an OXA β-lactamase (see below) which is capable of hydrolysing TZP (Dulyayangkul *et al.*, 2023)**.** No isolates were resistant to the carbapenem meropenem.

3.2.2 Mobile ABR genes

ResFinder identified five mobile β-lactamase genes: $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1A}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{OXA(uncharacterised)}}$. Both $bla_{\text{CTX-M-15}}$ and bla_{OXA} genes are associated with CAZ/FEP and FEP/TZP resistance respectively (Hirvonen, Spencer and Van Der Kamp, 2021). Table 6 shows the four isolates containing mobile β-lactamase genes and their resistance phenotypes to CAZ, FEP and TZP. The presence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ in isolates E19 and E23 accounts for their resistance to CAZ, FEP and TZP. Isolate C21 is susceptible to FEP despite the presence of the bla_{OXA-1} gene. Studies in *E. coli* have shown that bla_{OXA-1} expression levels can vary (Dulyayangkul *et al.*, 2023)**.** Therefore, whilst OXA-1 has been associated with FEP and TZP resistance, it is possible that it is not produced at a high enough level in isolate C21 to confer FEP resistance, though it is TZP-R. A lack of $bla_{CTX-M-15}$ in isolate C21 explains its CAZ susceptibility. We would also expect to see CAZ susceptibility in isolate C17 due to the lack of CTX-M-15 however this is not the case. The mechanism of CAZ-resistance is unclear however we noted the presence of a previously uncharacterised OXA enzyme (Table 6). Whilst OXA enzymes do not normally give CAZ-resistance, it is possible that this one does, as well as TZP-R and FEP-R.

Antibiotic	Mobile ABR gene	Gene presence (phenotype) in isolates			
		C17	C21	E19	E23
Ceftazidime (CAZ)	*bla*$_{CTX\text{-}M\text{-}15}$	N (**R**)	N (S)	Y (R)	Y (R)
Cefepime	*bla*$_{OXA\text{-}1}$	-	Y (**S**)	Y (R)	Y (R)
(FEP)	*bla*$_{OXA\ (uncharacterised)}$	Y (R)	-	-	-
Piperacillin/	*bla*$_{OXA\text{-}1}$	-	Y (R)	Y (R)	Y (R)
tazobactam (TZP)	*bla*$_{OXA\ (uncharacterised)}$	Y (R)	-	-	-

Table 6 The presence of mobile β-lactamase genes results in resistance to a broader spectrum of β-lactam antibiotics. Two isolates have unexpected phenotypes given the genes present. Four isolates contain mobile β-lactamase genes; each mobile gene is listed with the antibiotic it can confer resistance to. Isolate C17 is CAZ-R but lacks *bla*$_{CTX\text{-}M\text{-}15}$ which would ordinarily results in CAZ-susceptibility. Isolate C21 is FEP-S despite the presence of *bla*$_{OXA\text{-}1}$ which often results in FEP-resistance. (N = absence of gene, Y = presence of gene and "-" indicates that particular gene is not present. Each mobile gene is listed with the antibiotic to which it can confer resistance. The phenotype of each isolate to these antibiotics are in brackets, with R being resistant and S being susceptible).

Aminoglycoside resistance genes (and their substrate) identified by ResFinder in the four isolates that carry mobile ABR genes are as follows: *aph(6)-Id*, *aph(3")-Ib and aph(3')-Ib* (streptomycin), *aadA1* and *aadA2* (spectinomycin, streptomycin), *aac(3)-Ia* (gentamicin), *aac(3)-IIa* (gentamicin, tobramycin). The resistance phenotypes for these antibiotics were concordant with the presence of the relevant genes (Table 7). The *aac(6')-Ib-cr* gene, which is carried with OXA-1 (Mounsey *et al.*, 2023) confers resistance to tobramycin and is associated with amikacin resistance when expressed at high levels (Dulyayangkul *et al.*, 2023)**.** Therefore, it is likely that isolates C21 and E23, which are amikacin resistant, have high levels of *aac(6')-Ib-cr* expression compared to isolate E19 which is susceptible.

Antibiotic	Mobile ABR gene	Gene presence (phenotype)			
		C17	C21	E19	E23
Streptomycin	*aph(6)-Id*	Y (R)	N (R)	Y (R)	Y (R)
	aph(3'')-Ib	Y (R)	N (R)	Y (R)	Y (R)
	aph(3')-Ib	Y (R)	N (R)	-	-
	aadA1	Y (R)	Y (R)	Y (R)	Y (R)
	aadA2	-	Y (R)	-	-
Spectinomycin	*aadA1*	Y (R)	Y (R)	Y (R)	Y (R)
	aadA2	-	Y (R)	-	-
Gentamicin	*aac(3)-Ia* or *aac(3)-IIa*	Y (R)	Y (R)	Y (R)	Y (R)
Tobramycin	*aac(3)-IIa* only	N (S)	Y (R)	Y (R)	Y (R)
	aac(6')-Ib-cr	N (S)	Y (R)	Y (R)	Y (R)
Amikacin	*aac(6')-Ib-cr*	N (S)	Y (R)	Y (S)	Y (R)
Ciprofloxacin (CIP)	*aac(6')-Ib-cr*	N (R)	Y (R)	Y (R)	Y (S)
	qnrA1	Y (R)	-	-	N (S)
	qnrB1	-	Y (R)	Y (R)	N (S)
	qnrS1	-	-	Y (R)	N (S)

Table 7 The presence of mobile aminoglycoside and ciprofloxacin resistance genes results in resistance to a broad range of non β-lactam antibiotics. Four isolates contain mobile β-lactamase genes; each mobile gene is listed with the antibiotic to which it can confer resistance. There is no unexpected resistance or susceptibility in any isolates. (N = absence of gene, Y = presence of gene and "-" indicates that particular gene is not present. The phenotype of each isolate to these antibiotics are in brackets, with R being resistant and S being susceptible).

Ciprofloxacin (CIP) resistance has been shown to be multifactorial with contributing factors including increased efflux, reduced drug entry, the presence of plasmid mediate quinolone resistance (PMQR) genes *(aac-6'-Ib-cr; qnr*) and mutations in genes encoding target enzymes, *gyrA* and *gyrB* (DNA gyrase) and *parC* (topoisomerase) (Wan Nur Ismah *et al.*, 2018). Table 7 summarises the presence of the PMQR genes *aac(6')-Ib-cr* and *qnr* variants in the four CIP-resistant isolates as identified by ResFinder. The CIP resistance in isolate C17 is explained by the presence of *qnrA1* and mutations in *gyrA* and *parC* leading to amino acid substitutions (GyrA T83I, ParC S80I) which have previously been shown to be associated with CIP resistance (Yoshida *et al.*, 1990; Everett *et al.*, 1996; Deguchi *et al.*, 1997). CIP resistance in isolate C21 is likely due to the high expression of *aac(6')-Ib-cr* indicated by amikacin resistance, in

combination with the presence of *qnrB1* (Table 7). Isolates E19 and E23 are good examples of CIP resistance being multifactorial. Amikacin susceptibility in isolate E19 suggests that expression of *aac(6')-Ib-cr* is low, however the presence of *qnrB1* and *qnrS1* in addition to this gene confer CIP resistance. Isolate E23 is resistant to amikacin, suggesting high levels of *aac(6')-Ib-cr* expression, however there are no other PMQR genes present, likely resulting in the observed CIP susceptibility.

ResFinder also identified the following mobile ABR genes: *sul1* and *sul2* (sulfamethoxazole); *dfrA*12, *dfrA14* and *dfrA19* (trimethoprim); *tetA* (tetracycline) and *catA1* and *catB3* (chloramphenicol). Resistance to these antibiotics was concordant with the presence of the relevant genes and there was no resistance to any other antibiotics which is unexplained.

3.3 Phylogeny

3.3.1 Species identification

Along with antibiograms for the clinical isolates, the Severn Pathology diagnostic lab provided predictions of species causing these infections based on MALDI-TOF spectrometry. This data was then compared to predictions based on WGS data (Table 8). MALDI-TOF is a common tool used by clinical laboratories to identify species causing infection however it is has been shown to be unreliable for precise identification of *Enterobacter* spp. (Sutton *et al.*, 2018; Wu, Feng and Zong, 2020). This was apparent in this dataset: MALDI-TOF predicted that 87% (48) of *Enterobacter* spp. isolates were of the species *E. cloacae* (Table 8). However, WGS revealed that none of these isolates were *E. cloacae* and many were instead more closely related to the reference genome of *E. hormaechei.* Furthermore, maximum likelihood phylogenetic trees made using WGS data determined there to be two distinct groups of isolates clustering with the reference genomes of two subspecies of *E. hormaechei: E. hormaechei* subsp*. hoffmannii* and subsp*. steigerwaltii* (Figure 14). Similar results have been shown in previous studies, whereby only 3% of isolates originally identified as *E. cloacae* were later described as *E. cloacae* through molecular tests (Hoffmann and Roggenkamp, 2003).

Overall, there were 43 clinical isolates belonging to *E. hormaechei* and *E. hormaechei* subspecies. The other 12 *Enterobacter* spp. isolates were also misidentified by MALDI-TOF but

WGS reveals they were a mixture of the following species: *E. kobei, E. ludwigii, E. roggenkampii* and *E. bugandensis* (Table 8).

Genus	Species	MALDI-TOF prediction	WGS final classification
Enterobacter	*cloacae*	87% (48)	0% (0)
	other	11% (6)	0% (0)
	kobei	2% (1)	7% (4)
	hormaechei	0% (0)	2% (1)
	ludwigii	0% (0)	7% (4)
	roggenkampii	0% (0)	5% (3)
	hormaechei subsp. *steigerwaltii*	0% (0)	36% (20)
	hormaechei subsp. *hoffmannii*	0% (0)	40% (22)
	bugandensis	0% (0)	2% (1)

Table 7 MALDI-TOF species prediction is less precise than using WGS. MALDI-TOF often incorrectly predicted organisms to be *E. cloacae.* The percentage (and number) of the 55 *Enterobacter* spp. clinical isolates, as identified initially by MALDI-TOF and the final species classification by WGS.

On the whole, identification of *Citrobacter* spp. by MALDI-TOF was more accurate than for *Enterobacter* spp. MALDI-TOF correctly identified all 12 *C. koseri* isolates and six of eight *C. freundii* isolates. Table 9 shows the 13 *Citrobacter* spp. isolates misidentified by MALDI-TOF and their correct species identification attributed by WGS. Hence, the most common species in this dataset was *C. koseri* (48%) followed by *C. freundii* (32%).

Isolate	MALDI-TOF prediction	WGS final classification
C13	*Escherichia coli*	*C. amalonaticus*
C14	*C. freundii*	*C. freundii*
C15	*C. freundii*	*C. freundii*
C16	*C. freundii*	*C. freundii*
C17	*C. freundii*	*C. freundii*
C18	*Enterobacter cloacae complex*	*C. freundii*
C19	*C. freundii*	*C. freundii*
C20	*C. freundii*	*C. freundii*

C21	*C. koseri*	*C. freundii*
C22	*C. freundii*	*C. braakii*
C23	*Citrobacter species*	*C. pasteurii*
C24	*Klebsiella oxytoca*	*C. werkmanii*
C25	*C. freundii*	*C. portucalensis*

Table 9 MALDI-TOF was more accurate in identifying *Citrobacter* spp. than *Enterobacter* spp. Of the 13 ampC containing *Citrobacter* spp. isolates (C13-C25), MALDI-TOF was most accurate in identifying *C. freundii* isolates.

3.3.2 Relationships between clinical isolates

From this point, we focussed on the 67 isolates carrying chromosomal *ampC* β-lactamase genes, since one of the primary clinically-important questions that arose from our initial analysis was to understand the mechanisms of CAZ resistance in the absence of mobile ABR genes, which was only seen in *ampC*-positive isolates (Table 5). Phylogenetic analysis was conducted to determine relationships between isolates, with the aim of addressing whether CAZ resistance was confined to a particular species or clade, or if resistance occurred sporadically. Species-specific maximum likelihood phylogenetic trees showed that there is no clustering of CAZ-R isolates separately from CAZ-S isolates. This rules out the possibility of CAZ resistance being attributable to only a particular subspecies of *Enterobacter* or *Citrobacter* spp., or that it is clonal and arose from a particular isolate. It was not possible to make phylogenetic trees for the following species due to having only one isolate of each species: *C. braakii, C. werkmanii, C. portucalensis, C. pasteurii, E. bugandensis.*

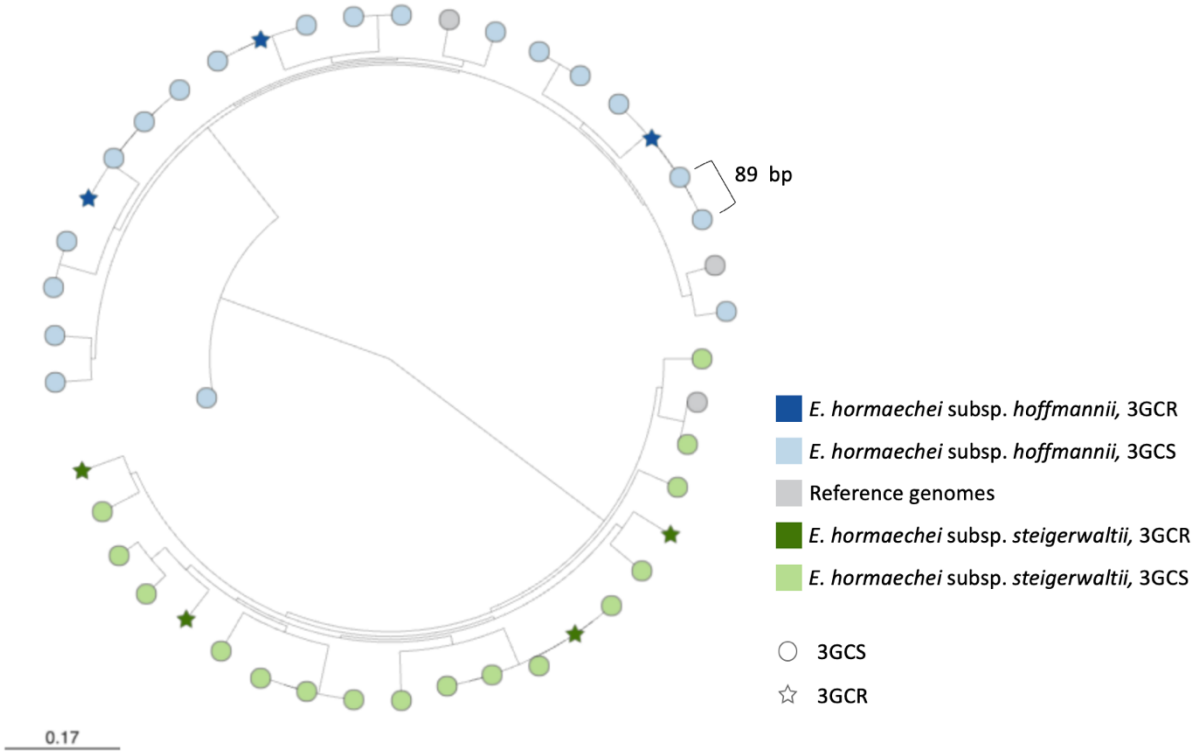

Figure 8 **WGS identified two subspecies of *E. hormaechei* present in the dataset, within which the emergence of CAZ-resistance is sporadic.** Core genome alignment of the 42 isolates belonging to the two subspecies of *E. hormaechei: E. hormaechei subsp. steigerwaltii* (green) and *E. hormaechei subsp. hoffmannii* (blue). (CAZ-R isolates are represented by a darker shade and a star. CAZ-S isolates are represented by a lighter shade and a circle). There is no clustering of CAZ-R isolates within a particular species

Phylogenetic analysis quantified the number of single nucleotide polymorphisms (SNPs) within the core genome of isolates. This can be used to determine if transmission events may have occurred. Table 10 shows the average number of SNPs between all isolates of a given species. The large number of SNPs within each species demonstrates the sporadic nature of the opportunistic *ampC* positive *Enterobacter* and *Citrobacter* spp. in contrast to clonal *E. coli* infections which generally see smaller SNP distances between sequence types. We therefore used an arbitrary cut off of 100 SNPs to determine isolates for which the relationship should be further investigated. This is well below the average for each species, indicating that at some point in the recent past, the two isolates have diverged from each other.

