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Mechanisms of cephalosporin resistance in Escherichia coli from cattle and humans

Mechanisms of cephalosporin resistance in *Escherichia coli* from cattle and humans

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ABSTRACT

Antibacterials of the β-lactam class (including penicillins, cephalosporins and carbapenems) are the most commonly used antibacterials in human and veterinary medicine. Understanding the underlying mechanisms of β-lactam resistance is therefore important. The overarching aims of the current project were i) to characterise cephalosporin resistance mechanisms in *Escherichia coli* isolates from dairy farms and urinary tract infection (UTI) in the absence of cephaosporinases, and to assess evidence for farm to human (zoonotic) transmission, and, ii) to generate *E. coli* mutants with reduced susceptibility to cephalosporins or to the carbapenem ertapenem, and to determine how the mutations acquired induce their phenotypic effects.

This study demonstrates that the main cause of 3rd generation cephalosporin (cefotaxime) resistance in farm and UTI isolates lacking mobile cephalosporinases is the hyperproduction of chromosomal AmpC due to promoter mutations; different promoter mutations were shown to induce different levels of AmpC production, and some of these additionally led to cefoperazone resistance. Cefotaxime resistance was extended to 4th generation cephalosporin (4GC), cefoperazone and ceftazidime resistance in some cases where AmpC was mutated, resulting in an expanded-spectrum variant. Cefoperazone resistance was also caused by MarR-mutation mediated AcrAB-TolC efflux pump activation in one AmpC hyperproducer isolate or TEM-1 hyperproduction in another. 4GC resistance was associated with additional production of OXA-1 in several AmpC hyperproducers.

Phylogenetic analysis revealed no evidence for acquisition of farm-related AmpC hyperproducer *E. coli* isolates by members of the local human population, though farm to farm

transfer was common. Presence of AmpC hyperproducers on farms was associated with the use of amoxicillin/clavulanic acid, and not with the use of cephalosporins.

Resistance to the first-generation cephalosporin cefalexin was found not to be β-lactamase mediated in a large proportion of farm and UTI isolates. In these cases, we identified that the phenotype was caused by OmpF porin disruption or downregulation. Importantly, multiple regulatory mutations that cause OmpF downregulation were identified. In addition to mutation of OmpR, already known to downregulate OmpF and OmpC porin production, we report a *rseA* mutation, which strongly activates the Sigma E regulon, greatly increasing DegP production leading to degradation of OmpF and OmpC porins. Furthermore, we showed for the first time that mutations affecting lipopolysaccharide structure, exemplified by the loss of GmhB, essential for lipopolysaccharide heptosylation, and even the essential lipopolysaccharide biosynthetic committed enzyme LpxB, also activate DegP production, resulting in OmpF degradation. Remarkably, given the critical importance attached to such systems for normal *E. coli* physiology, we find evidence of *gmhB*, *lpxB* and *rseA* mutations in *E. coli* isolates derived from human infections. Finally, we show that these regulatory mutations enhance the ability of group 1 CTX-M β-lactamase to confer reduced ertapenem susceptibility, particularly those mutations that cause OmpC in addition to OmpF downregulation and that OmpC loss confers ertapenem reduced susceptibility on AmpC hyperproducer *E. coli*, particularly if the AmpC has an expanded spectrum of activity.

Overall, this work has identified important, previously unknown mechanisms of cephalosporin resistance in *E. coli*, and has added to our understanding of how multiple mechanisms come together to confer late generation cephalosporin and carbapenem resistance. These findings are important for the utility of WGS analysis as a tool to predict antibacterial susceptibility.

DEDICATION

I dedicate this achievement to my family. A special gratitude to my loving parents for their constant love, prayers, caring and helping in whatever way they could during this challenging period.

To my sister Halima for her unconditional love and endless support not only during the PhD journey but throughout my whole life; her unhesitatingly support has helped my — and her own — dreams come true.

To my brothers, for their continuous support and for reminding me that I was awesome, even when I did not feel that way.

To all those who made this achievement possible: love and unlimited gratitude.

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After an intensive period of six months, today is the day: writing this note of thanks is the final touch to this thesis. While a completed thesis bears the name of a single student, its completion is always through the dedicated work of many people. I would like to reflect on those who have supported and helped me so much throughout this period.

I would like first to express my deep and sincere gratitude to my research supervisor, Professor Matthew Avison, for giving me the opportunity to undertake this research, and for providing invaluable guidance throughout. Thank you for your patience, support, and encouragement, during both the practical work and writing up of this thesis. Without your guidance and constant feedback, this work would not have been achievable. Thank you too for the friendship, empathy, kindness, and care that you gave to every member of your group, especially during the challenging times of the Covid-19 pandemic. It was a great privilege and honor to work and study under your supervision.

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Finally, my thanks go to all the people who have supported me to complete this research work directly or indirectly.

Thank you very much, everyone!

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 the candidate's 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: Maryam Mohammed Ali Alzayn DATE: 15 SEPT 2021

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Abbreviations

PBPs	penicillin-binding proteins
MRSA	Methicillin-resistant Staphylococcus aureus
MSSA	methicillin-sensitive Staphylococcus aureus
ESBLs	Extended Spectrum β-Lactamase
1st GCs	First generation cephalosporins
3rd GCs	Second generation cephalosporins
4th GCs	Fourth generation cephalosporins
CRE	Carbapenem Resistant *Enterobacteriaceae*
WHO	World Health Organisation
FDA	Food and Drug Administration
MFS	major facilitator superfamily
SMR	small multidrug resistance
RND	resistance-nodulation-cell division
MATE	multidrug and toxin extrusion
ABC	ATP-binding cassette
MDR	Multidrug resistance
pAmpC	plasmid-mediated *ampC* genes
Aph (3')	Aminoglycoside-3'-phosphotransferase
DHFR	dihydrofolate reductase
LB	Luria Bertani
WGS	Whole genome sequencing
CLIMB	Cloud Infrastructure for Microbial Bioinformatics
SNP	Single nucleotide polymorphism
CAMHB	Cation Adjusted Muller Hinton Broth
MIC	Minimal inhibitory concentration
CLSI	Clinical and Laboratory Standards Institute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
CTX-R	cefotaxime-resistant
LC/MS-MS	Liquid Chromatography/ Mass Spectrometry- Mass Spectrometry
HP-CIA	Highest priority critically important antimicrobial
RAPD	Random Amplified Polymorphic DNA
ESCR-EC	Extended spectrum cephalosporin resistant *E. coli*
STs	Sequence type
NCBI	National centre for biotechnology information

Chapter 1
Introduction

1.1 History of antibiotics and other antibacterials: An overview

The term “antibiotic” is used to describe natural products (chemicals produced by bacteria and fungi) while “antibacterial” is a wider term that includes synthetic chemicals as well. Since many clinically used antibiotics are produced synthetically, and since there is confusion about these two terms, “antibacterial” will be used in this thesis when referring to natural or synthetic chemicals with antibacterial activity, unless use of the “antibiotic” is specifically necessary. Antibacterials were administered many years before the advent of modern medicine. In ancient Egypt, the benefit of filamentous fungi growing on bread was known for treating wounds and burns (1). Chinese and Greek healers used musty texture to treat illness in the Middle Ages. In the 19th century, scientists started to notice that some bacteria could inhibit others and identified the ability of moulds to inhibit bacterial growth (2). In 1909, the first chemical-based antibacterial used commercially under the name Salvarsan discovered by Paul Ehrlich who identified the effectiveness of arsphenamine, an arsenic derivative, against *Treponema pallidum*, the causative of syphilis. This disease had reached an epidemic level and was incurable at that time in the USA and Europe. Salvarsan and Neosalvarsan (a more soluble and less toxic derivative) drugs were a great success despite some drawbacks. These antibacterials were most frequently prescribed until the 1940s when penicillin replaced them (3).

Penicillin was accidentally discovered and purified from *Penicillium notatum* by Alexander Fleming although the industrial production of this antibiotic was performed seven years after its discovery, in 1940, by Howard Florey and Ernst Chain, using *Penicillium chrysogenum* (4). In the same year, Selman Waksman developed a systematic research programme to identify the antibacterial activity of soil bacteria, particularly from filamentous Gram-positive bacteria

named *Streptomyces*, members of *Streptomycetes* (5). A wide range of major antibacterials and antifungals were then discovered, such as actinomycin (from Streptomyces spp.) (6), streptomycin (from *Streptomyces griseus*) (7), neomycin (from *Streptomyces fradiae*) (8), fumigacin (from *Aspergillus fumigatus*) and clavacin (from *Aspergillus clavatus*) (9) by using several culture techniques and strategies (e.g. the 'Waksman platform') (5). Some of these drugs, including streptomycin, actinomycin, and neomycin, are still in use. Inspired from the Waksman platform, more than 20 examples of present-day antibacterials were discovered from a large number of bacteria and fungi between the 1940s and 1970s (2).

1.2 Use of antibacterials and challenges of new discoveries

Development of antibacterials provided simple and effective treatments against bacterial infections, which were, prior to the widespread use of antibacterials, a major cause of morbidity and mortality, meaning that antibacterials have had significant effects on human health and longevity (10). For example, between 1938 and 1952, the mortality rate due to pneumonia, influenza, and tuberculosis was reduced by 8.2% per year in the USA, which can be primarily attributed to the introduction of antibacterial chemotherapy (11).

Most of the antibiotics from bacterial and fungal sources were discovered during the golden age, between the 1940s and 1960s. However, since the 1980s, no new classes of antibiotics have been found and brought to the clinic (Figure 1.1). This shows the importance of the development of new strategies in antibacterial drug production. For example, industry has turned to in vitro synthesis of antibacterial drugs, but only a few classes of synthetic antibacterials have been discovered and brought to the clinic (e.g., nitrofuran - 1953; quinolones - 1960; sulphonamides - 1961; oxazolidinones - 1987). In parallel, a number of already discovered antibacterials that had previously failed in the commercial pipeline, have

been approved for commercial use such as linezolid in 2003 and daptomycin in 2001, which had been known since 1955 and 1986, respectively (12). Although a large number of antimicrobial peptides (AMPs) have been developed from different origins include animals, invertebrates and plants; none of them have been used as systemic antibacterials, and instead they are used as topical agents (13).

A mainstay to provide diversity of antibacterial drugs has been the production of semisynthetic compounds from natural product antibiotics, such as ketolides and metronidazole which derived from macrolides, and a natural product of *Streptomyces* spp., respectively. The synthetic penicillin, cephalosporin and carbapenem derivatives are very diverse, as will be discussed later, and in recent years, cefiderocol, a new cephalosporin derivative modified to improve penetration into Gram-negative bacteria, was found to be effective against carbapenem-resistant bacteria (14).

Unfortunately, economic drivers have led pharma companies to shift their priority from identifying new antibacterials to the development of drugs for chronic diseases (12). Among the 20 companies that invested in antibacterial discoveries in the 1980s, only five companies remained by 2015 (15).

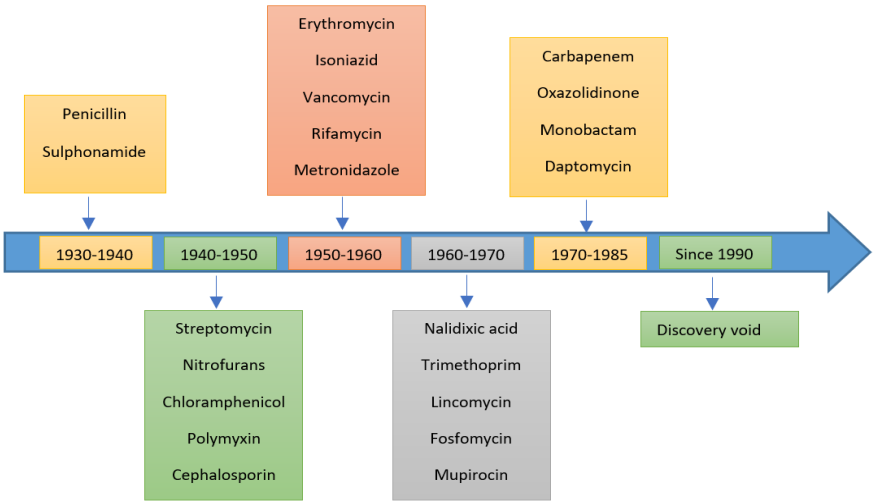


Figure 1.1: Pipeline of antibacterial development. Discovery void means the gap in antibacterials discovery. (Adapted from 16)

1.3 Antibacterial classes

Antibacterial agents include antibiotic natural products, semi-synthetic antibiotics, and fully synthetic antibacterial drugs. Generally, antibacterial agents are classified based on five broad categories (17), which are discussed in the following sections.

1.3.1 Type of action

Antibacterials can be classified into two based on the general effect that they have on cells: bacteriostatic and bactericidal. The antibacterial drugs that reversibly inhibit or slow down the growth of bacteria without killing them are termed as bacteriostatic (18) and those that kill bacteria are known as bactericidal. In fact, there is some overlap between these designations

for most antibacterials, dependent on the concentration of the drug and the time that it interacts with cells.

1.3.2 Source of antibacterial agents

Antibacterial drugs can be produced either from natural resources such as neomycin, cephalosporin, cefamycin and benzylpenicillin, or in the synthetic and semi-synthetic way. In the last form, the drugs are developed by chemically altering natural products. The semi-synthetic process of antibacterial production helps to overcome factors such as toxicity, insolubility, low permeation rates, and therefore increases the clinical effectiveness of natural product antibiotics. Fully synthetic antibacterials have the added benefit of not being exposed to bacteria until they are released, meaning that mechanisms that have pre-evolved to resist antibacterials, which have been present in nature for millennia, are less likely to be relevant. They can also be selected to have greater efficacy (lower antibacterial doses) and less toxicity than is true for many antibacterials, including semi-synthetic derivatives (19).

1.3.3 Activity spectrum

Based on their target specification, antibacterial agents can be classified into narrow or broad spectrum. The narrow spectrum antibacterial drugs are those that can work against narrow range of microorganisms either Gram-positive or Gram-negative bacteria only, or in some cases, only against anaerobes or against *Mycobacteria*. On the other hand, broad spectrum antibacterial agents are effective on both Gram-negative and positive bacteria meaning that their effects include wider range of pathogenic bacteria, and so they are more suitable for use when the bacterial cause of an infection is not known. However, narrow spectrum

antibacterials have the advantage that they do not kill as many normal-floral microorganisms in the body and the fact that fewer bacteria are targets means that resistance is being selected in fewer bacteria in total. Therefore, narrow spectrum antibacterials cause less superinfection and less resistance, which make these drugs preferred over broad spectrum antibacterials (18).

1.3.4 Function

The cellular functions essential for bacterial growth such as cell wall synthesis, cell membrane function, protein synthesis and nucleic acid synthesis are considered important targets for antibacterial agents. These processes are also either non-existent, or markedly different in Eukaryotic cells meaning that toxicity is likely to be selective. Therefore, antibacterial agents can be subdivided into four groups: cell wall (peptidoglycan) synthesis inhibitors, membrane function/structure inhibitors/disruptors, protein synthesis inhibitors, and inhibitors of nucleic acid synthesis, though this last class is diverse and includes agents that damage DNA (18) (Figure 1.2).

1.3.5 Chemical structure

Classifying antibacterial agents based on their chemical structure is particularly important as each group with similar structure have similar modes of actions and origins (Figure 1.2), though factors such as toxicity, spectrum of activity and efficacy can be dramatically affected by subtle changes to the core structure. In the context of Gram-negative bacteria, most relevant to this thesis, the key, clinically important antibacterial classes are β-lactams, β-

lactam/β-lactamase inhibitor combinations, aminoglycosides, and fluoroquinolones. In this study, cephalosporin resistance is the focus, which are members of the highly diverse β-lactam antibacterial group, so these will be considered in detail in the following sections (18).