Species	Average number of SNPs between isolates	Smallest pairwise distance
C. freundii	34382	513
E. hormaechei* subsp. *hoffmannii	20511	54
E. hormaechei* subsp. *steigerwaltii	26081	67
E. kobei	14199	243
E. ludwigii	36799	34992
E. roggenkampii	48578	47213

Table 8 SNP distances show that there are some pairs of isolates more closely related than the average relationship between isolates. For some species *(C. freundii, E. hormaechei subsp. hoffmannii, E. hormaechei subsp. steigerwaltii and E. kobei*), the smallest pairwise SNP distances are much lower than the average number of SNPs between isolates, suggesting that pair is more closely related.

There were three pairs of isolates with <100 SNPs between them. In all three pairs, each isolate was collected on a different hospital ward. Two *E. hormaechei* subsp. *hoffmannii* isolates collected 10 months apart and had 67 SNPs difference (Figure 9). Two *E. hormaechei* subsp. *steigerwaltii* isolates were 92 SNPs different (collected one month apart) and another two differed by 54 SNPs (collected five months apart) (Figure 10). All these values are significantly lower than the average for the species (Table 10) and so, suggest that these pairs of isolates have at some point in the more recent past diverged from a single source. Isolate pair E3 and E5 are particularly interesting as they are only 54 SNPs different but have different ABR phenotypes; one was CAZ-R (E3) and the other was CAZ-S (E5).

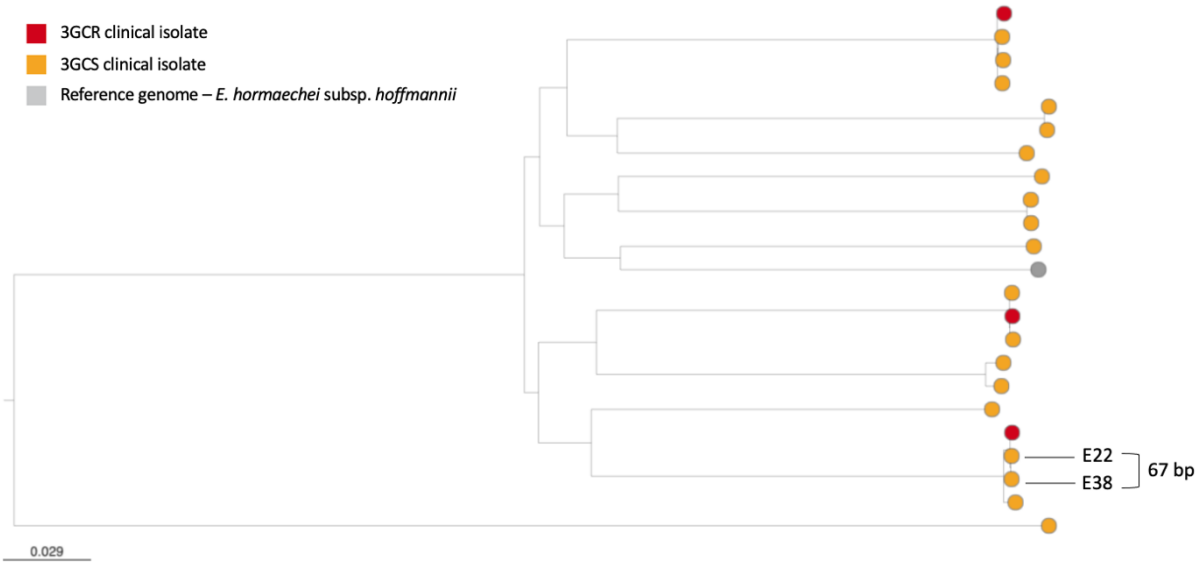

Figure 9 WGS and SNP dist analysis showed a pair of closely related *E. hormaechei* subsp. *hoffmannii* isolates and no clustering between CAZ-R and CAZ-S isolates. Core genome alignment shows that the CAZ-R (red) isolates do not cluster separately to the CAZ-S isolates (orange), highlighting the sporadic nature of *Enterobacter* spp. infections. One pair of isolates (E22/E38) has <100 SNPs different and so was investigated further.

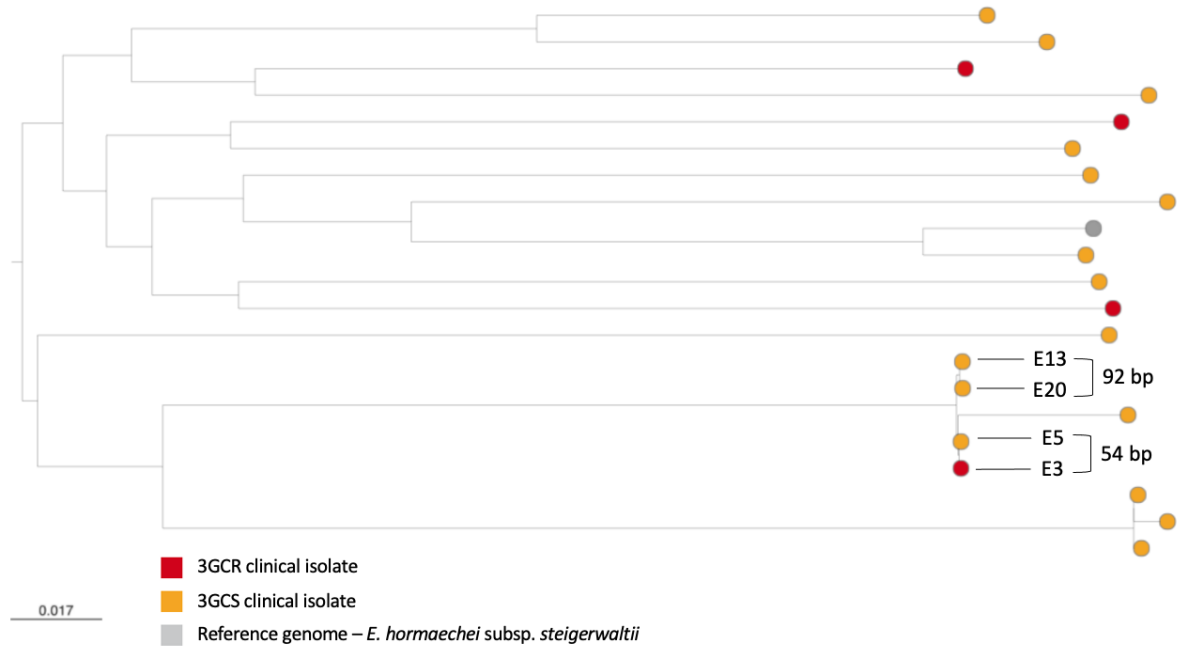

Figure 10 WGS and SNP dist analysis showed two pairs of closely related *E. hormaechei* subsp. *steigerwaltii* isolates and no clustering between CAZ-R and CAZ-S isolates. Core genome alignment shows that the CAZ-R (red) isolates do not cluster separately to the CAZ-S (orange) isolates, highlighting the sporadic nature of *Enterobacter* spp. infections. Two pairs of isolates (E13/E2) and E5/E3) have <100 SNPs different and so were investigated further.

3.4 CAZ-R isolates

Twelve of these clinical isolates collected in 2020 were CAZ-R. Three isolates contained mobile ABR genes accounting for this resistance (Table 6), leaving 9 isolates with an undetermined

mechanism for CAZ-resistance. We hypothesised that this was caused by hyper-production of chromosomally encoded AmpC. To test this hypothesis, we performed liquid chromatography-tandem mass spectrometry (LC/MS-MS) whole cell proteomics on 5 CAZ-R isolates, in comparison with 3 CAZ-S isolates from the same species as a control. The other CAZ-R isolates were not tested because there was no available CAZ-S comparator or because they contained mobile ABR genes conferring CAZ-resistance. All of the five CAZ-R/CAZ-S pairs showed greater AmpC production in the CAZ-R isolate (Table 12). Hence, we conclude that AmpC hyper-production is the predominant mechanism in the 12 CAZ-R isolates studied here. It was not possible to validate this statistically as the levels of AmpC in the CAZ-S control isolates were undetectable in the assay in all three replicates, as perhaps expected because under wild type (WT) conditions, AmpC is produced at very low levels.

FEP resistance is present in 9/12 CAZ-R isolates. In three of these isolates, resistance can be explained by the presence of a mobile β-lactamase gene (Table 6). The cause of resistance in the other six isolates is less clear. Correlating FEP disc diffusion zones against AmpC levels from proteomics show that greater levels of AmpC does not correlate to decreased FEP susceptibility (Table 11). However, FEP resistance must be at least in part attributable to AmpC hyper-production since there are no CAZ-S isolates exhibiting FEP resistance, and previous work shows that *ampC* constitutive expression increases FEP MIC (Kohlmann, Bähr and Gatermann, 2019).

Isolate	Mean AmpC level in isolate (relative to ribosomal proteins)	Mean AmpC level in CAZ-S control (relative to ribosomal proteins)	FEP disc diffusion zone (mm)	FEP phenotype
E3	3.40	0.00	21	resistant
E38	1.54	0.00	23	resistant
E33	6.16	0.00	32	susceptible
E50	16.46	0.00	24	intermediate
E54	9.01	0.06	25	intermediate

Table 9 Cefepime resistance does not correlate with the extent of AmpC production. LC-MS/MS proteomics was conducted on five CAZ-R *Enterobacter* spp. isolates. The mean level of AmpC production in the three replicates of each CAZ-R isolate is compared that in the CAZ-S control isolate of the same species, showing that AmpC is much more greatly produced in the CAZ-R isolates. The disc diffusion zone of cefepime does not correlate with the level of AmpC production.

The literature shows that aside from ESBLs and some OXA enzymes, another cause of FEP resistance is reduced drug entry through loss of porins. Two major porins, OmpC and OmpF *(E. coli)* and their *K. pneumoniae* homologs, OmpK35 and OmpK36, are associated with FEP resistance (Liu *et al.*, 2012; Sugawara, Kojima and Nikaido, 2016). Mutations in *ompR* encoding the transcriptional activator of *ompC* and *ompF* can lead to downregulation of these porins (Russo, Slauch and Silhavy, 1993). However, the OmpR sequences in all nine FEP-R isolates were identical to the WT sequence of those species. OmpC and OmpF sequences from FEP-R isolates were aligned against those from FEP-S isolates and a reference sequence. This showed that in five of the six FEP-R isolates with no other explanation for FEP-resistance, there were no amino acid substitutions unique to FEP-R isolates. The exception was in *E. roggenkampii* isolate E54 where there is a 12 amino acid insertion within the OmpC sequence. However, porins are known to have variable sequences (Fernández and Hancock, 2012) and there are OmpC sequences within the BLAST database that have 100% identity to this sequence. Therefore, it is not clear whether this insertion affects the function of OmpC or not.

Proteomics show that OmpC was actually overproduced ($p < 0.001$) in three of the four FEP-R isolates compared to a FEP-S control (Table 12). OmpC fold change could not be accurately calculated in the fourth pair (isolate E54) as two of the three replicates of the control isolate had a value of zero, suggesting some kind of technical error. FEP-R isolates E1 and E38 were compared against the CAZ-R/FEP-S isolate E33 because the CAZ-S/FEP-S control isolate had a genetically inactivated OmpC which was undetectable in the proteomics data. This is interesting in itself as it confirms that OmpC loss does not cause FEP-resistance in the absence of other mechanisms. It could be that there is a mutation elsewhere at an unknown site that causes FEP-resistance in these isolates, and results in OmpC overproduction, for example as a physiological response to compensate for the loss of another FEP porin. The only significant ($p < 0.05$) OmpF abundance change was downregulation in isolate E50, suggesting that reduced production of OmpF alone also does not confer FEP resistance. The true mechanism(s) remains undefined.

Isolate	FEP phenotype	FEP disc zone (mm)	Fold change (*p*-value)		
			OmpC	OmpF	OmpR
E1	**R**	17	19.38* **(<0.001)**	0.652 (> 0.05)	1.13 (> 0.05)
E38	**R**	23	25.46* **(< 0.001)**	0.649 (> 0.05)	1.62 **(< 0.05)**
E33	S	32	-	0.932 (> 0.05)	1.88 **(< 0.05)**
E50	**I**	24	1.54 **(< 0.001)**	0.324 **(< 0.05)**	1.47 **(< 0.05)**
E54	**I**	25	-	-	0.036 **(< 0.05)**

Table 10 There is no correlation between porin (OmpC/OmpF) or porin regulator (OmpR) levels and cefepime resistance phenotype. Porin loss is usually associated with FEP resistance however OmpC is upregulated in FEP-R isolates. OmpF is not significantly downregulated in FEP-R isolates. OmpR is significantly upregulated in FEP-R isolates (E38,E50) and FEP-S isolates (E33) and is also significantly downregulated in a FEP-R isolate (E54). The FEP phenotype (R=resistant, S=susceptible and I=intermediate) and the FEP disc zone (mm) is shown along with the fold change of each protein compared to a CAZ-S control. The p-value from an unpaired t-test is shown, with significant values highlighted in red. * indicates that these two isolates were compared against a CAZ-R/FEP-S control (E33). – indicates that the protein in the control isolate was undetectable.

TZP resistance was seen in all 12 CAZ-R isolates, although resistance in three of these is due to mobile β-lactamase genes (Table 6). It is possible that TZP-R is therefore caused by AmpC hyperproduction in parallel with CAZ-R. Analysis of the CAZ and TZP disc diffusion zones of these isolates showed no correlation. However, all the CAZ zones were very small and therefore CAZ MICs may reveal a correlation between the two, although this was not within the scope of this project.

3.5 Comparison of clinical dataset to national infection data

3.5.1 Species prevalence

Data from the English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) shows that the most common cause of monomicrobial *Citrobacter* spp. BSIs in 2020 was *C. koseri* (65.01%) followed by *C. freundii* (25.25%). Our dataset reflects this, with 48% of *Citrobacter* spp. isolates being *C. koseri* and 32% being *C. freundii.* ESPAUR data reports that

nationally, the most common species contributing to monomicrobial *Enterobacter* spp. infections was *E. cloacae* (68.18%) and members of the *E. cloacae* complex (12.53%). However as discussed, the identification of *Enterobacter* spp. by MALDI-TOF mass spectrometry is notoriously unreliable. Our dataset shows that subspecies of *E. hormaechei* were by far the most common cause of *Enterobacter* spp. infections (78.18%), corroborating previous literature (Hoffmann *et al.*, 2005; Sutton *et al.*, 2018).

3.5.2 Antibiotic resistance

The percentage of CAZ, CTX and TZP resistance within the 2020 clinical *Enterobacter* isolates was compared with corresponding statistics from ESPAUR reports. Chi-squared analysis found that the frequency of resistance occurring within the clinical dataset and ESPAUR data was not statistically different ($p>0.05$): 20% of clinical *Enterobacter* spp. isolates were resistant to CAZ and CTX and 11% to TZP compared to 24%, 26% and 17% from ESPAUR data respectively (Table 14). The statistical similarity between these datasets shows that the 2020 clinical isolates are representative of national infections. However, it is interesting to note the discrepancies between the data: the clinical isolates show slightly less resistance to 3GC and slightly more resistance to TZP than is shown in national figures (Table 13). This clinical dataset also shows statistically similar, but slightly lower rates of gentamicin and CIP resistance than national figures.

Comparing ABR in clinical *Enterobacter* spp. isolates to ABR *E. coli* (Table 13) from national statistics also sheds light on the resistance mechanisms and distribution of resistance amongst these species. 3GC and TZP resistance in *Enterobacter* spp. is greater than in *E. coli* due to chromosomally encoded inducible (and so hyper-producible following mutation) *ampC* genes. Whereas resistance to non-β-lactams is greater in *E. coli* than *Enterobacter* spp. due to the highly plastic nature of the *E. coli* genome.

	% of resistant *Enterobacter spp*. (clinical isolates)	% of resistant *Enterobacter spp*. infections (national data)	χ^2 *p*-value	% of *E. coli* infections resistant (national data)
Ceftazidime (CAZ)	20%	24%	0.48	14% *
Cefotaxime (CTX)	20%	26%	0.35	14% *
Piperacillin/ tazobactam (TZP)	20%	17%	0.61	9.3%
Meropenem	0%	1%	0.38	-
Gentamicin	4%	6%	0.47	10.4%
Ciprofloxacin (CIP)	2%	6%	0.23	18.4%

Table 11 **Prevalence of ABR within the *Enterobacter* spp. clinical isolates are reflective of national statistics.** The percentage of clinical *Enterobacter* spp. isolates that are resistant to the mentioned antibiotics is statistically similar to that of national *Enterobacter* spp. infections. In both clinical and national data, β-lactam resistance is higher in Enterobacter spp. than in *E. coli* but resistance to non β-lactam antibiotics is higher in *E. coli.* (* indicates resistance to one of the 3GC CAZ, CTX, cefpodoxime and ceftriaxone. – indicates there was no data available. All data for national statistics came from ESPAUR data.

3.6 Conclusion and Discussion

Characterisation of these 80 clinical *Enterobacter* and *Citrobacter* spp. bloodstream isolates show that they all contain either class-A or class-C β-lactamase enzymes. Both these enzymes give intrinsic resistance to AMX, explaining the resistance in 100% of the isolates. All inducible *ampC* containing isolates have intrinsic resistance to AMC because clavulanic acid is not an inhibitor of AmpC enzymes. However, AMC does inhibit class-A enzymes, explaining the AMC sensitivity in 100% of the class-A β-lactamase containing isolates. Resistance to 3GC, CAZ and CTX, was higher in *Enterobacter* spp. than in *Citrobacter* spp. Although the sample size of AmpC producing *Citrobacter* spp. was smaller than that of the *Enterobacter* spp. however, there were no *Citrobacter* spp. isolates exhibiting AmpC mediated CAZ-R. This reflects previous work showing that *ampC* positive *Enterobacter* spp. are more likely to develop resistance to CAZ during treatment than *ampC* positive *Citrobacter* spp. (Chow *et al.*, 1991; Kaye *et al.*, 2001; Choi *et al.*, 2008).

Within this dataset, lower rates of resistance to FEP than 3GC are expected since FEP is more difficult for AmpC to hydrolyse. OXA β-lactamases are capable of hydrolysing FEP (Hirvonen, Spencer and Van Der Kamp, 2021), and account for FEP resistance in three isolates. The cause of FEP resistance in *Enterobacter* spp. without the presence of ESBLs is unclear. FEP resistance is always accompanied by CAZ resistance however CAZ resistance is not always accompanied by FEP resistance. This suggests that FEP resistance is occurring by a mechanism in addition to CAZ-R, not unique to it. FEP resistance is associated with changes in membrane permeability in some species, however our sequence alignments and proteomics data investigating OMPs does not reveal any strong evidence for this, suggesting that FEP resistance may be multifactorial. It would be interesting to investigate this further by cloning genes into isogenic environments and investigating their effect on FEP resistance.

Mobile ABR genes in four isolates account for their resistance to other classes of antibiotics in addition to β-lactams. Aminoglycoside (streptomycin, spectinomycin, gentamicin, tobramycin) resistance is fully explained by the presence of the relevant aminoglycoside modifying genes. Amikacin and CIP resistance have been linked to expression levels of the *aac(6')-Ib-cr* gene in *E. coli* (Dulyayangkul *et al.*, 2023). When looking at amikacin resistance in conjunction with CIP resistance profiles of these isolates, they seem to corroborate the previous literature showing links to gene expression. Expression assays of *aac(6')-Ib-cr* could be measured to investigate amikacin and CIP resistance, and gene knockouts of the different PMQR genes could show whether CIP resistance is indeed multifactorial, as mentioned in section 3.2.2.

The ABR profiles of these 80 clinical isolates are reflective of what we see nationally. Resistance to select antibiotics (CAZ, CTX, TZP, meropenem, gentamicin, CIP) amongst clinical isolates occurs at a similar frequency to national *Enterobacter* spp. data. However, there are subtle differences between the two – the clinical isolates show slightly less resistance to 3GC and more resistance to TZP. These phenotypes point towards a greater occurrence of ESBLs within national infections than is shown in the Bristol isolates (since CTX-M-15 will give resistance to CAZ but not TZP). Therefore, it is possible that Bristol has reduced rates of ESBL-mediated β-lactam resistance compared to other areas of England. This clinical dataset also

show slightly lower rates of gentamicin and CIP resistance than national figures. Since CTX-M-15 is often carried in association with gentamicin and CIP mobile resistance genes, this could also point to Bristol having lower rates of ESBLs and mobile ABR genes than the national average. This comparison was not able to be made for *Citrobacter* spp. isolates since there was only one that exhibited resistance to the tested antibiotics.

One aim of this study was to investigate whether CAZ resistance in *ampC*-positive *Enterobacter* and *Citrobacter* spp. is more prominent within certain species or clades. WGS data corroborated previous studies showing that MALDI-TOF species prediction used in clinical settings frequently misnames *Enterobacter* spp. However, phylogenetic analysis showed that CAZ-R was not confined to particular species or clades and that there was no clustering of CAZ-R isolates separate to CAZ-S isolates in any species. There were three pairs of isolates with SNP dists below our threshold of 100 SNPs. This value was well below the average SNP dist value for each species, and therefore suggests that the isolate pairs diverged from a common ancestor much more recently than other isolates. One isolate pair was particularly interesting as the two isolates showed different CAZ susceptibility phenotypes (E3: CAZ-R; E5: CAZ-S) but were only 54 SNPs different. This suggests that the cause of CAZ-R in E3 is either something outside of the core genome (though these two isolates do not carry any mobile ABR genes), insertional inactivation with non-core genomic DNA, or is actually caused by one of the 54 SNPs. This is discussed further in section 5.3.

Proteomics data shows that AmpC hyper-production is occurring in a subset of the CAZ-R isolates. Combined with the general lack of mobile ABR genes and the lack of clonality amongst CAZ-R isolates suggests a mutational cause of CAZ-R, sporadically emerged within the population of bacteria sampled here.

4. Results Chapter 2: Characterisation of CAZ-R clinical *Enterobacter* spp and *Citrobacter* spp isolates from children in 1983-1990 and comparison to CAZ-R 2020 isolates

To establish whether mechanisms conferring ceftazidime (CAZ) resistance have changed among *Citrobacter* spp and *Enterobacter* spp clinical isolates between the 1980s and 2020, CAZ-resistant (CAZ-R) isolates from each time frame were compared phenotypically and using whole genome sequencing (WGS). All the isolates discussed in this chapter were collected during the 1980s because of their CAZ resistance. We can therefore compare them against the subset of 2020 CAZ-R isolates discussed in chapter 3. Prevalence of resistance to other antibiotics among these CAZ-R isolates was compared across the two sample datasets to identify areas where there were significant differences either between the timeframes, or between genera. Since the only CAZ-R *Citrobacter* isolate from 2020 contained CTX-M-15 as the CAZ resistance mechanism, comparisons between genera were made only using data from the 1980s and comparisons between timeframes were made only using data from *Enterobacter* spp. We were particularly interested in the levels of piperacillin/tazobactam (TZP) resistance. CAZ and TZP were introduced clinically in 1985 and 1994 respectively, and therefore TZP use was limited, or non-existent at the time the early samples were collected. Conversely in 2020, TZP was the most commonly prescribed antibiotic for bloodstream infections (BSIs) with usage of CAZ having significantly reduced.

4.1 Whole genome sequencing

19 clinical isolates, 10 *Citrobacter* spp. and 9 *Enterobacter* spp*.,* collected from patients being treated at the Bristol Children's Hospital from 1983-1990 were sent for WGS. These isolates were originally selected specifically for their CAZ-R phenotype. Therefore, it is important to note that, unlike in the 2020 dataset, there were no *Citrobacter* spp. isolates with class A penicillinase enzymes. All chromosomal *ampC* cephalosporinase genes found in these isolates produced matches to the following mobile variants identified by ResFinder: bla_{ACT} in *E. hormaechei* species, bla_{MIR} in *E. roggenkampii* and bla_{CMY} *in Citrobacter* spp., as seen in the 2020 isolates. No mobile antibiotic resistance (ABR) genes associated with resistance to any antibiotics were identified.

4.2 ABR phenotype

4.2.1 β-lactam resistance

All 19 isolates collected from 1980-1993 contain a chromosomal *ampC* with an AmpR regulator associated with *ampC* induction by some β-lactams, and so they are intrinsically resistant to amoxicillin (AMX) and amoxicillin/clavulanate (AMC). They were all resistant to the third-generation cephalosporins (3GCs) cefotaxime (CTX) and CAZ as expected based on their selection criterion (Table 14). Nine (47%) were cefepime (FEP) resistant: eight (89%) *Enterobacter* spp. and one (10%) *Citrobacter* spp. This is a significantly higher distribution of FEP resistance within *Enterobacter* spp. than *Citrobacter* spp. (chi-squared analysis, $p < 0.05$) (Table 15). Only two isolates (11%), one *Enterobacter* spp. and one *Citrobacter* spp., were TZP-R. All isolates were resistant to aztreonam and only a single isolate exhibited resistance to meropenem.

	Enterobacter spp.		*Citrobacter* spp.	
	2020	**1980-1993**	**2020**	**1980-1993**
Amoxicillin (AMX)	100% (11)	100% (9)	100% (1)	100% (10)
Amoxicillin/clavulanate (AMC)	100% (11)	100% (9)	100% (1)	100% (10)
Ceftazidime (CAZ)	100% (11)	100% (9)	100% (1)	100% (10)
Cefepime (FEP)	73% (8)	89% (8)	100% (1)	10% (1)
Piperacillin/tazobactam (TZP)	100% (11)	11% (1)	100% (1)	10% (1)
Aztreonam	100% (11)	100% (9)	100% (1)	100% (10)
Meropenem	0%	11% (1)	0%	0%
Ciprofloxacin	9% (1)	0%	100% (1)	0%
Cotrimoxazole	18% (2)	0%	100% (1)	0%
Gentamicin	18% (2)	0%	100% (1)	0%
Amikacin	9% (1)	0%	0%	0%
Tobramycin	18% (2)	0%	0%	0%

Table 12 Antibiotic susceptibility data from CAZ-R clinical isolates in 2020 and the 1980s shows highest levels of resistance to β-lactam antibiotics in both timeframes, and no resistance to non-β-lactam antibiotics in the 1980s. The percentage (and number) of CAZ-R isolates from each time frame, broken down by genus, that show resistance to the listed antibiotics. All isolates collected from the 1980s were CAZ-R whereas only a subset of the 2020 isolates were. There was no resistance to non-β-lactam antibiotics within the 1980s dataset whereas there was evidence of this in 2020, due to the presence of mobile ABR genes.

Interestingly, levels of TZP resistance within *Enterobacter* CAZ-R isolates were significantly higher in 2020 (100%) than in 1980 (11%) ($p < 0.0001$) (Table 15). It should be noted that TZP-resistance in two of the 10 isolates from 2020 was due to OXA β-lactamase, however this does not explain the full extent of the increase in resistance. In contrast to this, there is no significant difference between levels of FEP resistance within CAZ-R *Enterobacter* spp. between 1980s and 2020 (Table 15).

	Timeframe comparison (*Enterobacter* spp. only)		Genus comparison (1980s dataset only)	
	***p*-value**	**significant difference?**	***p*-value**	**significant difference?**
Cefepime (FEP)	$p > 0.05$	no	$p < 0.05$	more in *Enterobacter*
Piperacillin/ tazobactam (TZP)	$p < 0.0001$	more in 2020	$p > 0.05$	no
Aztreonam	$p > 0.05$	no	$p > 0.05$	no
Meropenem	$p > 0.05$	no	$p > 0.05$	no
Ciprofloxacin	$p > 0.05$	no	$p > 0.05$	no
Cotrimoxazole	$p > 0.05$	no	$p > 0.05$	no
Gentamicin	$p > 0.05$	no	$p > 0.05$	no
Amikacin	$p > 0.05$	no	$p > 0.05$	no
Tobramycin	$p > 0.05$	no	$p > 0.05$	no

Table 13 TZP resistance within *Enterobacter* spp. significantly increased from 1980 to 2020 and FEP resistance is significantly higher in *Enterobacter* spp. than *Citrobacter* spp. Chi-squared analysis of the prevalence of resistance between a) timeframes (2020 vs 1980s) and b) genus shows that the only significant changes are TZP between timeframes and FEP between genera. Comparisons between timeframes were made only within *Enterobacter* spp. Comparisons between genera were made only within the 1980s dataset

4.2.2 Non-β-lactam antibiotic resistance

There were no isolates from the 1980s resistant to any of the non-β-lactam antibiotics tested (Table 14). This is unsurprising given the fact that ResFinder did not identify any mobile ABR genes in these isolates, unlike the three CAZ-R isolates containing mobile ABR genes from 2020. It is not possible to statistically validate these results, as there are 0 of 19 CAZ-R isolates from the 1980s with ABR genes. However, comparing this to 3 of 11 CAZ-R isolates from 2020 suggests an increase in the prevalence of ABR genes between the time periods.