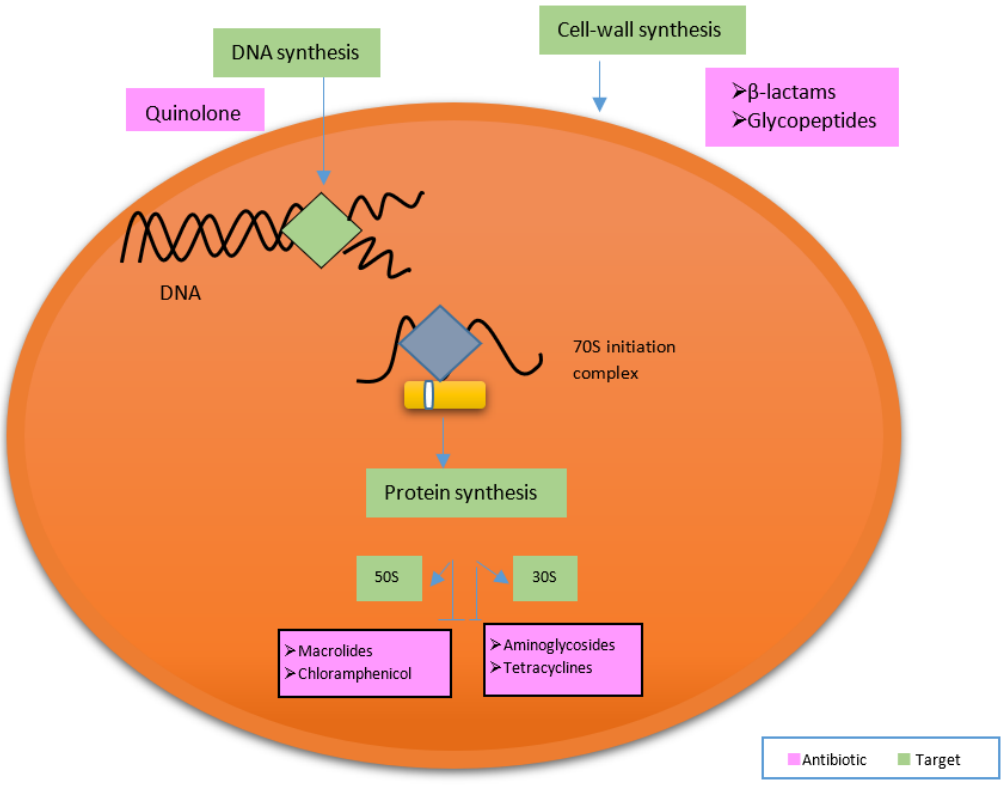


Figure 1.2: Antibacterial classes and their targets. Each antibacterial group can target and inhibit one of the three main targets: cell wall/envelope synthesis, protein synthesis (the ribosome and associated proteins, 50S and 30S subunits) and the DNA-RNA synthesis pathways (adapted from 12).

1.4 β-lactams

β-lactams are the most prescribed class of antibacterial agents world-wide, and include penicillin, the first antibacterial to be developed for clinical use. They all contain a four-membered ring known as the β-lactam ring although members of this class might vary based on the side chain attached or additional ring structures, which are core structures belonging to the antibacterial starting point that has been subjected to semi-synthetic modifications to generate four major sub-classes: penicillins, monobactams, cephalosporins, and carbapenems (Figure 1.3).

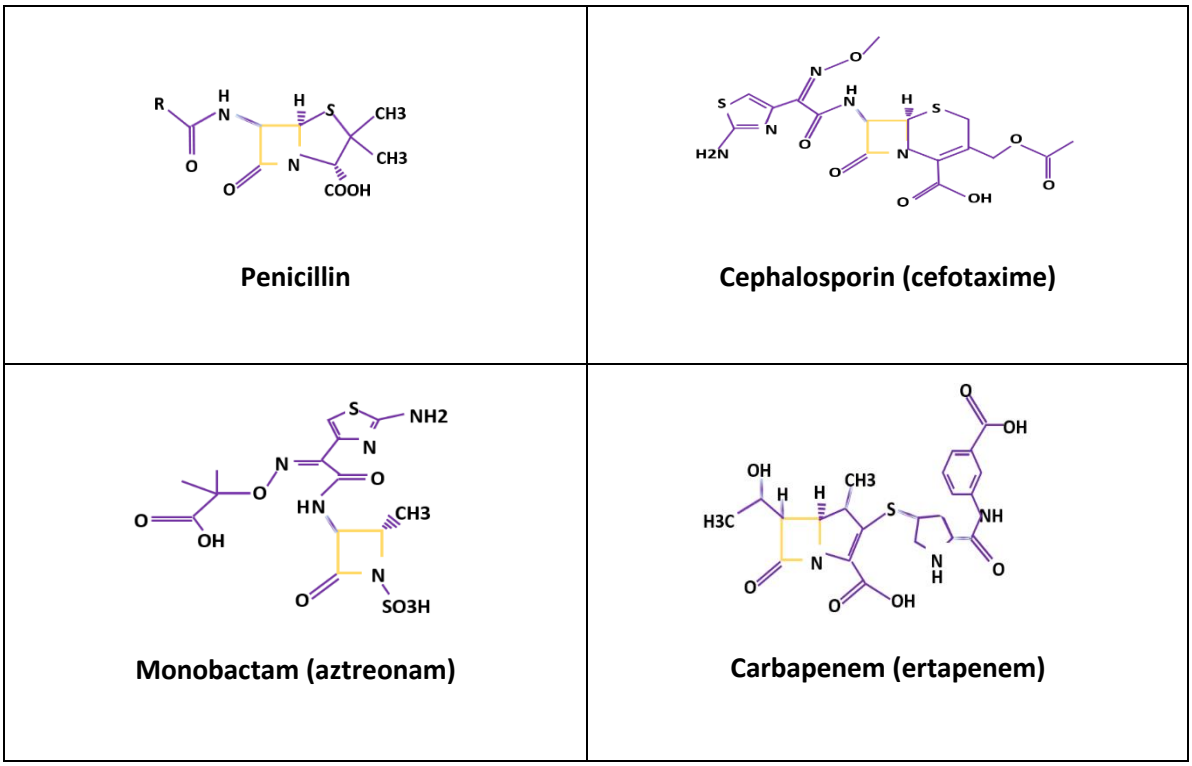


Figure 1.3: Four major sub-classes of β-lactams (penicillin, cephalosporins, monobctam, carbapenem). They all contain a β-lactam ring in their core structure (shown in yellow).

All these antibacterials work by inhibiting the synthesis of the bacterial cell wall, which is central to cell integrity. They do this by covalently binding (and so inhibiting) essential enzymes known as penicillin-binding proteins (PBPs). The bactericidal effects of β-lactams are by inhibiting transpeptidase PBPs involved in the terminal steps of peptidoglycan cross-linking (trans-peptidation) in both Gram-negative and Gram-positive bacteria (Figure 1.4). Thus, β-lactam antibacterials are bactericidal, though cell death is not rapid, because lethality is driven by gradual breakdown of the existing cell wall, whilst the β-lactams prevent its replacement (20,21).

PBPs, and peptidoglycan synthesis more generally are very important target for antibacterials (22,23) due to (i) the critical role of peptidoglycan in bacterial survival, (ii) the complex peptidoglycan biosynthetic pathway, meaning there are multiple potential protein targets, many of which, like PBPs are in the periplasm, reducing the impact of envelope permeability issues, and (iii) the absence of similar pathways in Eukaryotes that decrease the probability of off-target effects (24).