4.3 Phylogeny

4.3.1 Species identification

Isolates from this dataset had been identified by 16S rRNA analysis at the time of collection. Other than the *E. roggenkampii* isolate, all *Enterobacter* spp. isolates had been identified as

E. cloacae. At the time, *E. cloacae* was one of the few defined species of *Enterobacter* spp. therefore this identification was accurate at the time. The species in this study were only described recently, in 2005 and 2018, (Hoffmann *et al.*, 2005; Sutton *et al.*, 2018) and WGS allowed us to reassign these isolates to the new species. There were two *Enterobacter* spp. present in this dataset which were not present in the 2020 dataset: *E. hormaechei* subsp. *oharae* and *E. hormaechei* subsp. *xiangfangensis.* WGS corroborated the initial identification of *Citrobacter* spp. isolates from the 1980s. There were two *Citrobacter* spp. present in this dataset which were not present in the 2020 dataset: *C. murliniae* and *C. youngae.* Within the 2020 dataset, *C. koseri* was the most prevalent *Citrobacter* species however there are no *C. koseri* in the 1980 dataset since they were selected for their CAZ-resistance and *C. koseri* has a class A penicillinase.

4.3.2 Relationships between isolates

Maximum likelihood phylogenetic trees of isolates collected during the 1980s were made for the following species: *E. hormaechei* spp. (Figure 11), *C. murliniae (*Figure 12) and *C. freundii.* It was not possible to make phylogenetic trees for other species present in the 1980s dataset *(E. roggenkampii* and *C. youngae)* as there were too few isolates of each species. Single nucleotide polymorphism (SNP) distance analysis identified two pairs of isolates within the 1980s dataset with <100 SNPs between them. One of these pairs was two *C. murliniae* isolates which differed by 91 bp (Figure 12). The second pair of isolates with <100 SNPs were isolates E58 and E56 which differ by 97 bp (Figure 12). Since the two *C. murliniae* organisms were isolated 8 years apart, we suggest that both of these pairs diverged from a common ancestor in a matter of years prior to isolation, rather than months or decades.

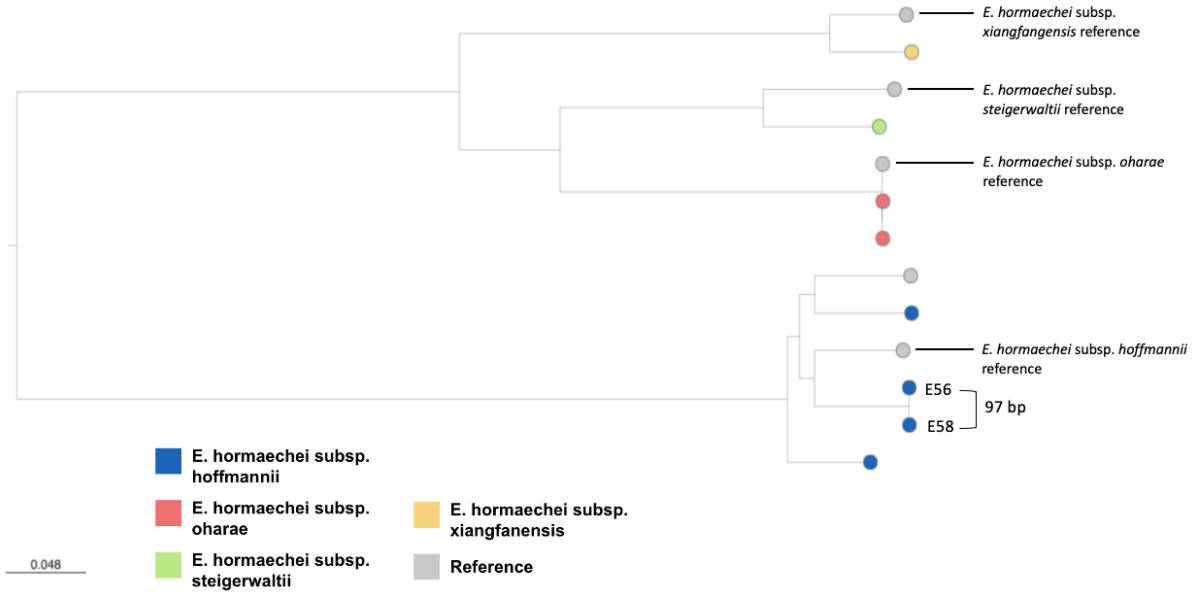

Figure 11 There are four different subspecies of *E. hormaechei* present within the 1980s isolates as reclassified by WGS, along with a pair of closely related *E. hormaechei* subsp. *hoffmannii* isolates. WGS and core genome alignment showed that the isolates previously identified by 16S rRNA analysis as *E. cloacae* are in fact subspecies of *E. hormaechei.* SNP dist analysis showed a pair of isolates (E56/E58) with <100 SNPs different.

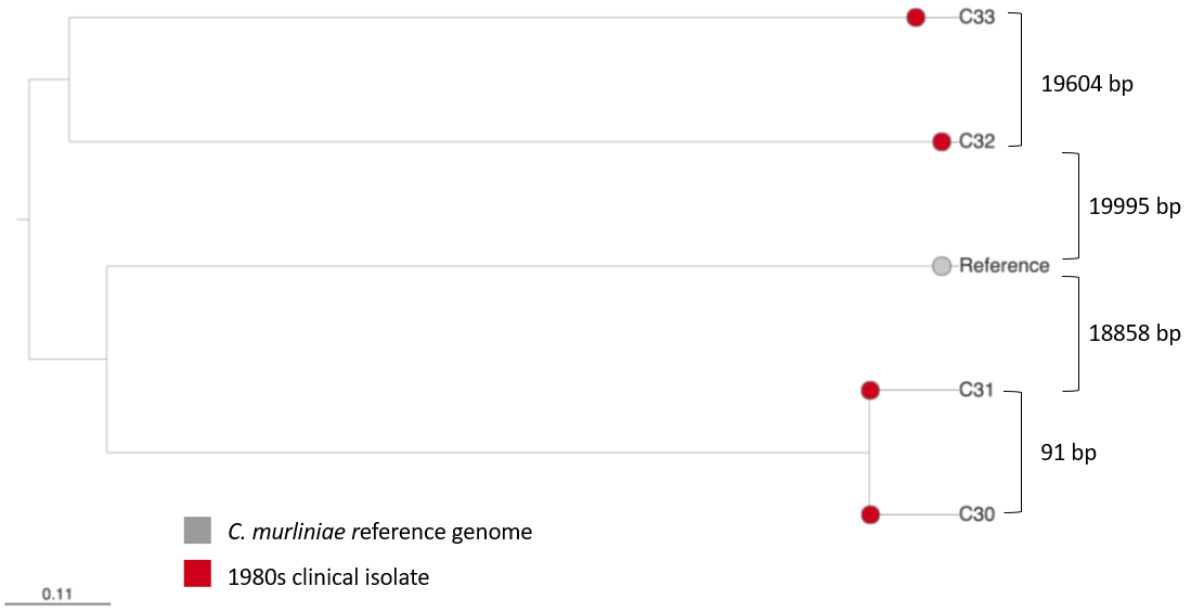

Figure 12 WGS and SNP dist analysis showed a pair of closely related *C. murliniae* isolates. Core genome alignment compared with the *C. murliniae* ATCC 51118 reference genome showed that there was a pair of isolates (C30/C31) that differ by only 91 bp, these organisms were investigated further.

Phylogenetic trees were also made for species which were present in both the 1980s and 2020 dataset (whether CAZ-R or not in 2020), these were: *C. freundii, E. roggenkampii* and *E. hormaechei* subspp. (Figures 13-15). This analysis revealed two main findings: that there was i) no clustering between CAZ-R separate from CAZ-S isolates and ii) no clustering between isolates from the two timeframes.

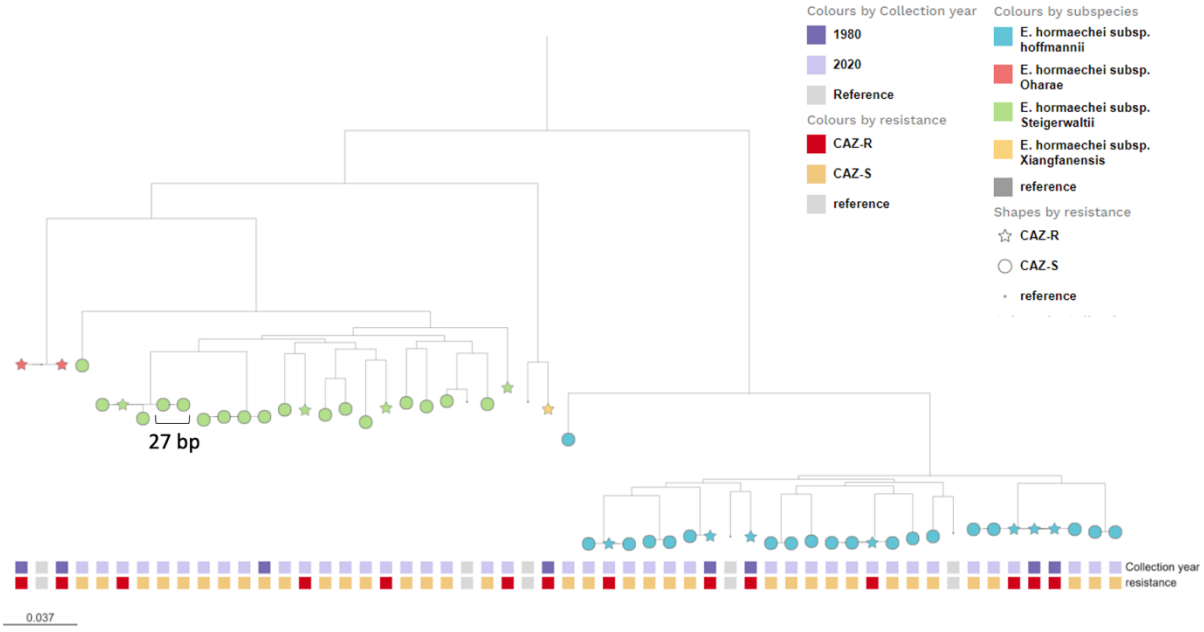

Figure 13 Within *E. hormaechei* subspp., there is no clustering of CAZ-resistance to particular subspecies, or to a particular collection period. Core genome alignment of all CAZ-S (circle) and CAZ-R (star) isolates from the 1980s and 2020 of different *E. hormaechei* subspecies: *hoffmannii* (blue), *oharae* (pink), *steigerwaltii* (green), *xiangfangensis* (yellow). Metadata blocks below each isolate show whether it was collected in the 1980s (dark purple) or 2020 (light purple) and whether the isolate was CAZ-S (yellow) or CAZ-R (red) and show that there was no clustering of CAZ-R isolates to a particular timeframe. Pairwise SNP distances (bp) are labelled between the two most closely related isolates for scale.

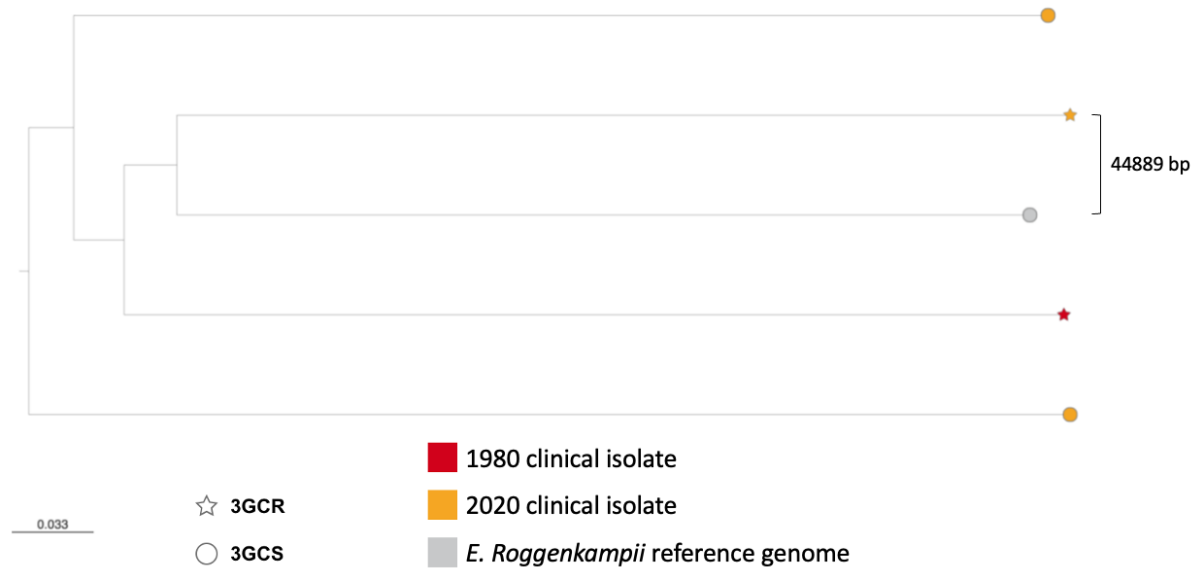

Figure 14 The two CAZ-R *E. roggenkampii* isolates from the 1980s and 2020 are not clonal. Core genome alignment of all four *C. murliniae* isolates from 2020 (orange) and the 1980s (red) against the *C. murliniae* reference genome and their CAZ phenotype (CAZ-R (star) or CAZ-S (circle)) show that the two CAZ-R isolates do not cluster together separately from other pairs of isolates. Pairwise SNP distances (bp) are labelled between the two most closely related isolates for scale.

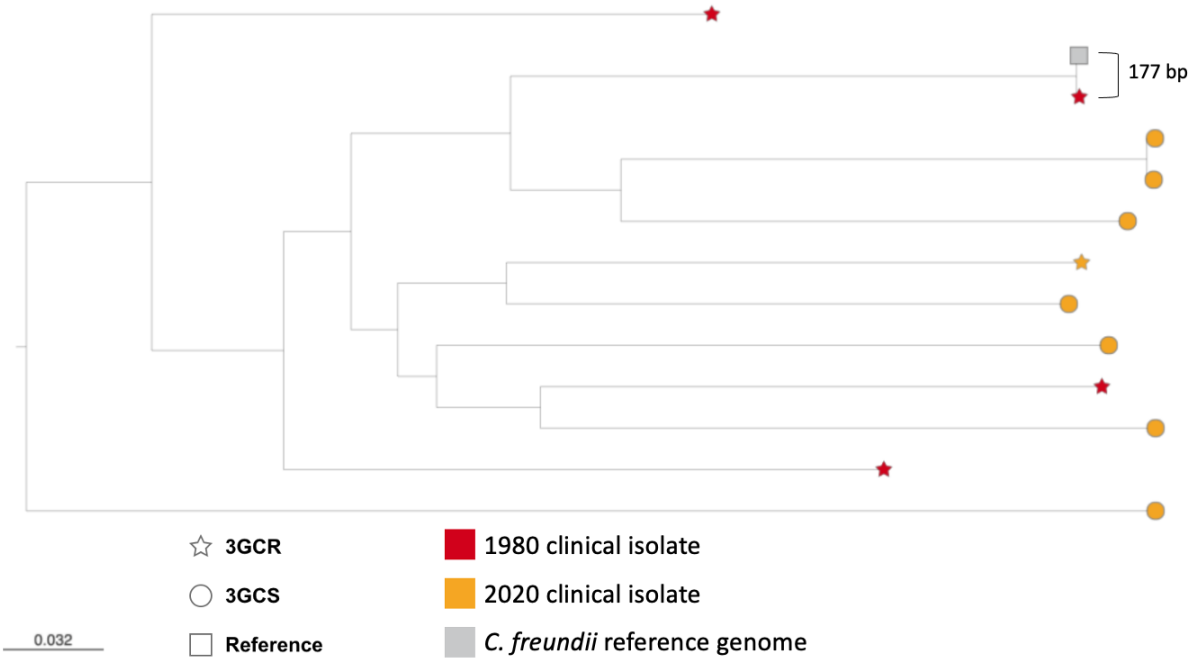

Figure 15 There is no clustering of CAZ-R *C. freundii* isolates separately to CAZ-S isolates. Core genome alignment of all four *C. freundii* isoaltes from 2020 (orange) and the 1980s (red) and their CAZ phenotype (C-R (Star) or CAZ-S (circle)) show that there is no clustering of the two, highlighting the sporadic nature of *Citrobacter* spp. infections. Pairwsie SNP distances (bp) are labelled between the two most closely related isolates for scale.

4.4 Conclusion and discussion

The isolates in this dataset collected between 1983 and 1990 were selected specifically for their CAZ resistance that previous work has shown to be due to AmpC hyper-production (Avison, 2004). As such, their ABR phenotypes reflected this in showing high levels of resistance to β-lactam antibiotics AMX and AMC, but also 3GC CTX and CAZ. One isolate exhibited resistance to meropenem, a last resort β-lactam antibiotic. Since meropenem was only introduced in 1996, it is interesting that a bacterium isolated before this exhibits resistance. Given the lack of carbapenemases present, a possible mechanism for this is decreased drug intake through porin loss, or increased drug efflux through efflux pump overproduction. In the presence of AmpC hyper-production, both of these mechanisms have been shown to confer meropenem resistance in *Enterobacterales* species (Meletis *et al.*, 2012; Hao *et al.*, 2018).

Levels of TZP resistance amongst CAZ-R isolates within and between these datasets is interesting. Our data shows that in *Enterobacter* spp. there is more TZP-R in 2020 than in the

1980s and that there is no difference between TZP-R rates in *Enterobacter* spp. and *Citrobacter* spp. TZP was first approved for medical use in 1993 so was not in use when the 1980s samples were collected. In stark contrast, it is now the most commonly prescribed antibiotic for BSI (Walker *et al.*, 2022)**.** This suggests that the selection pressure of increased TZP use is driving this emergence of resistance. Proteomics could be conducted on the 1980s isolates in order to rule out any underlying differences in AmpC hyper-production between the 1980s and 2020 isolates. If there is no significant difference between the two, it is likely that the greater TZP-resistance observed in 2020 isolates is due to a mutational effect acting in addition to AmpC hyper-production.

Interestingly, patterns of FEP resistance between genera and across timeframes show the opposite trends to TZP: there is more FEP resistance in *Enterobacter* spp. than *Citrobacter* spp. but similar levels of resistance across the timeframes. This suggests that the *Enterobacter* AmpC is better than the *Citrobacter* AmpC at hydrolysing FEP. Hence, when AmpC is hyper-produced, there is more likely to be FEP resistance in *Enterobacter* spp. than *Citrobacter* spp. FEP was introduced for clinical use in 1994 but has rarely been used as a treatment in the UK. The absence of selection pressure may explain the lack of resistance to FEP as there is no selection to drive the emergence of mechanisms associated with FEP resistance.

In contrast to the 2020 isolates, no isolates from the 1980s contained mobile ABR genes. This may be because *Enterobacter* spp. (which account for most of the CAZ-R isolates in 2020) were less likely to be a pathogen in the 1980s and so less likely to be acquiring mobile genes. Since then, the use of CAZ and AMC has created a selection pressure to increase the likelihood of *Enterobacter* spp. causing opportunistic infections, making them more likely to be present in a healthcare environment rich in mobile ABR genes, which they can then acquire.

Phylogenetic analysis of the 2020 and 1980s isolates together revealed similar conclusions in terms of the lack of clustering of CAZ-R isolates separate to CAZ-S isolates. Furthermore, the 1980s isolates did not cluster separately to the 2020 isolates. This highlights the sporadic nature of *Enterobacter* and *Citrobacter* spp. infections.

5. Results Chapter 3: AmpD loss of function as a cause of CAZ-R

Characterisation of the ceftazidime-resistant (CAZ-R) isolates from the 1980s and 2020 led to the conclusion that AmpC hyper-production is the most likely cause of CAZ-R in 28 isolates. As discussed in the introduction, chromosomally encoded *ampC* genes are inducible through the AmpR transcriptional regulator, linked to the peptidoglycan recycling pathway. Mutations in genes involved in this pathway are the most common cause of *ampC* overexpression in AmpC hyper-producing organisms.

To investigate this within the CAZ-R clinical isolates, amino acid sequence alignment was conducted to identify substitutions within a set of proteins (as outlined in methods section 2.3) using ceftazidime-susceptible (CAZ-S) isolates as controls. These proteins were: AmpC, AmpD, AmpR, AmpE, DacD (PBP6b), and Mpl. As mentioned in section 1.5, these proteins (other than AmpC) are linked to *ampC* expression through their involvement in the peptidoglycan recycling pathway. The frequency of substitutions in these proteins was compared between CAZ-R and CAZ-S isolates and revealed two main findings: Firstly, of all proteins tested, AmpD has the largest ratio of the total number of amino acid substitutions relative to the reference sequence in CAZ-R isolates versus CAZ-S isolates (Table 16). Secondly, AmpD has a higher frequency of loss of function mutations than all other proteins and these mutations occurred solely in CAZ-R isolates, unlike in other proteins where loss of function mutations were also found in CAZ-S isolates (Table 17). This corroborates previous literature showing that *ampD* mutations are the leading cause of *ampC* overexpression (Jacoby, 2009; Guérin *et al.*, 2015a) and rationalised the decision to investigate AmpD further in these clinical isolates.

Average number of amino acid substitutions relative to the reference sequence, normalised by protein length						
	AmpC	**AmpR**	**AmpD**	**AmpE**	**DacD**	**Mpl**
CAZ-R isolates	0.0090	0.0045	0.0073	0.0025	0.0031	0.0018
CAZ-S isolates	0.0088	0.0062	0.0045	0.0021	0.0039	0.0017
CAZ-R:CAZ-S ratio	1.02	0.72	1.63	1.22	0.78	1.09

Table 16 AmpD has the largest ratio of amino acid substitutions in CAZ-R isolates compared to CAZ-S isolates. When comparing the average number of amino acid substitutions within different proteins involved in AmpC hyperproduction in both CAZ-R and CAZ-S isoaltes compared to a reference sequence, AmpD CAZ-R isolates have the largest increase in amino acid substitutions. Values were calculated using only species with inducible *ampC* and excluding isolates with mobile β-lactamase genes.

Percentage of isolates with loss of function mutations	AmpC	AmpR	AmpD	AmpE	DacD	Mpl
CAZ-R isolates	0%	0%	50%	0%	7%	0%
CAZ-S isolates	4%	0%	0%	2%	2%	2%
Total	**2%**	**0%**	**17%**	**1%**	**4%**	**1%**

Table 14 AmpD has the highest frequency of loss of function mutations. Within proteins involved in AmpC hyperproduction, loss of function mutations happen most frequently in AmpD. In this context, loss of function mutations are either frameshifts, nonsense mutations or insertional inactivation. Values were calculated using only species with inducible *ampC* and excluding isolates with mobile β-lactamase genes.

Of the 28 CAZ-R isolates from across the two timeframes, 14 contained AmpD loss of function mutations. The remaining 14 CAZ-R isolates contained amino acid substitutions within AmpD. Comparisons of the types of mutations occurring between datasets showed that loss of function and missense mutations occurred at similar frequencies across timeframes (1980s versus 2020) and genera (Table 18). The types of mutations occurring and how they affect AmpD and CAZ resistance is discussed below.

Mutation type	Genus		Timeframe	
	Enterobacter	***Citrobacter***	**2020**	**1980**
Loss of function	44% (8)	60% (6)	44% (4)	53% (10)
Missense	56% (10)	40% (4)	56% (5)	47% (9)

Table 15 There are similar rates of *ampD* loss of function and missense mutations within CAZ-R isolates between timeframes and genera. The percentage (and frequency) of *ampD* loss of function and missense mutations in the 28 AmpC hyper-producing CAZ-R isolates show that there are similar rates between a) *Enterobacter* and *Citrobacter* spp. and b) the 1980s and 2020 datasets. This was not statistically validated due to the small number of samples. Loss of function mutations are categorised as frameshifts, nonsense mutations and insertional inactivation.

Isolate	Sample collection date	Species	AmpD substitution	Other cause of CAZ-R
E1	2020	*E. hormaechei* subsp. *steigerwaltii*		AmpD ins. G156 [frameshift]
E3	2020	*E. hormaechei* subsp. *steigerwaltii*		*ampD* insertional inactivation
E6	2020	*E. hormaechei* subsp. *steigerwaltii*	H154N	-
E19	2020	*E. hormaechei* subsp. *steigerwaltii*		CTX-M-15
E23	2020	*E. hormaechei* subsp. *hoffmannii*		CTX-M-15
E33	2020	*E. hormaechei* subsp. *hoffmannii*	H154L	-
E38	2020	*E. hormaechei* subsp. *hoffmannii*		AmpD R93Stop [truncation]
E49	2020	*E. kobei*		*ampD* insertional inactivation
E50	2020	*E. kobei*	G166S	-
E54	2020	*E. roggenkampii*	W95R	-
E55	2020	*E. bugandensis*	P89L T122I Q131H	
C17	2020	*C. freundii*		Mobile ABR genes
E56	1990	*E. hormaechei* subsp. *hoffmannii*		*ampD* insertional inactivation
E57	1985	*E. hormaechei* subsp. *hoffmannii*	I78S	-
E58	1986	*E. hormaechei* subsp. *hoffmannii*	G166S	-

E59	1990	*E. hormaechei* subsp. *hoffmannii*	L117Q	-
E60	1989	*E. hormaechei* subsp. *steigerwaltii*	I78S	-
E61	1988	*E. hormaechei* subsp. *xiangfangensis*		AmpD del. R93-W95 [frameshift]
E62	1986	*E. hormaechei* subsp. *oharae*		AmpD W7Stop [truncation]
E63	1988	*E. hormaechei* subsp. *oharae*	S100L	-
E64	1983	*E. roggenkampii*		AmpD W95Stop [truncation]-
C26	1990	*C. freundii*		*ampD* insertional inactivation
C27	1990	*C. freundii*		*ampD* insertional inactivation
C28	1990	*C. freundii*		*ampD* insertional inactivation
C29	1988	*C. freundii*		AmpD Q86Stop [truncation]
C30	1984	*C. murliniae*		*ampD* ins 4bp [frameshift]
C31	1990	*C. murliniae*	P39S L123M	-
C32	1990	*C. murliniae*	I78S	-
C33	1988	*C. murliniae*		*ampD* ins. 4bp [frameshift]
C34	1990	*C. youngae*	S73F	-
C35	1984	*C. youngae*	I78S	-

Table 16 Genetic inactivation of AmpD was the cause of CAZ-R in 28 of 31 CAZ-R isolates. The species of each of the 31 CAZ-R isolates with the year in which they were collected is listed. 28 of these 31 isolates had either AmpD amino acid substitutions or loss of function mutations within *ampD.* The other 3 isolates contained mobile β-lactamase genes resulting in their CAZ-resistance.

5.1. Insertions and deletions

An insertion or deletion (indel) of nucleotide(s) can result in the addition or loss of amino acids, or disruption of the reading frame if the indel is not in a multiple of three. One isolate had a single glycine insertion at position 155 of AmpD (Figure 16). This may have disrupted protein function since glycine residues are small and often cause the protein chain to kink, altering the folding pattern (Dong *et al.*, 2012), but this is not certain. A second isolate, E61, contained a deletion of three amino acids, R93-W95 (Figure 16). These residues have previously been described as important for the function of AmpD (Stapleton, Shannon and

Phillips, 1995; Carrasco-López *et al.*, 2011), suggesting that their loss in this isolate is the cause of CAZ-R in this isolate.