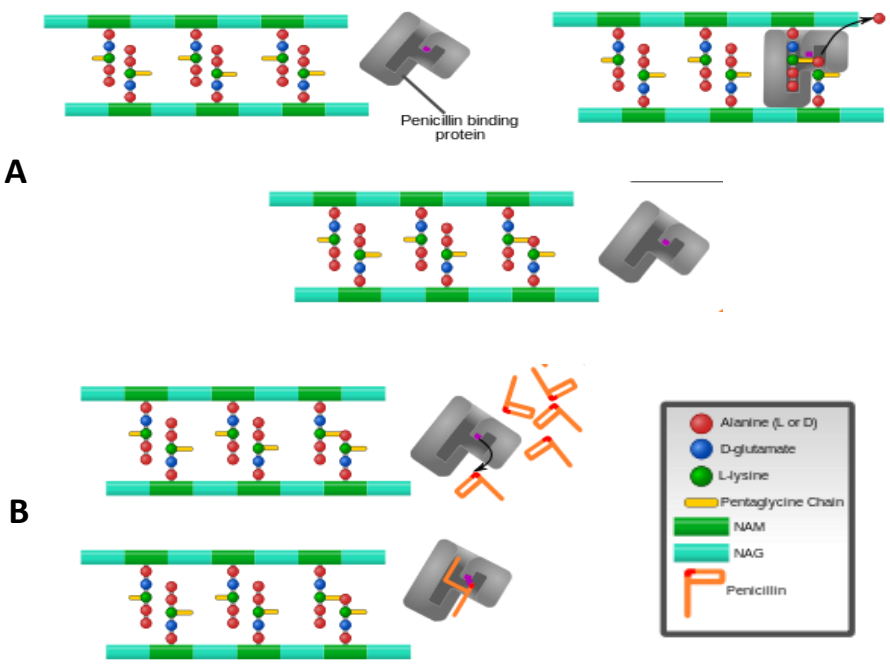


Figure 1.4: B-lactam mode of action. A. Terminal steps of peptidoglycan biosynthesis involves cross-linking (trans-peptidation) by transpeptidase PBPs. B. In the presence of B-lactam drug (e.g penicillin), this step is inhibited following the binding of B-lactam drug into PBPs and therefore inhibit the cell wall synthesis. Taken from 25

1.4.1 Penicillins

Penicillins, the most common class of antibacterials used in paediatrics, can generally be grouped into four: the naturally occurring penicillins, penicillinase-stable penicillins, amino-penicillins and the extended-spectrum penicillins (26). The naturally occurring Benzylpenicillin or Penicillin G was the first β lactam agent utilised clinically. It was most frequently used to treat streptococcal infections due to its high potency (27,28). Penicillin V is another example of a natural penicillin, which is still in use as an oral formulation for treating mild to moderate susceptible *Streptococcus* spp. Infections (29). Unfortunately, the use of penicillin G decreased due to the emergence of penicillin-resistant staphylococci that produced penicillinase

enzymes. This issue raised the urgent need for new penicillin drugs with higher stability to the staphylococcal penicillinase (30,31).

To overcome the obstacle of penicillin-resistant *Staphylococcus aureus*, semisynthetic penicillins such as oxacillin, methicillin, and nafcillin were developed. These drugs are active against bacteria producing these staphylococcal penicillinases, but they are not active against Gram-negative pathogens, partly because of the production of a penicillin amidase, and partly because of low penetration across the Gram-negative outer membrane. Therefore, the semisynthetic penicillins known as aminopenicillin (e.g., ampicillin and amoxicillin) which were effective against Gram-negative bacteria because they penetrate better across the outer membrane were developed (26).

Extended-spectrum semisynthetic penicillins have two major classes: acylureidopenicillins (piperacillin) and the carboxypenicillins (ticarcillin and carbenicillin). They were developed to enhance the activity of penicillins against Gram-negative organisms including *Enterobacter*, *Pseudomonas* and *Klebsiella* spp that produce penicillinases capable of breaking down aminopenicillins. The current use of aminopenicillins and even extended-spectrum penicillins as monotherapy is limited due to the increasing number of β-lactamases present in Gram-negative bacteria with activity against these agents. In combination with an appropriate β-lactamase inhibitor, however, amoxicillin (used with clavulanic acid), piperacillin (used with tazobactam), and ticarcillin (used with clavulanic acid) are still useful, and extensively used. Nonetheless, penicillins as a single agent, even penicillin G and penicillin V are still effective against the few bacterial species that lack β-lactamase production such as *Treponema pallidum* and Group *A Streptococci* (32).

1.4.2 Cephalosporins

Cephalosporins were the second class of β-lactam antibacterial to be discovered. Since the discovery of cephalosporin C as a naturally occurring penicillinase-stable antibacterial, hundreds of novel semi-synthetic cephalosporins have been developed (33,34). They were first used to treat infections caused by penicillin resistant penicillinase-producing bacteria. Many cephalosporin agents were introduced into clinical practice to be used parenterally or orally (34). These drugs can be classified by their antibacterial spectrum into groups or generations. In this classification system, first-generation cephalosporins are active against Gram-positive organisms such as *S. aureus* (MSSA), including penicillinase producers. They have limited activity against Gram-negative bacilli, including *E. coli* or *K. pneumoniae*. Second generation drugs have increased spectrum of action against aerobic Gram-negative bacilli, while having variable effectiveness in clinical use agaist Gram-positive cocci (35,36). Generally, the next two generations (third and fourth) have broader, though frequently variable, spectra of activity compared with previous generations. Further efforts to introduce advanced-generation cephalosporin for clinical use is ongoing. Two drugs have been approved as fifth-generation agents, although this terminology remains controversial, that offer a treatment option for MRSA (see below) (37). This includes Ceftobiprole (2002), a drug which demonstrated in vitro activity against Gram-positive cocci and Gram-negative bacilli including AmpC-producing *E. coli* and *P. aeruginosa*, as well as ceftaroline, which has broad spectrum activity against Gram-positive and many Gram-negative pathogens (2005) (38). Neither drug is active against extended-spectrum β lactamase-producing ESBLs Gram-negative bacteria (37,38) as will be described below.

1.4.3 Carbapenems

Carbapenems have the broadest spectrum of activity of all the β-lactam antibacterials (39) and are stable to almost all bacterial β lactamases seen commonly in the clinic (40). Therefore, they are effective agents in treating severe infections caused by ESBLs producing pathogens or chromosomally mediated AmpC β-lactamases (41).

Carbapenems are derivatives of thenamycin, which is the first natural carbapenem compound identified and is produced by the soil fungus *Streptomyces cattleya* (42). The subgroup includes semi-synthetic derivatives meropenem, imipenem, doripenem, ertapenem, biapenem and panipenem. The activity of carbapenems covers both Gram-positive pathogens, including penicillinase-producing MSSA but not MRSA, as will be described below, as well as Gram-negative pathogens including *Pseudomonas* spp. Worldwide, carbapenem use is increasing mainly due to increasing resistance to cephalosporins in the *Enterobacteriaceae* (43). However, the presence of Carbapenem Resistant *Enterobacteriaceae* (CRE) that produce carbapenem-destroying β-lactamases is common in some regions of the globe and is emerging in them all (44).

1.4.4 Monobactams

Monobactams are β-lactams with a monocyclic nucleus, i.e., lacking any other ring structure. Monobactams are naturally occurring drugs isolated from *Ckromobacteruim violaceum* and subsequently modified chemically to produce the only available agent in this class, aztreonam (26 ,35). Clinically, aztreonam has good safety profile and has been used as a monotherapy or in combination with other antibacterials to treat many infections such as lower respiratory infections, urinary tract infections, gynaecological infections, skin/soft tissue infections and intra-abdominal infections. It can be effective against infections caused by susceptible Gram-

negative aerobic bacilli (35). This drug can also be used strictly in paediatric clinics for community-acquired infections when the use of aminoglycosides is not appropriate or adequate therapy. It is also approved by the US Food and Drug Administration (FDA) for children aged seven years or older who suffer from cystic fibrosis, to treat *P. aeruginosa* lower respiratory tract infection as an inhalational antibacterial (26). Generally, aztreonam is mostly used for patients who have a major hypersensitivity to other β-lactams if they are in need of anti-pseudomonal β-lactam treatment (35).

1.5 Antibacterial drug resistance

1.5.1 Resistance mechanisms

Antibacterial resistance can be broadly classified as intrinsic or acquired. Bacterial intrinsic resistance is a naturally occurring phenomenon, independent of mutaton or horizontal gene transfer, which means that all members of some bacterial species are not susceptible to the actions of a given antibacterial (45,46).

Intrinsically resistant bacteria sometimes lack the target site for the antibacterial used, thus rendering it ineffective. An example is the absence of a typical peptidoglycan cell wall in *Mycoplasma* spp., making them intrinsically resistant to β-lactam antibacterials and glycopeptides. Furthermore, intrinsic resistance can be caused by the inability of the drug to penetrate the envelope and to access its target (47). Penicillin cannot penetrate the *E. coli* outer membrane very efficiently, as mentioned above, meaning that *E. coli* is intrinsically penicillin resistant. Finally, some bacterial species can produce enzymes that inactivate the antibacterial, such as *P. aeruginosa* that produces an AmpC β-lactamase whose production is inducible in the presence of certain β-lactams, and so confers intrinsic resistance to cefoxitin, amoxicillin and early generation cephalosporins (48). *E. coli* also produces an AmpC enzyme,

but this is not inducible, and instead production is at levels too low to give intrinsic resistance to β-lactams.

In contrast, naturally susceptible bacteria can acquire resistance to some antibacterials via chromosomal mutations and by horizontal gene transfer (45). Acquired resistance can be divided into four main mechanisms: (i) antibacterial drug modification or inactivation, (ii) reduction of antibacterial drug accumulation by reducing entry (via porin downregulation or disruption) or enhancing efflux (via increased efflux pump production), (iii) modification of the antibacterial drug target due to mutation or post-translational alteration to reduce the attraction between the drug and its target (Figure 1.5), (iv) acquisition by horizontal transfer of a pre-mutated drug target, or over-production of a susceptible target allowing target activity to continue in the presence of drug (45,47).

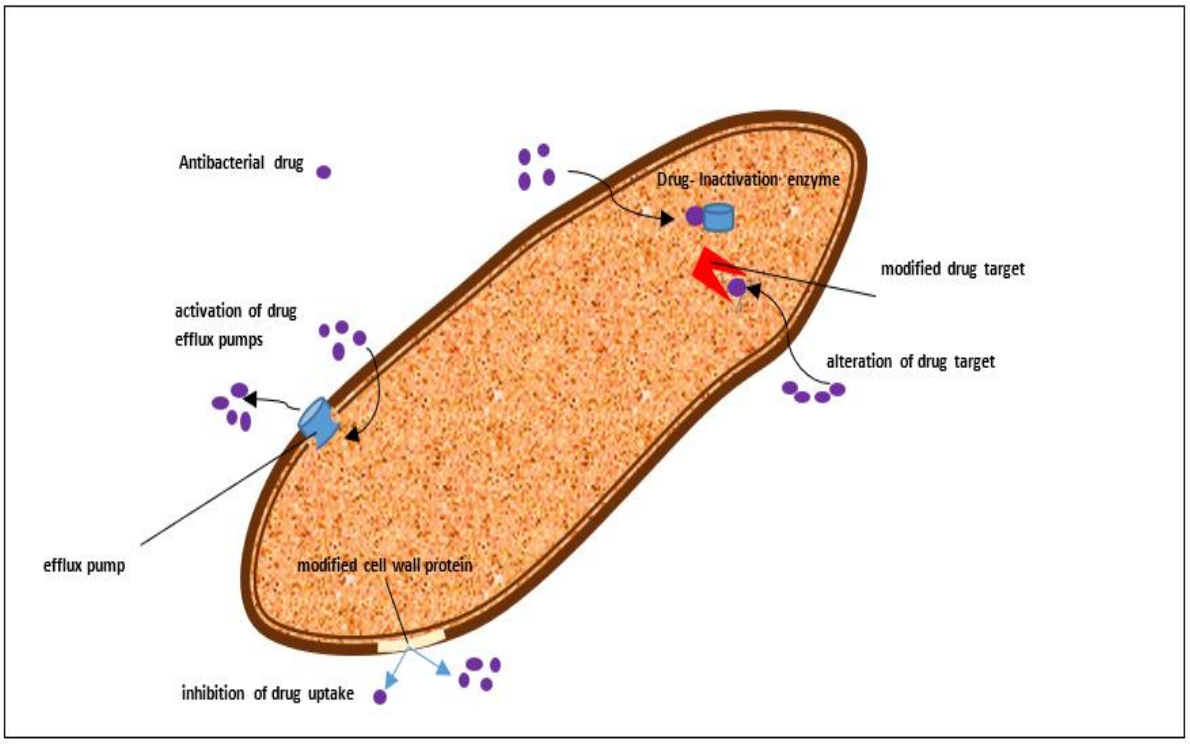


Figure 1.5: Different mechanisms of antibacterial drug resistance in bacteria including pumping a drug out the cell, reduction of the drug uptake through porin mutations, alteration of the drug target, or acquisition of a modified target and enzymatic inactivation or modification of the drug. The purple circles represent antibacterial drugs. Adapted from (49)

1.5.2 Inactivation of antibacterial drugs

The most common acquired resistance mechanisms used by bacteria is to render the antibacterial inactive either by chemical modification of its structure or destruction of the drug itself. Inactivation mechanisms are well-known in both Gram-negative and -positive bacteria. Enzymatic modification can be catalysed by various biochemical reactions including phosphorylation (chloramphenicol, aminoglycosides), acetylation (streptogramins, aminoglycosides, chloramphenicol) or adenylation (aminoglycosides, lincosamides).

β-lactamases are the only clinically-relevant antibacterial destruction mechanisms known. They were identified before penicillin was introduced into clinical practice and they likely

evolved due to the presence of β-lactams in nature for millennia. β-lactamases work by breaking the β-lactam ring that renders the antibacterial drug incapable of binding to its PBP targets. Since the first emergence of β-lactamase mediated β-lactam resistance, new types of β-lactams or those modified to reduce the rate of destruction by β-lactamases have been developed, as described above. This has since led to the emergence of new or mutated β-lactamases and further modifications of β-lactams (50).

Today, there are several hundred known β-lactamases that are classified broadly under two classification systems:

1. **Ambler** classification system based on amino acid sequence homology of β-lactamases. Under this system there are four classes (A-D), where classes A, C and D are serine β-lactamases (utilize an active-site serine for hydrolysis), and class B is metallo-β-lactamases (use at least one divalent zinc atom for hydrolysis),
2. **Bush-Jacoby-Medeiros** classification system where they are classified, based on functional similarities (substrate, inhibitor profiles etc), into four main groups and multiple subgroups (51).

The first reported β-lactamase was the chromosomally-encoded AmpC from *E. coli* (52). The first plasmid-mediated β-lactamases in Gram-negative bacteria to be identified were TEM-1 and OXA-1, in *E. coli* and later in many different members of the family *Enterobacteriaceae, P. aeruginosa, Haemophilus influenzae*, and *Neisseria gonorrhoeae* (53,21). Other early examples of plasmid-mediated β-lactamases include SHV-1 (53,54). These enzymes belong to class A except OXA-1 which belongs to class D, and they have similar resistance patterns against ampicillin, amoxycillin, ticarcillin and carbenicillin (55). To combat their emergence, clincians switched to the use of cephalosporins, particularly the third generation cephalosporins (3GCs)

to which these enzymes cannot confer resistance. This was particularly relevant for example, to treat infections caused by ampicillin-resistant *E*. *coli* and *K. pneumoniae* producing TEM-1 or SHV-1. However, the emergence of ESBLs has blocked the efficacy of 3GCs (51). ESBLs are capable of hydrolysing penicillins, the 1st, 2nd, 3rd and 4th generation cephalosporins and aztreonam. They can however be inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam, mentioned above. The first ESBL enzymes evolved from TEM-1 and SHV-1 by mutations causing two amino acid substitutions. However, the main source of ESBLs today is the CTX-M types, which are also class A enzyme (56). Unlike TEM and SHV ESBLs, the source of CTX-M is the chromosomal β-lactamase genes of *Kluyvera* spp., a rarely encountered member of *Enterobacteriaceae*, where they have, before mobilisation, ESBL activity (57).

CTX-M enzymes comprise a large group of ESLBs that a subdivided into five clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) according to their amino acid sequence identities. Members within these clusters have >94% amino acid identities and ≤90% relatedness between groups (58). Generally, CTX-M enzymes confer clinical resistance to cefotaxime and ceftriaxone but not to ceftazidime. However, some CTX-M enzymes (e.g., CTX-M 15) can hydrolyse ceftazidime at higher rates than others (e.g., CTX-M-14). This ability is thought to be one reason for the spread of CTX-M 15 amongst bacteria causing serious human infections, against which ceftazidime is frequently used (59). The rapidly emerging nature of ESBLs, CTX-M is also believed to be due to the co-resistance of genes encoding ESBLS alongside those encoding resistance to other drug classes (for example, aminoglycosides) on the same plasmid thereby limiting antibacterial treatment options and providing co-selection using any number of drug classes, for resistance to them all. Carbapenems are increaseingly the treatment of choice for serious ESBL-positive infections, particularly if aminoglycoside resistance although carbapenem-resistant isolates have since emerged (51).