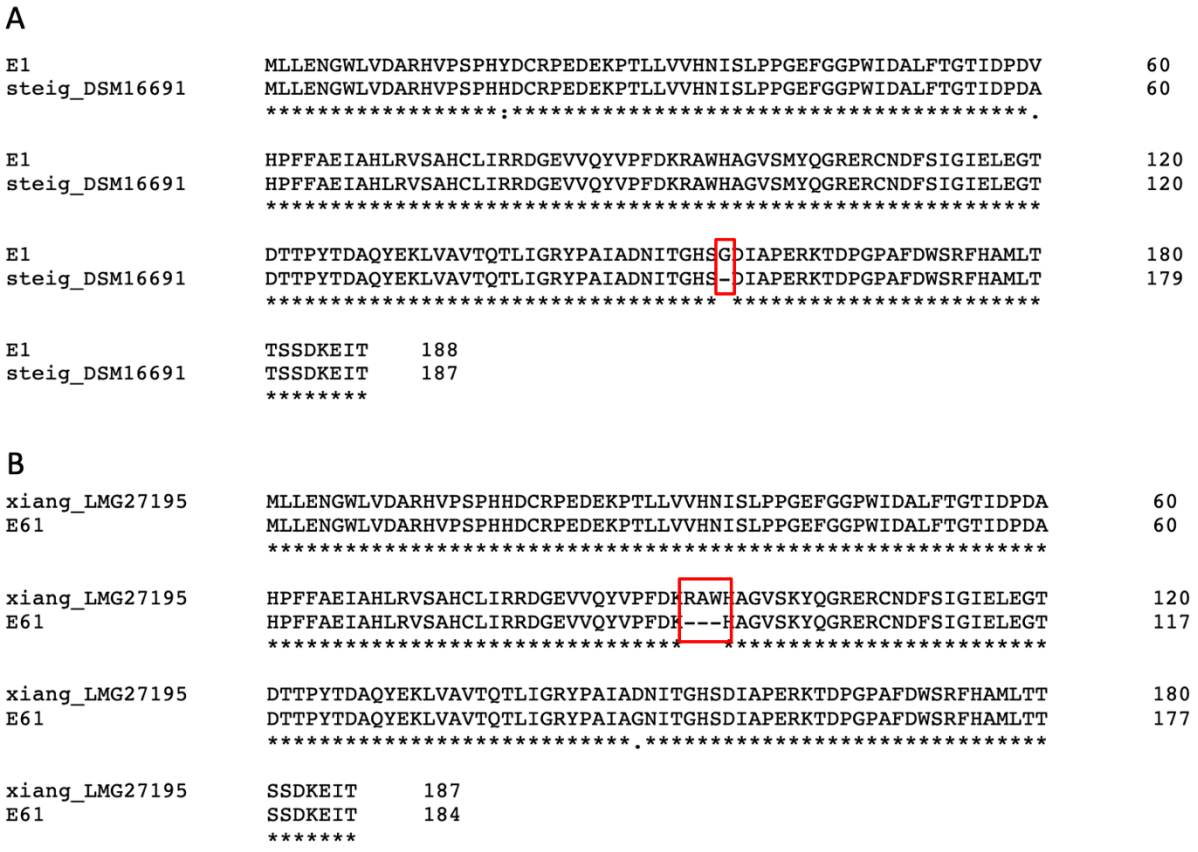

Figure 16 AmpD amino acid insertions and deletions leading to CAZ-R were present in two isolates. Sequence alignments of AmpD from two CAZ-R isolates compared with the AmpD sequence from the reference genome of that species show.an amino acid insertion (A) in isolate E1 and a deletion (B) in isolate E61. Amino acid changes are highlighted in red.

Frameshift mutations due to nucleotide deletions were present in two *C. murliniae* isolates (Figure 17). These two isolates are previously discussed in section 4.3.2 whereby their core genome differs by only 91 SNPs. This four nucleotide deletion would cause a frameshift that would disrupt translation and abolish the function of AmpD leading to *ampC* overexpression and consequently, CAZ resistance.

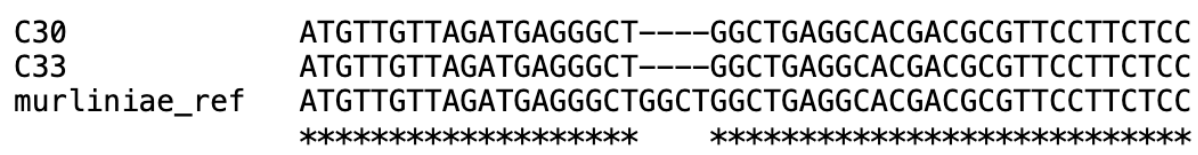

Figure 17 Two isolates contained frameshift mutations in *ampD* leading to CAZ-R. Partial *ampD* nucleotide sequence alignment of two isolates, (C30, C33) and the species type strain, show a four-nucleotide deletion leading to a frameshift which will result in non-functional AmpD.

5.2. Nonsense mutations

A nonsense mutation occurs when a nucleotide substitution results in a stop codon instead of a codon for an amino acid, leading to premature termination of protein translation and a non-functional protein. There were four clinical isolates which had *ampD* nonsense mutations. Their sequences were truncated after residues 6, 85, 92 and 94 (Figure 18) compared to the full-length reference sequences of 183 or 187 residues, depending on the species. These nonsense mutations would therefore result in much smaller peptides than wild type (WT) AmpD, resulting in non-functional protein that causes *ampC* overexpression.

```
E64             MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA  60
roggenkampii    MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA  60
E38             MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA  60
hoffmannii      MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA  60
E62             MLLENG------------------------------------------------------  6
oharae          MLLENGWLVDARHVPSPHHDCRPEDEKPTLLVVHNISLPPGEFGGPWIDALFTGTIDPDA  60
C29             MLLDEGWLAEARRVPSPHYDCRPDDENPSLLVVHNISLPPGEFGGPWIDALFTGTIDPNA  60
freundii        MLLDEGWLAEARRVPSPHYDCRPDDENPSLLVVHNISLPPGEFGGPWIDALFTGTIDPNA  60
                ***::*

E64             HPFFAEIAHLRVSAHCLIRRDGEVVQYVPFDKRA-------------------------  94
roggenkampii    HPFFAEIAHLRVSAHCLIRRDGEVVQYVPFDKRAWHAGVSMYHGRERCNDFSIGIELEGT  120
E38             HPYFAEIAHLRVSAHCLIRRDGEVVQYVPFDK---------------------------  92
hoffmannii      HPYFAEIAHLRVSAHCLIRRDGEVVQYVPFDKRAWHAGVSMYQGRERCNDFSIGIELEGT  120
E62             ------------------------------------------------------------  6
oharae          HPFFAEIAHLRVSAHCLIRRDGEVVQYVPFDKRAWHAGVSMYQGRERCNDFSIGIELEGT  120
C29             HPYFAGIAHLRVSAHCLIRRDGEIV-----------------------------------  85
freundii        HPYFAGIAHLRVSAHCLIRRDGEIVQYVPFDKRAWHAGVSSYQGRERCNDFSIGIELEGT  120

E64             ------------------------------------------------------------  94
roggenkampii    DTTPYTDSQYQQLAALTRTLIGLYPAIADNITGHSDIAPARKTDPGPAFDWPRFRAMLTA  180
E38             ------------------------------------------------------------  92
hoffmannii      DTTPYTDAQYQKLVAVTQTLVGLYPAIADNITGHSDIAPERKTDPGPAFDWSRFHAMLTT  180
E62             ------------------------------------------------------------  6
oharae          DTTPYTDAQYEKLVAVTQTLIGRYPAIADNITGHSDIAPERKNDPGPAFDWSRFHAMLTT  180
C29             ------------------------------------------------------------  85
freundii        DTLAYTDAQYQQLAAVTNALITRYPAIANNMTGHCNIAPERKTDPGPSFDWARFRALVTP  180

E64             -------      94
roggenkampii    SSE----      183
E38             -------      92
hoffmannii      SSDKEIT      187
E62             -------      6
oharae          SSDKEIT      187
C29             -------      85
freundii        SSHKEMT      187
```

***Figure 18* Four isolates contained truncated AmpD leading to CAZ-R.** Sequence alignment of AmpD from the four isolates with *ampD* nonsense mutations leading to truncation of the protein, against the AmpD sequence from the reference strain of that species. The premature end to the protein is marked in red.

5.3. Insertion sequences

Insertion sequences (IS) are small transposable elements which can easily move across genomes, sometimes carrying with them other genes (Saedler and Heiß, 1973; Heffron *et al.*, 1975). If an IS inserts itself into either the promoter or coding region of a gene, it will prevent transcription. Hence, the presence of an IS in either *ampD* or its promoter, in six clinical isolates is causing AmpD loss of function and leading to AmpC mediated CAZ-R (Table 19). Figure 28 shows the WT genetic arrangement around *ampD* and effect on the arrangement when an IS is present within the gene.

Genus	Species	Isolate	Location of IS (gene/promoter)	Residue at which AmpD differs to WT
Citrobacter	*freundii*	C26	gene	148
		C27	gene	80
		C28	gene	163
Enterobacter	*kobei*	E49	gene	147
	hormaechei subsp. *steigerwaltii*	E3	promoter	-
	hormaechei subsp. *hoffmannii*	E56	promoter	-

Table 17 Six isolates contained insertion sequences within *ampD* or its promoter that resulted in CAZ-R. The location of insertion of the IS (either the *ampD* gene or its promoter) is shown, along with the amino acid residue at which the AmpD sequence differs to the WT of that species, indicating where in the gene the IS has entered.

To identify the IS present in these isolates, query nucleotide sequences were run through a BLAST database using IS Finder (Siguier, 2006) to ascertain matches. Due to the assembly method of short-read whole genome sequencing (WGS) data, when an IS is present it will often be at the start or end of a contig. In isolates where there is an IS within *ampD,* the gene is essentially split in two, with one part at the start of a contig and the other part at the end of another. The short nucleotide sequence (~130bp) from the *ampD* gene to the start/end of the contig is the IS DNA and was used as the query sequence for IS Finder. When the IS

sequence was present in the *ampD* promoter, the nt sequence upstream of *ampD* to the start/end of the contig was used as the query sequence.

Table 21 shows the family from which each IS belongs and the *E*-value of the match – the lower the *E*-value, the closer the identity of the query and result. This shows that the nature of the IS occurring has changed throughout the timeframes: isolates from 1990 generally contain elements which were described earlier than those present in the 2020 isolates.

Isolate	Sample collection year	IS family	E- value
C26	1990	IS5	6.00E-60
C27	1990	IS1	1.00E-64
C28	1990	IS6	0
E56	1990	IS110	7.00E-63
E3	2020	IS110	7.00E-63
E49	2020	IS200	0.78

Table 18 Two isolates contained the same insertion sequence that is causing CAZ-R. Isolates E56 and E3 have the same IS sequence (IS110 family member) despite being isolated 30 years apart. Data was obtained from IS Finder, showing the family to which each IS belongs, the *E-value* of the highest match within the IS Finder database, and the species from which the match originated from. A smaller *E*-value represents a closer match between the query IS sequence and database match.

There were three *C. freundii* isolates which contained IS sequences within *ampD* (Table 21). These isolates were unrelated and contained different IS – members of the IS1, IS5 and IS6 families. Isolate C26 contained a member of the IS5 family. Alignment of the terminal nucleotide sequence (122nt) from the two contigs containing *ampD* show that they are inverted repeats of an IS suggesting that this is a simple insertion of IS5 into *ampD* (Figure 19). Isolate C27 contains an IS1 family IS. The terminal nucleotide sequences from the two contigs containing *ampD* do not align, suggesting that the insertion has been more complex than the previous example.

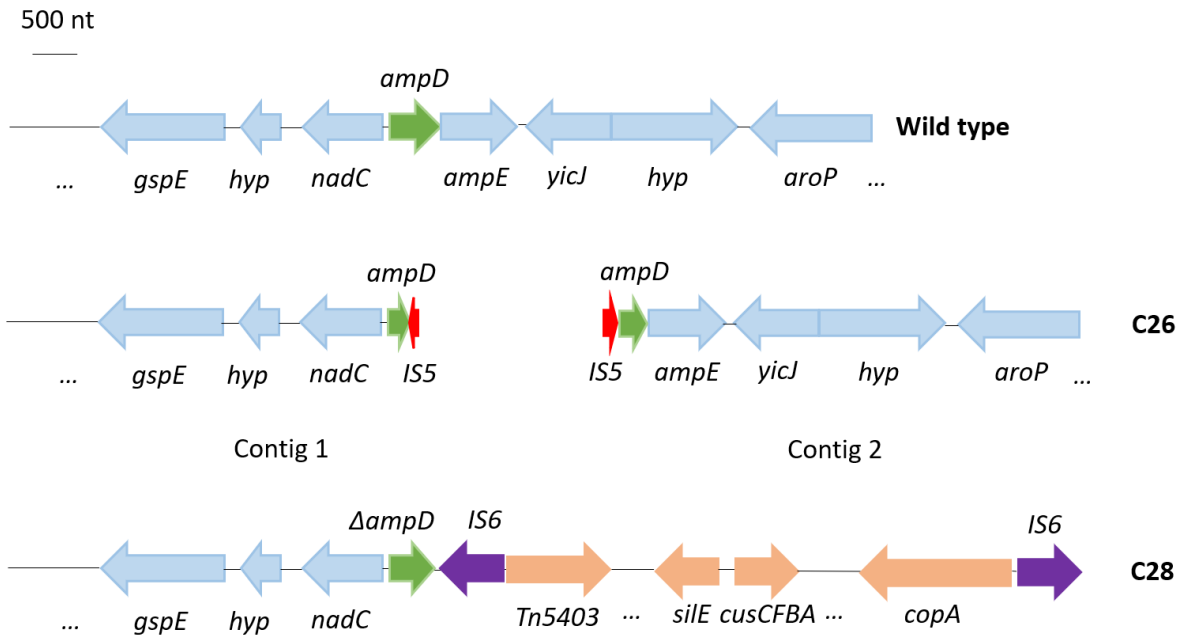

Figure 19 Isolate C26 contains an IS5 within *ampD* and isolate C28 contains a large transposable element, inserted into ampD, that is causing CAZ-R. The genetic environment around *C. freundii ampD* (green) in the wild type genome (ATCC 8090) (top). Isolate C26 (middle) has an IS5 inserted into the *ampD* gene and isolate C28 has an IS6 (purple) carrying a large transposable element with heavy metal resistance genes inserted into the *ampD* gene (bottom).

Isolate C28 contains a large transposable element inserted at the 3' end of *ampD.* IS Finder shows the IS belongs to the IS6 family (Table 21), with results showing an E-value of 0 for both IS26 and IS15. Two identical matches have occurred because the query nucleotide sequence is short, however IS26 is dominant in antibiotic resistance (ABR)– it is the element which mobilises bla_{SHV} as well as CTX-M-15 and aac(6')-Ib-cr (Ford, 2004; Mounsey *et al.*, 2023). This transposable element contains genes associated with heavy metal resistance, including those encoding silver binding proteins (SilE, SilP), copper resistance protein (CopA) and cation efflux system proteins (CusCFBA), along with other genes not found on the *C. freundii* chromosome (Figure 19). There is another copy of these genes found elsewhere on the chromosome of isolate C28, and they are also present in CAZ-S and reference isolates however an additional copy, with slightly differing protein sequences, is found on this transposable element.

Isolate E49 contains an IS with a low identity compared to others in the database; the closest match was an IS200 family member, however the *E*-value was 0.78 (Table 24). The IS inserted itself into the *ampD* gene at a similar position to the IS5 found in isolate C26 (Table 24), disrupting the transcription of *ampD.*

Two isolates (E3 and E56) collected 30 years apart contained identical IS from the IS110 family each present in an identical position relative to the start of *ampD*. These isolates were different subspecies of *E. hormaechei* and so unrelated, making this an interesting finding which should be investigated further. Figure 29 shows how the IS has disrupted the *ampD* promoter sequence in E3 and E56 compared to the WT reference sequence. The AG rich region upstream of the *ampD* ATG start codon is the probable ribosome binding site. However, the presence of this IS within the promoter has abolished the ribosome binding site, preventing *ampD* transcription.