Carbapenem resistance is mediated by carpabenemase production. Carbapenemases involve members of β-lactamase molecular classes A, B, and D. A particularly prevalent class A carbapenemases is KPC, found mostly in *K. pneumoniae* plasmids where it confers resistance to all β-lactam classes except the cephamycins. Class D carbapenemases include the increasingly prevalent OXA-48-like enzymes, which are penicillinases with some carbapenemase activity, but rarely confer resistance to cephalosporins or monobactams. Class B carbapenemases (metallo-β-lactamases) include IMP, VIM, SPM, GIM, and SIM families, but my far the most common is the NDM type. These enzymes give resistance to all β-lactams except the monobactam aztreonam (60). It should be noted that β-lactamases have been extensively studied and described by the research community (21,61,62).

AmpC cephalosporinases belong to molecular class C and can confer resistance not only to penicillins and early cephalosporins but also to 3GCs such as ceftazidime, cefotaxime, and monobactams, though this spectrum varies from enzyme to enzyme, and they do not normally confer resistance to 4th generation cephalosporins, though see below (63,64). Furthermore, they are not inhibited by inhibitors such as clavulanic acid and tazobactam. Although a chromosomally-encoded AmpC is produced by many species of Gram-negative bacteria (64), several families of plasmid-encoded AmpC (pAmpC) variants have been reported (e.g., CMY-1, CMY-2 and FOX). In addition to AmpC production, porin mutations in the outer membrane can also reduce susceptibility to cephalosporins, and to carbapenems in already 3GCR chromosomal or pAmpC producers (65,66).

In *Enterobacteriaceae*, *ampC* genes are found in the chromosomes of many members, including *Enterobacter* spp., *Citrobacter freundii, E. coli, Providencia* spp. *Serratia marcescens, Morganella morganii* and *Shigella*. The production of AmpC β-lactamase in all these except *E. coli* and *Shigella* is induced in the presence of many β-lactams (though only weakly with 3GCs

and the monobactam aztreonam, so such isolates are rarely 3GCR) via a transcriptional regulator AmpR. Over-expression of *ampC* gene can result from a mutation in *ampR*, activating transcription of *ampC*, or mutations in a repressor gene, *ampD*, which activate AmpR. This is the primary mechanism of acquired 3GCR in these species (67). In *E. coli,* however, since *ampR* does not exist, the chromosomal *ampC* is not inducible and is constitutively expressed at a low level, making wild-type *E. coli* susceptible even to amoxicillin. The *ampC* promoter is highly polymorphic, however, and dictates basal expression. Many different mutations can lead to AmpC hyper-production and 3GCR, alongside amoxicillin and amoxicillin/clavulanate resistance (67). In most cases, plasmid-mediated pAmpC genes are expressed constitutively at high levels, while some have been reported to be inducible via a co-resident *ampR* (66). Various nucleotide polymorphisms within the *ampC* gene have been reported to produce an extended spectrum of AmpC (ESACs) variant which can confer resistance to 4th generation cephalosporins (e.g. cefepime) (68,69). ESACs have been reported in *Enterobacteriaceae*, including *Enterobacter aerogenes* (70), *P. aeruginosa* and *Acinetobacter baumanii* (71). In *E. coli* production of ESAC has been reported in both human clinical isolates (72,73) and those derived from animals (74).

Amino acid chenges within the active site of AmpC associated with an ESAC phenotype include deletions, insertions, or substitution on the R2 loop (H-10 or H11) (residues from 280 to 312) or on the omega loop (residues from 175 to 225), leading to conformational changes that increase the flexibility of the active site and therefore extend its hydrolytic activity (75,76). Examples of specific amino acids substitutions within the R2 loop include Leu293Pro in clinical *E. coli* isolates (77) as well as Val298Leu, His296Pro, Ala292Val and Ser287Asn or Ser287Cys which have been reported in *E. coli* isolates from both humans and animals (68,74).

The chromosomal *ampC* from *E. coli* is a focus of this project and it is discussed in detail in results chapters alongside the various β-lactamase most relivant to this work.

1.5.3 Reduction of antibacterial accumulation

One of the mechanisms used by bacteria to survive in the presence of antibacterial drug is to reduce the level of drug accumulation inside the cell (i.e. at the site of the drug's target) by either reducing the rate of drug entry or enhancing drug efflux. The effects of these two phenomena are subtly different but commonly occur together, and can enhance other mechanisms of resistance, particularly those involving antibacterial modifying enzymes.

1.5.3.1 Decreased drug entry

To exert their effects, many antibacterial agents must reach their intracellular bacterial targets (some actually target the outside of the cell, so not antibacterials are relevant here) by penetrating the outer and/or cytoplasmic membrane. Thus, decreasing the rate of antibacterial drug entry is a mechanism used by bacteria to reduce the degree of inhibition of the antibacterial's intracellular or periplasmic target (47). This is only a relevant mechanism of antibacterial resistance in Gram-negative bacteria due to the presence of outer membrane that plays a role as a selective permeability barrier to many antibacterials (45).

The main route of entry into Gram-negative bacteria for hydrophilic antibacterial drugs such as β-lactams, tetracyclines and some fluoroquinolones is through the water-filled diffusion channels (porins) formed in the outer membrane. Thus, the rate of antibacterial drug permeability and consequently, the susceptibility of the bacterium can be determined by the

number and type of porins present in the outer membrane. This is particularly important if the cell also produces an enzyme that modifies or destroys the antibacterial drug (see above), or produces an efflux pump that pumps out the cell (see below), because lowering the rate of entry on its own does not lower the final concentration of drug that is achieved, but allows modifying enzymes or efflux pumps to "keep up" with the entry rate, maintaining drug concentration at a lower level than in the wild-type state. A mutational alteration that affects the expression and/or function of porins can therefore directly influence the susceptibility of bacteria to antibacterials. Modification of porins can occur by reducing the level of porin production (downregulation), or most commonly via complete loss of the porin production due to, for example, a frameshift mutation (porin loss) or through a mutation that reduces the size of the porin channels being produced (Figure 1.6) (78).

Previous studies have shown that modification in the production of porins in *Enterobacteriaceae, Acinetobacter* spp. and *Pseudomonas* spp. can remarkably lower susceptibility to cephalosporins and carbapenems (79-82). For instance, clinically relevant resistance to carbapenem can emerge in *Enterobacteriaceae* due to reducing/loss of porin production by mutation in combination with other mechanisms (e.g., β-lactamases with weak carbapenemase activity and/or efflux pump over-production) (79,83).

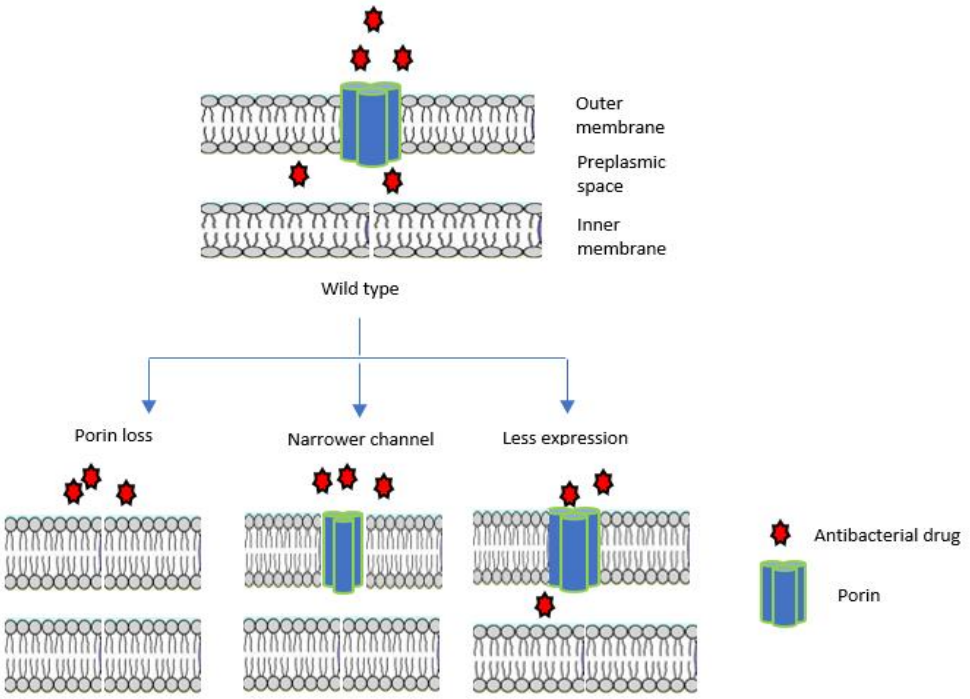


Figure 1.6: Different types of mutations which affect porins and, consequently, decrease the permeability of the cell. Permeability reduction helps bacteria to survive in the presence of the antibacterial drugs (adapted from 78).

1.5.3.2 Increased drug efflux

Bacteria have an array of energy-dependent proteins located in the inner membrane that are capable of actively pumping out intracellular molecules across the membrane(s) and in most cases, out of the cell. The result is reduced accumulation of toxic compounds in the cytoplasm and/or periplasm. Efflux pumps have an important role in intrinsic and acquired antibacterial drug resistance as many pumps have included within their substrate profile one or more classes of antibacterial and can therefore act to limit the intracellular accumulation of these drugs (84).

Efflux proteins that contribute to antibacterial drug resistance can be divided to five families: the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance-nodulation-cell division (RND) family, multidrug and toxin extrusion (MATE) family, and ATP-binding cassette (ABC) family (85). These superfamilies are differentiated based on the source of energy required for transport: using the proton-motive force made as a result of respiration (includes efflux pumps belong to SMR, MFS, MATE and RND families) and the ABC family that gains energy from the hydrolysis of ATP (78,84).

Members of the RND transporters family are important in both intrinsic and acquired resistance in Gram-negative bacteria. The AcrAB-TolC efflux pump found in *E. coli* is a well-known example of this type. RND-type efflux pumps are composed of the following elements: an efflux protein located in the inner membrane that is powered by the proton motive force and can pump molecules from the cytoplasm or from the periplasm; a linker protein known as a membrane-fusion protein located in the periplasmic space, which facilitates the removal of periplasmic chemicals, and links the efflux protein with the third component, and outer membrane channel protein. AcrB, AcrA and TolC, respectively, are examples of each type of protein (86-88). All are produced in clinical-relevant isolates of *E. coli* (88,89) but they are not normally produced at levels high enough to give resistance to important antibacterial drugs.

RND-type efflux pumps can contribute to acquired resistance due to mutations in regulatory genes, which results in the overexpression of the genes encoding the three components of the pump, therefore leading to an increase in antibacterial export rate, and a resultant decrease in accumulated concentration. Upregulation of RND-type efflux pump production is mediated by different transcriptional regulator families including AraC-type transcriptional activators in Enterobacteriaceae. These include MarA, SoxS, Rob, RamA, and RarA, and they are commonly encoded beside a gene encoding a repressor of the “Multiple Antibiotic Resistance” (MarR)

protein family. Following the loss of the MarR-family repression due to inactivation of its gene, the increased transcription of the AraC-family transcriptional activator gene (and so production of this activator protein) confers multi-drug resistance due to the resulting overexpression of efflux pumps and, in some cases the repression of porin protein production (45,90).

MarA is an AraC-family transcriptional activator which encoded alongside marR (encoding the archetypal MarR repressor) in the chromosomal *mar* locus of *E. coli*. Expression of *marA* is repressed by MarR. Inactivation of *marR* via mutation or insertional inactivation, or mutation of the MarR repressor binding site upstream of *marA* causes increased *marA* transcription, resulting in the differential regulation of numerous chromosomal genes within the MarA regulon, causing the Mar phenotype (91). This includes the upregulation of AcrAB-TolC efflux pump which reduces susceptibility to structurally unrelated antibacterials such as fluoroquinolones, chloramphenicol, tetracycline, and some β-lactams (92), as well as organic solvents (93), oxidative stress agents (94), and some disinfectants (95). Additionally, MarA activates the expression of *micF*, which encodes an encodes an *ompF* antisense RNA, resulting in OmpF downregulation through the inhibition of *ompF* mRNA translation (96).

Furthermore, acquired resistance mediated by efflux transporters more generally can result from the horizontal acquisition of genes encoding transporter proteins able to export antibacterials from the cell (84). For instance, the recently discovered resistance mechanism carried on an IncH1 plasmid found in a *Citrobacter freundii* strain. The plasmid carries a combination of the NDM-1 carbapenemase, the 16S RNA methylase gene *arm*A and a cryptic prophage carrying the RND efflux pump (97). This is a worrying development as it shows that antibacterial resistance mediated by RND efflux pumps can be transmissible and could therefore be rapidly shared by other clinically relevant pathogens.

1.5.4 Protection of the antibacterial target

An antibacterial drug recognises and specifically binds to its target with high affinity through specific contact. Therefore, any alteration in the target structure reducing antibacterial/target affinity can confer resistance. Target alteration can be achieved by mutation in the gene encoding the target. One example is resistance to rifampicin, which results from a single point mutation in *rpo*B, the gene that encodes the target of rifampicin. However, since antibacterial targets are essential proteins, they are not highly mutable, particularly in the active site area that is usually targeted by an antibacterial drug. Overall, therefore, this lack of relative mutability reduces the rate at which target site mutations emerge (84). An alternative strategy to overcoming target inhibition is to elevate production of the target protein which increases the concentration of antibacterial drug required to inhibit sufficient target proteins to have the expected antibacterial effect. An example of this is overproduction of dihydrofolate reductase (DHFR), an enzyme that has central role in the synthesis of nucleic acid precursors and is the target of trimethoprim, which can render bacterium *E. coli* resistant to trimethoprim (98). Thirdly, rather than mutating the antibacterial target (and some targets are not actually protein products of genes) enzymatic modification of the target, or protection of the target by binding of a protein, can result in reduced antibacterial drug binding and resistance. One example here is ribosomal RNA methylation, which affects the target binding affinity of certain protein synthesis inhibitor antibacterials. Another example is Qnr, which binds to the DNA gyrase target of fluoroquinolones, protecting it. Target protection differs from target modification with an enzyme as it does not cause a permanent chemical change of the target but instead resistance mediated by target protection requires a continuous, direct physical interaction between protection protein and the antibacterial target (84).

Finally, if a gene encoding an antibacterial drug target previously evolved to have reduced affinity for the antibacterial drug is acquired on a mobile genetic element, this acquired target can replace the chromosomally-encoded target, which is inhibited as normal. An example of this is MecA in MRSA, encoding a modified PBP2, which is less sensitive to β-lactams (99).

1.6 Antibacterial drug resistance in farms

Antibacterials are usually administrated to food animals in one of three ways: firstly, when the symptoms of an illness are detected, an antibacterial is prescribed (therapeutic use); secondly, antibacterials can be used to suppress infections prior to clinical symptoms, as prophylactic or non-therapeutic use; and finally, to promote growth in food animals, a widespread practice in many countries throughout the world (100). Antibacterials for use as growth promoters are given to animals in sub-therapeutic dosage to increase productivity (101). Despite the beneficial impacts of antibacterial use in animals such as enhancing production, infectious diseases control and to improve the overall health and welfare of the animals, antibacterial drug resistance can also be selected in bacteria associated with these animals (102).

Efforts to reduce the consumption of antibacterials on farms began in the mid-1980s, due to concerns related to its effect on resistance. At this time, Sweden outlawed the use of all antibacterials as growth promoters. Avoparcin and virginiamycin were banned in Denmark in 1995. In Europe, growth-promoting antibacterials have not been allowed since 2006 (103,104). In the U.S., this step was delayed until 2017, when medically important antibacterial drugs (as defined by the U.S. Food and Drug Administration (105) were outlawed for growth promotion.