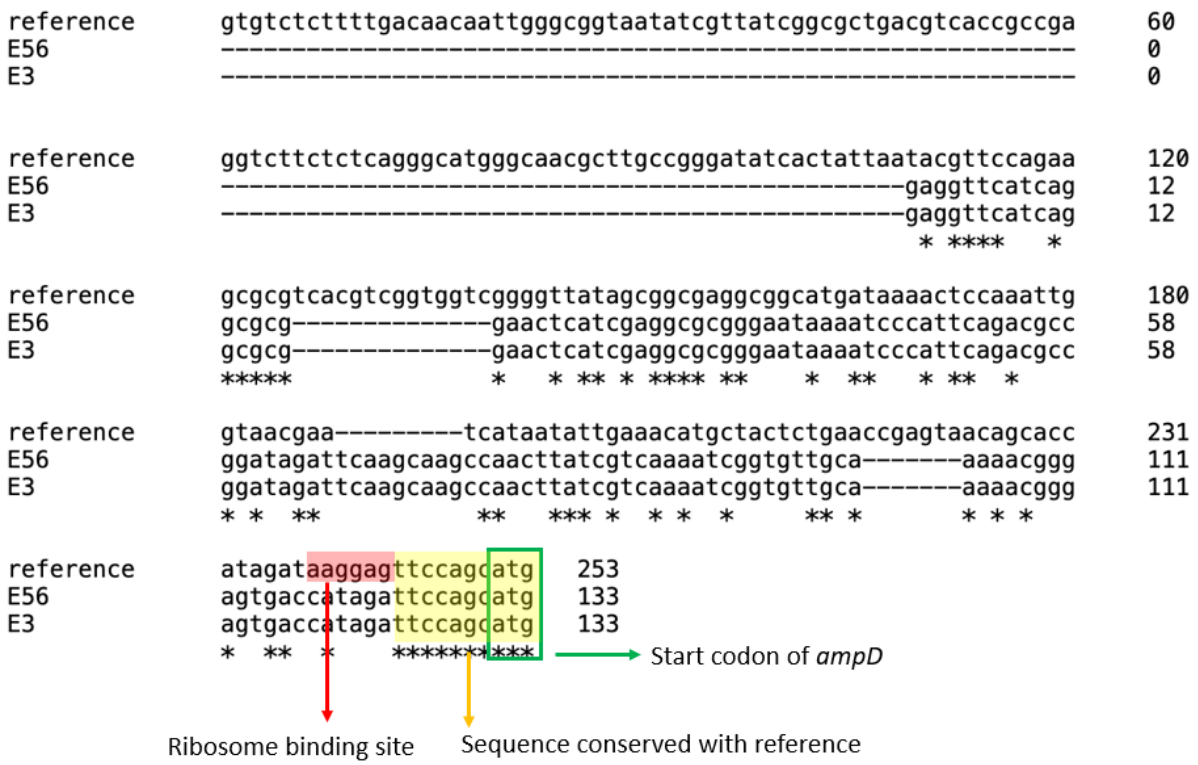

Figure 20 Isolate E56 and E3 contain identical IS that have inserted at the same position within the *ampD* promoter. Alignment of the nucleotide sequence upstream of the *ampD* start codon (green) of two clinical isolates against the *E. hormaechei* reference sequence. The alignment shows that the sequence is identical in isolates E3 and E56 however there is only a small sequence conserved between all three (yellow). This yellow region is the WT sequence whereas the rest of the sequence in E3 and E56 is the IS110 element.

As alluded to in section 3.3.2, a pair of *E. hormaechei* subsp. *steigerwaltii* isolates from 2020 exhibiting different CAZ susceptibility phenotypes were <100 SNPs apart (Figure 10). The CAZ-R isolate (E3) contained an IS embedded in the *ampD* promoter whereas the CAZ-S isolate

contained a WT genetic arrangement around *ampD* (Figure 19). The IS is not part of the core genome so will not contribute to the SNP distance calculation which shows that there is only 54 base pairs different between the isolates across the core genome. This provides further evidence that this IS causing AmpD loss of function is the cause of CAZ-R in this isolate and suggests the emergence of resistance in situ.

5.4. Missense mutations

Missense mutations are a nucleotide substitution that leads to an amino acid change within the protein. Missense mutations are common and do not always affect protein function. The resulting amino acid substitution can be either conservative, or non-conservative. Conservative substitution – when the original and replacement amino acid have similar chemical properties – generally do not negatively affect the protein. Non-conservative substitutions, whereby the new amino acid has different chemical properties tend to have more serious effects, often leading to reduced or total loss of activity.

Missense mutations in *ampD* unique to CAZ-R isolates (i.e. not found in CAZ-S isolates) were identified in 14 isolates, making missense mutations the most common type of *ampD* mutation observed. Twelve of these 14 isolates had a single amino acid substitution in AmpD. One isolate had two substitutions, and one had three (Table 19). Further analysis on isolates which had more than one substitution was not carried out due to the difficulty in attributing cause of CAZ-R to a specific substitution. Across the 12 isolates with a single amino acid change, there were seven different substitutions as three occurred in more than one isolate (Table 19). Figure 30 highlights the locations of these residues within AmpD, showing that they are all broadly near the substrate binding site, suggesting they play an important role in protein function.

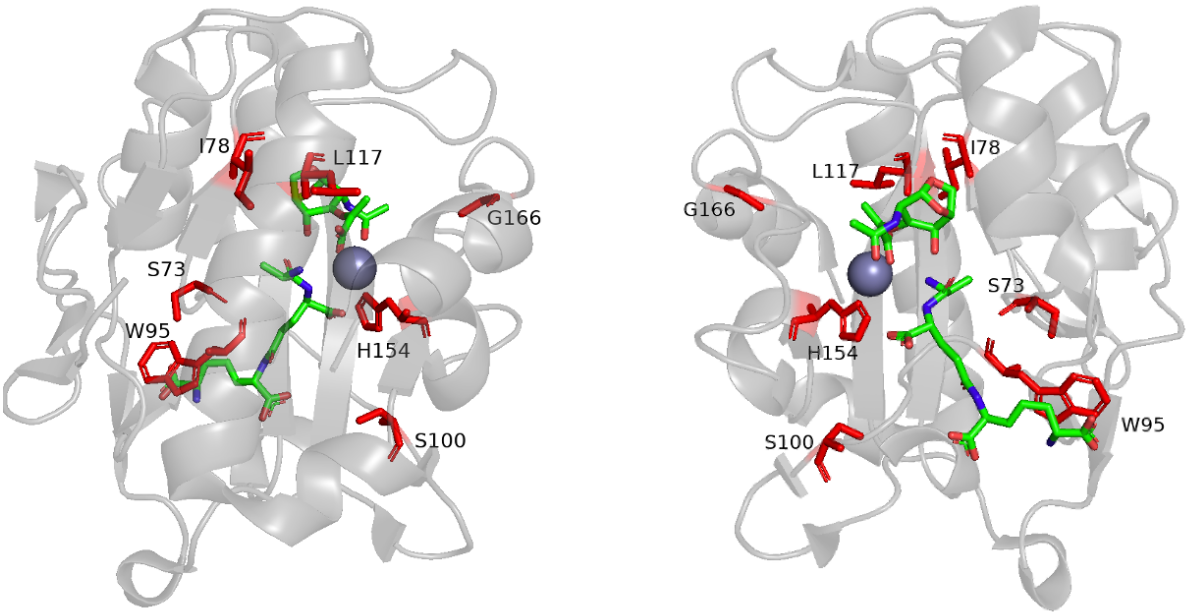

Figure 21 Substitutions at seven amino acid residues in AmpD are proposed to cause CAZ-R. Two orientations of the 3D structure of *C. freundii* AmpD (PDB code: 2Y2B) with the 1,6-anhydro-muropeptide substrate shown in green. The seven amino acids positions where substitutions occur only within CAZ-R clinical isolates are in red. Substitutions at four of these positions have previously been shown to cause CAZ-R, the other three are being reported for the first time.

5.5. Previously described AmpD amino acid substitutions

From these seven amino acid substitutions unique to CAZ-R isolates, substitution of four of the residues have been previously reported to disrupt AmpD function (H154, W95, L117 and G166) and are hence implicated in AmpC hyper-production leading to a CAZ-R phenotype. In this study, substitutions of H154 occurred in two clinical isolates: H154N (E6) and H154L (E33). In isolate E33, there were no amino acid substitutions in other proteins relevant to AmpC hyper-production analysed and proteomics showed AmpC hyper-production (Table 12), likely confirming the role of H154L as a loss of function AmpD mutation.

Cephalosporin resistant isolates with both substitutions and premature stop codons at residue W95 in cephalosporin resistant isolates have been described numerous times in the literature (Stapleton, Shannon and Phillips, 1995; Carrasco-López *et al.*, 2011; Xie *et al.*, 2022). Isolate E54 with a W95R substitution shows AmpC mediated CAZ-R as it had significantly higher ($p < 0.05$) AmpC levels than the CAZ-S control (Table 12). This suggests that the W95R substitution affects AmpD function.

Another position at which an amino acid substitution identified in my clinical isolates has been previously reported is L117. Isolate E59, from the 1980s, has a L117Q substitution (Table 19). The final residue identified in this study that has been implicated in literature as 3GCR-causing is G166. Two isolates in this study, of different species and from different timeframes, have G166S substitutions (Table 19), the implications of which is discussed later.

5.6. Novel AmpD amino acid substitutions

In addition to those previously described, there were three residues at which amino acid substitutions occurred in these clinical AmpC hyper-producing isolates which are not mentioned in current literature: S73, I78 and S100. In general, AmpD sequence is highly conserved across genera (Figure 22) (Petrosino *et al.*, 2002). Therefore, the conservation of these residues was investigated to determine their substitution rates among species with inducible *ampC* in comparison with species having no *ampC* or a non-inducible *ampC*. By comparing rates of substitution (how often an amino acid at a particular position was conserved and how often any other amino acid was present across the entire database for a given species) in these species (across the NCBI database), an estimation of the importance of the residue to AmpD function can be made. Expression of inducible *ampC* is affected by AmpD, and so mutation might be under selection for AmpC hyper-production and CAZ-R, which might mean over-representation of a database biased towards clinical isolates, but in bacteria with no *ampC,* or a non-inducible *ampC,* substitutions in AmpD should not be under the same selective pressure in nature, and so also in terms of their representation in the database. Therefore, higher substitution rates in species with inducible *ampC* relative to species that do not, may indicate that this AmpD substitution is a positive selection pressure.

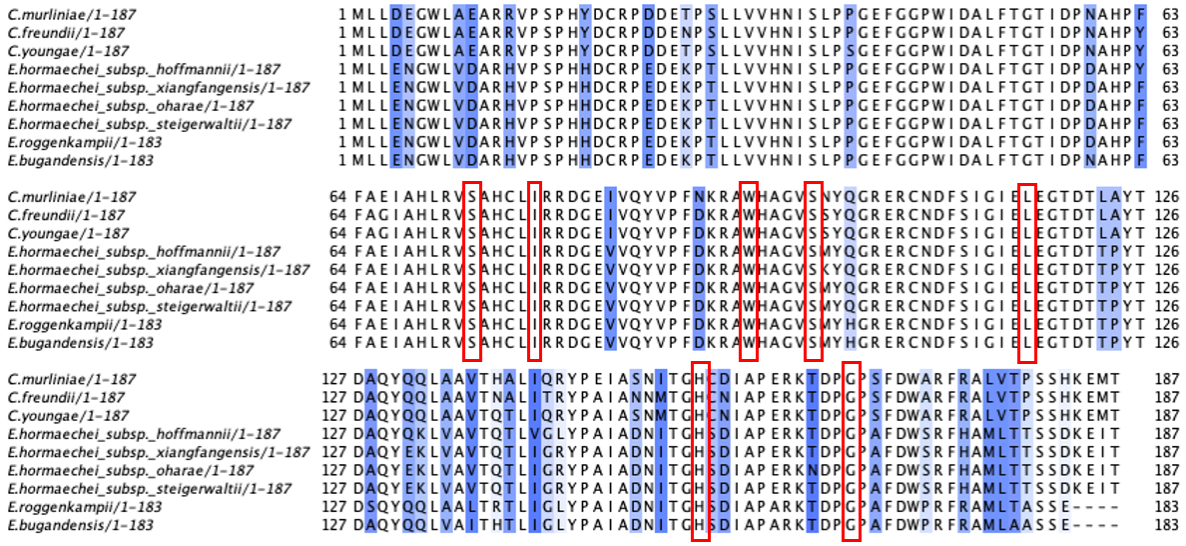

Figure 22 AmpD sequences are highly conserved between *Enterobacter* and *Citrobacter* spp. AmpD sequence alignment of reference sequences from different *Enterobacter* spp. and *Citrobacter* spp. found in this study show that the sequence is highly conserved not only within, but between, genera. Positions highlighted in blue are those where the consensus sequence is below 90% similarity threshold across all sequences. Positions outlined in red are the seven positions where substitutions unique to CAZ-R clinical isolates are found. Made using CLUSTAL for alignment and JalView for visualisation.

To do this, the conservation of residues within AmpD sequences of 1812 entries on the NCBI database (all those identified using a blastp search as outlined in section 2.5) across a variety of inducible *ampC* and non-inducible *ampC* containing (and *ampD* negative) *Enterobacteriales* species were analysed (Table 22). Table 22 shows the higher conservation rate of residues S73, I78 and S100 across all 1812 database entries (so irrespective of *ampC* inducibility status) compared to residue A60, a residue which was frequently substituted in the AmpD of both CAZ-S and CAZ-R clinical isolates seen in this study. There is absolute conservation of these three residues across the (CAZ-S) reference sequences of *Enterobacter* spp. and *Citrobacter* spp. found in isolates in this study (Figure 22). This puts into context the high level of conservation of these three residues, suggesting they may be important to AmpD. Further evidence for this is that all three residues are located near to the AmpD substrate binding site (Figure 23).

Amino acid	Frequency at which each amino acid is conserved in All species	Inducible *ampC* species	Non-inducible *ampC* species	χ^2 *p*-value
A60	95.36%	94.76%	97.75%	-
S73	99.72%	99.58%	99.82%	0.345
I78	99.28%	99.91%	98.32%	9.13E-05
S100	99.89%	99.86%	99.91%	0.954

Table 19 Conservation rates of specific amino acids within AmpD show that the three residues at which substitutions occur in CAZ-R isolates are highly conserved. Substitution rates of certain amino acid residues within AmpD obtained from a blastp search with the input sequence of AmpD from different species (listed in table 2). The percentage of NCBI AmpD database entries with the amino acid at that position in a) all species, b) species with an inducible *ampC* (defined by the presence of *ampR)* and c) species with a non-inducible *ampC* (without the presence of *ampR).* Residue A60 is frequently substituted in both CAZ-S and CAZ-R clinical isolates whereas S73, I78 and S100 are only substituted in CAZ-R clinical isolates. The conservation rate of A60 is much lower than that of the other three, putting into context the high level of their conservation.

A serine substitution at I78 was present in four CAZ-R clinical isolates of different species from the 1980s (Table 19). Within the BLAST database, there was significantly higher substitution rates amongst species with inducible *ampC* (99.91%) than those with non-inducible *ampC* (98.32%) at this residue (Table 22). This suggests that a substitution at this residue may be under positive selection among inducible *ampC* species. The sequences comprising the 0.09% (12) of results of from the inducible *ampC* group with a substitution at I78 were all from *E. hormaechei* isolates*.* At surface level, this clustering may suggest that this was a clonal outbreak or that it is genetic variation unique to *E. hormaechei.* The sample records revealed that two of 12 isolates were the same, however the rest were unrelated. Furthermore, substitution at I78 is present in two different *Citrobacter* spp. in this study showing that it also occurs in other genera. Interestingly, I78S occurred four times in 1980s isolates however it was not found at all in 2020 isolates. Due to the sporadic emergence of this substitution, it is intriguing as to why this previously occurred so frequently but is no longer observed. Possibilities include that there was selection pressure for I78S then but not now, or that there was a possible fitness cost caused by it then and not now. However what this could be is undetermined.

Although I78 substitutions have not been previously reported in CAZ-R isolates, the literature has described residues R79 and R80 as being involved in a key bonding network which

provides structural stability during conformational changes of AmpD between its active and inactive state (Carrasco-López *et al.*, 2011). Indeed, they show that R80A substitutions result in this salt-bridge network being locked in the open conformation and that these interactions are with residue E42 which mutagenesis shows is crucial for AmpD structure (Carrasco-López *et al.*, 2011). As well as this, these arginine residues on the surface of the protein, along with nearby R12 and R13, could represent an area of the protein involved in sensing triggers of conformational change (Carrasco-López *et al.*, 2011). Therefore, it is unsurprising that our data suggests a substitution at I78 affects AmpD activity.

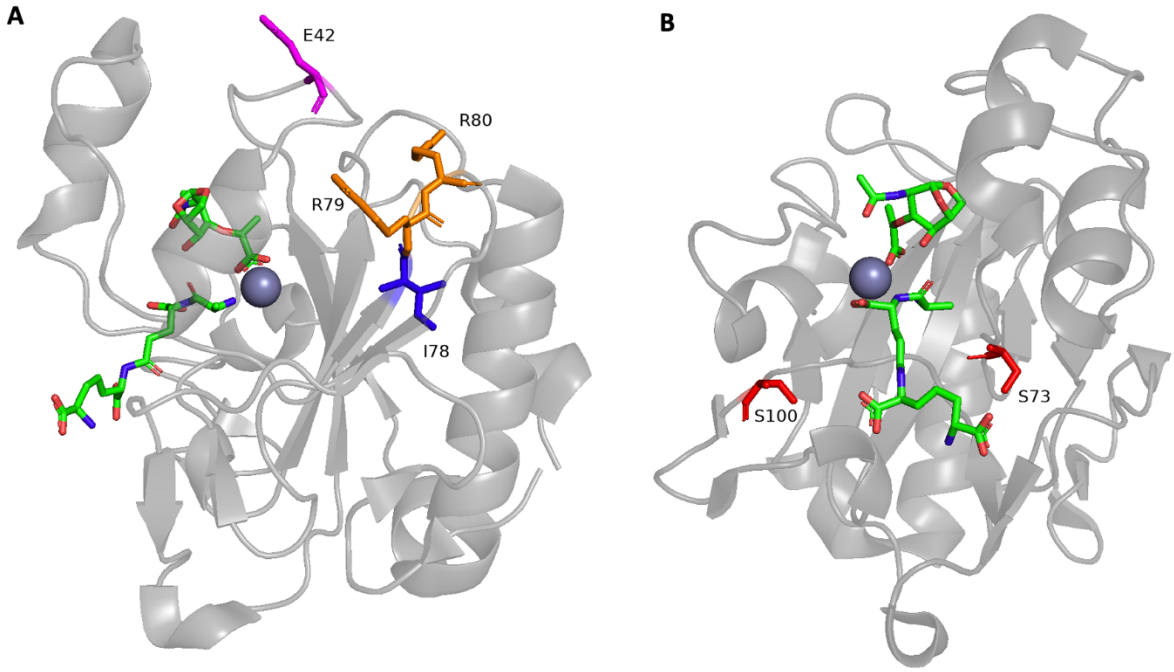

Figure 23 Residues S100, S73 and I78 which are substituted in CAZ-R isolates are either near the substrate binding site or close to residues involved in a key bonding network. 3D visualisation of *C. freundii* AmpD (PDB code: 2Y2B) with the 1,6-anhydro-muropeptide in green shows the three residues, which are substituted in CAZ-R isolates, that have not been previously described. (A) Four residues of interest are highlighted – I78, substituted in four CAZ-R clinical isolates (blue). R79 and R80 (orange) and E42 (pink) near this residue are residues involved in a key bonding network. (B) Two residues, S100 and S73 (red) are near to the substrate binding site.

One *C. youngae* isolate from 1990 (C34) has a unique substitution of S73F. Within the other proteins analysed the only other substitution present was T325K in the Mpl protein. There is no evidence in the literature of T325 substitutions affecting Mpl function and given that AmpD mutations are the leading mutational cause of AmpC mediated CAZ-R, this S73F substitution is more likely to be a cause of *ampC* overexpression. However, when looking at substitution rates of S73, there was no significant difference ($p > 0.05$) between the subtitution rate in

inducible and non-inducible *ampC* species (Table 22). However, this residue is still highly conserved amongst AmpD sequences in the BLAST database (Table 22). Therefore, there is not enough evidence to suggest whether substitutions at this residue would be beneficial for inducible *ampC* species or not.

An *E. hormaechei* subsp. *oharae* isolate (E63) from 1988 has a S100L AmpD substitution and no other amino acid substitutions within the other protein sequences analysed. However, similarly to S73, mutability analysis showed that there was no significant difference *($p > 0.05$)* in the substitution rates of S100 between inducible and non-inducible *ampC* species (Table 22). S100 is also highly conserved amongst sequences in the BLAST database and therefore it is difficult to evaluate the potential role that a S100 substitution is having on AmpD function and its effect on *ampC* expression.

5.7. Conclusions and Discussion

Analysis of protein sequences from CAZ-R and CAZ-S isolates showed that mutations in *ampD* are the most common mutations leading to AmpC hyper-production in CAZ-R isolates, corroborating previous literature (Jacoby, 2009; Guérin *et al.*, 2015a). Our data shows that missense mutations are by far the most common *ampD* mutation, occurring in 50% of isolates. The remaining 50% of isolates contain nonsense and frameshift mutations and insertional inactivation due to IS which all lead to AmpD loss of function. The distribution of missense and loss of function mutations is similar between the *Enterobacter* and *Citrobacter* genera, and across the different timeframes of isolate collection.

Within the AmpD substitutions characterised in these isolates, there were four residues at which substitutions have been previously characterised in 3GC-R isolates: H154, W95, L117 and G166. Histidine 154 is one of three key residues (H34, H154 and D164) involved in binding of the catalytic zinc ion (Liepinsh *et al.*, 2003) Mutagenesis studies have shown that alanine substitutions at these three residues lead to inactive AmpD due to their loss of ability to bind zinc (Stapleton, Shannon and Phillips, 1995; Généreux *et al.*, 2004). Intriguingly, previous work has shown that although H154A is a loss of function substitution, an H154N mutant retains its

ability to coordinate the zinc ion, as well as its amidase activity (Généreux *et al.*, 2004). Our hypothesis is that the H154N is a loss of function causing mutation and that the *in vitro* method to measure amidase activity used in previous work rendered misleading results. It is possible that the H154N substitution would have a different effect *in vivo* which may not be apparent *in vitro.* This is particularly likely since our proteomics showed that, even in CAZ-S control isolates, AmpD is present at undetectable levels. Therefore since cellular levels are so low, only a small reduction in AmpD levels may cause *ampC* overexpression and this could well be within the error of an *in vitro* experiment. Given our proteomics results showing H154L is causing AmpC hyper-production, the key role of H154 in protein function and the argument above, we hypothesise that this H154N mutation does lead to AmpD loss of function causing CAZ-R in this isolate.

W95 has been highlighted as an important hydrophobic residue due to its large, exposed surface area which helps to stabilise the 1,6-anhydro-*N*-acetylmuramul substrate (Carrasco-López *et al.*, 2011). Therefore, it is unsurprising that amino acid substitutions at this position will lead to AmpD loss of function and subsequent *ampC* overexpression.

Substitutions at residue L117 were initially reported in 2001 however the effect on AmpD was uncertain (Petrosino *et al.*, 2002). A subsequent study implicated L117 in affecting AmpD function due to its location within the protein core (Carrasco-López *et al.*, 2011). The neighbouring residue E116, found deep within the substrate binding cavity, is crucial for AmpD activity (Généreux *et al.*, 2004). E116A AmpD mutants are completely inactive, possibly because E116 may ensure correct orientation of the H34 residue which is required for zinc coordination (Généreux *et al.*, 2004). Therefore, it is possible that L117 substitutions may diminish the ability of E116 to do this, and so affect AmpD function.

Previous work generating spontaneous 3GCR AmpD mutants showed that a substitution of G166 (G166C, G166D) occurred in three of their 32 mutants (Xie *et al.*, 2022). Residue 166 is nearby D164, a key zinc coordinating residue, mutants of which are inactive (Stapleton, Shannon and Phillips, 1995). Therefore, it is possible that substitution of G166 disrupts the orientation of D164, reducing its capacity to bind zinc and hence the amidase activity of AmpD.

Some missense mutations identified in CAZ-R isolates had been previously described in the literature however there were three which had not. Evidence from this study and literature around the surrounding residues suggests that I78S is a loss of function mutation in AmpD. The effect that substitutions at position S73 and S100 have on AmpD is less clear. It is difficult to draw conclusions about the effect that a substitution has on AmpD function from substitution rates obtained from the BLAST database. It is possible that comparisons between the substitution rates in inducible and non-inducible *ampC* species are biased. Since the database is a clinical database it may contain more *ampD* mutants in inducible species since they are under the selection pressure of emerging resistance to CAZ. Therefore, this data must be taken in context with other factors. In order to further investigate the effects of these substitutions, the *ampD* gene could be cloned and transformed into competent cells containing a non-functional AmpD. CAZ-MIC testing would then show whether or not the transformed gene would recover any AmpD activity and increase the susceptibility of the recombinant to CAZ.

6. General Discussion

Characterisation of ABR profiles in 80 isolates from 2020 and 19 isolates from the 1980s show that the highest levels of resistance was to β-lactam antibiotics. All isolates exhibited the expected resistance phenotypes to amoxicillin and amoxicillin clavulanate depending on the class of β-lactamase enzyme present. In 2020, of the 55 *Enterobacter* spp. containing AmpC (class C) β-lactamase enzymes, 20% were resistant to third generation cephalosporin (3GC), ceftazidime (CAZ). This reflects what we see in national statistics, making our dataset representative. All isolates from the 1980s were collected due to their CAZ resistance. In 2020 a small number of isolates were resistant to non β-lactam antibiotics. Interestingly, in the 1980s no isolates showed resistance to these agents, highlighting the lack of mobile antibiotic resistance (ABR) genes then, and the presence of them now.

These antibiotic phenotypes are suggestive of AmpC mediated β-lactamase resistance, and proteomics data reveals that this is indeed the dominant CAZ-resistance mechanism in these isolates. In the 1980s isolates it was the only ABR mechanism observed and was associated

with CAZ-resistance but low levels of TZP resistance. In isolates from 2020, AmpC hyper-production was associated with CAZ resistance and high levels of TZP resistance. The other mechanism conferring ABR in 2020 isolates were mobile ABR genes. Of not is bla_{OXA} for cefepime (FEP) and piperacillin/tazobactam (TZP) resistance and $bla_{CTX\text{-}M\text{-}15}$ for cefotaxime (CTX) resistance.

One of the most interesting observations from this study is the increased levels of TZP resistance in 2020 compared to the 1980s. We hypothesise that this is due to the selection pressure of increased TZP use (which did not start until 1994) driving the emergence of resistance in later years, however the mechanism through which this resistance is arising is unclear. There is no correlation between levels of AmpC based on proteomics data and TZP-disc diffusion zone, no correlation between specific substitutions in AmpC or AmpD occurring uniquely in TZP-R isolates and no correlation between the type of *ampD* mutation (frameshift, missense, nonsense, insertional inactivation) and TZP susceptibility. There is also no obvious population based explanation as there is no evidence of clonal switch over time towards a subgroup that is more TZP-R. Conducting proteomics on the 1980s isolates which are TZP-S would allow the comparison TZP-S and TZP-R isolates and may help to elucidate a mechanism.

One hypothesis to explain the significantly higher levels of TZP resistance in 2020 compared to the 1980s is that the old isolates may have lower levels of AmpC hyper-production than the new isolates. This leads to the hypothesis that mutations in the old isolates have a less severe effect on AmpD function than mutations occurring in 2020 isolates. Hence, this could explain the interesting observation as to why I78S is observed in the old, but not the new, isolates. However, we have shown that total loss of function mutations occur at similar rates across the timeframes, and that loss of function mutants from the 1980s are TZP susceptible, providing evidence against this hypothesis. Conducting proteomics on the 1980s isolates to determine AmpC levels would test this further as it may be that there is an unrelated mechanism at play in the 2020 isolates, such as overproduction of efflux pumps.

Maximum likelihood phylogenetic trees and single nucleotide polymorphism (SNP) dist analysis show that, for all species, there is no clustering of CAZ-R isolates separately to CAZ-S isolates. Furthermore, when considering only CAZ-R isolates, there is no clustering of isolates

into clades of the timeframes they were collected from. This highlights the sporadic nature of *Enterobacter* and *Citrobacter* spp. infections. It corroborates our findings that the primary cause of CAZ-resistance is a mutational cause rather than a genetic cause outside of the core genome which has resulted from clonal expansion from one isolate.

This study corroborates previous literature showing that this mutational cause is most frequently mutations occurring in *ampD* (Jacoby, 2009; Guérin *et al.*, 2015a). All the CAZ-R isolates in this study that are AmpC hyper-producing have mutations within *ampD,* either as a loss of function or, most frequently, missense mutations. In addition to previously described amino acid substitutions caused by these mutations, we characterise three novel AmpD substitutions which we propose to be causing CAZ-R.

Having a more detailed understanding of the underlying mechanisms driving ABR in *Enterobacter* spp. and *Citrobacter* spp. could help to guide the appropriate future treatment of these infections and therefore potentially preserve the use of critically important antibiotics, such as carbapenems, currently used to treat such infections.

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