Despite the fact that preventing growth promotion may greatly reduce the amount of antibacterial consumption in farmed animals, the use of antibacterials for treatment by veterinarians is still considerable and may even exceed consumption in human medicine (106).

This project relies, in part, on bacterial isolates collected as part of surveys of dairy farms in the Southwest of England. In dairy farms, antibacterials are used for prophylactic and therapeutic reasons. Therapeutic uses include treatments of common infections such as mastitis while in preventative use, antibacterials are used to prevent infection (such as dry cow therapy that is used between lactations to prevent mastitis) (107). According to (108), cefquinome, a 4th generation cephalosporin (4GC), was, at the time of publication, the most widely used antibacterial for dry cow therapy in England and Wales.

Antibacterial use in food animals may cause significant selective pressure for the emergence of resistant strains that could then theoretically be transferred to humans. This problem is also evident in clinically relevant bacteria, leading to public health hazards (109). The transmission between food animals to humans could occur through several ways which involve food supply chain (meat and dairy products), direct animal contact, or via environmental routes where animal waste contaminates the environment (Figure 1.7) (110,111). Many researchers have illustrated the relationships between antibacterial usage and the emergence of resistant strains in animals. Zoonotic transmission of resistant strains from food animal origins to humans could occur via direct or indirect human - animal interaction. People, including farmers, food handlers, and veterinarians, are more prone to get infected or colonised by resistant strains (103,112). Additionally, consumption of contaminated food products can expose consumers to resistant strains (111).

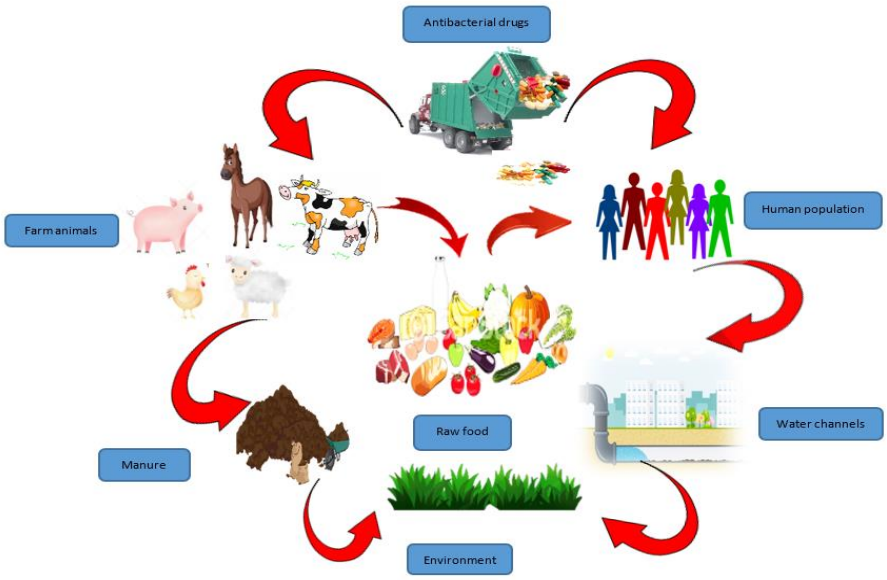


Figure 1.7: Representation of possible ways of zoonotic transmission: abtibacterial-resistant strains/genes can be transmitted among animals, human and their ecosystems (adapted from 111).

1.7 *Escherichia coli*

In 1885, Theodor Escherichia reported the identification of slender, short rods in the faeces of a child, which he named *Bacterium coli* commune; later the name was altered to *Escherichia coli*. This bacterium was identified as a harmless member of the gastrointestinal tract flora of humans and warm-blooded animals, worldwide. However, despite many strains being commensal, *E. coli* can also become pathogenic, which largely depends on the gain/loss of virulence genes though many infections in the developed world are entirely opportunistic. Strains of *E. coli* are responsible for many and different human diseases, ranging from gastrointestinal infections causing enteritis to extra-intestinal pathologies involving the central nervous system, bloodstream, urinary tract, and other tissues (113). Accordingly, *E. coli* are associated with cholecystitis, cellulitis, nosocomial pneumonia, osteomyelitis, infectious arthritis, gastroenteritis, peritonitis, and cholangitis, as well as neonatal meningitis (114,115).

Several *E. coli* strains are a common cause of intestinal pathology (e.g., enteropathogenic *E. coli*, enteroaggregative *E. coli*, enterotoxigenic *E. coli*, enterohemorrhagic *E. coli*, enteroinvasive *E. coli* and diffusively adherent *E. coli*) (115). Indeed, *E. coli* strains frequently occur in faeces and can survive in water and are thus utilised as an indicator of faecal contamination of food and water (113). *E. coli* can also acquire different mechanisms that confer resistance to commonly used antibacterials (114).

The ability of *E. coli* strains to survive in different ecological environments, as well as its genetic diversity, make this bacterium ideal for the transmission of antibacterial resistance studied between and within different sources (116,117). *E. coli* can be ingested after the consumption of contaminated water or food, or through direct contact with faecal matter. Indeed, many

food production processes (e.g. milk collection, animal slaughter, transport and processing) are associated with faecal, and so *E. coli* contamination of food.

Furthermore, *E. coli* is not only used as an indicator of faecal contamination, but the species is also widely used as a model organism (and molecular biology tool-box) in various fields and has been important for our understanding of bacterial life, metabolism, and disease. Genomic technologies have described the whole genome sequence of the organism, which further facilitate the molecular interrogation of the species in future (118,119).

1.7.1 Taxonomy, morphology and biochemical characteristics of *E. coli*

E. coli is a member of *Enterobacteriaceae* family and is one of six species belong to the genus *Escherichia*, along with *E. albertii, E. fergusonii, E. hermanii, E. blattae,* and *E. vulneris. E. coli* is the most diverse species among the genus *Escherichia*, with only a quarter of the genome being shared between all strains. This diversity allows *E. coli* to thrive on a variety of substrates and in commensalism with diverse species.

E. coli is a chemo-organotrophic microorganism. It can ferment lactose, glucose, D-mannitol, maltose, D-sorbitol, and arabinose as well as to reduce nitrate (113). Selective media, such as MacConkey agar, can be used to isolate *E. coli* from the faecal (or other) samples, and to distinguish non-lactose-fermenting strains from lactose-fermenting ones. Selected media with an antibacterial, as MacConkey agar supplement with cefotaxime, can be used to distinguish 3GC resistant (e.g. ESBL-producing *E. coli*) from those which are susceptible; pH reduction in MacConkey agar due to lactose fermentation results in a pink/red appearance of *E. coli* colonies(115).

E. coli is a Gram-negative, non-spore-forming, rod-shaped organism that lives as a single bacterium or in pairs or groups. Being facultative, Gram-negative anaerobes, they are also able

to inhabit low O_2 environments (such as the lower GI tract), accounting for their ability to inhabit diverse niches. Within a population itself, there is also a high degree of individual specialisation, depending on individual and population dynamics and constraints. For example, each bacterium may or may not produce microcapsules or capsules, have peritrichous flagella and be motile, or not. They may form part of the colony's interior or exterior. The typical cell is 2.0 µm in length and up to 1.0 µm wide, with optimal growth occurring at 37^{o}C, although strains commonly thrive at up to 49^{o}C (113).

1.7.2 Antibacterial drug resistance in *E. coli*

In 2014, WHO published a global prediction of antibacterial drug resistance prevalence and according to the report, *E. coli* constituted one of the three species most associated with the development of resistance along with *K. pneumoniae* and *S. aureus*. All three species are strongly associated with nosocomial infections with high degrees of mortality, and community cases are very common, e.g., urinary tract infection (120); demonstrating the remarkable ability of *E. coli* to quickly exploit the human niche. Over the past few years, multi-drug resistant Gram-negative bacteria have become more common, and special interest has centred on resistant strains expressing ESBLs and AmpC β-lactamases because they are resistant to 3GCs, which are the mainstay of therapy for serious infections, e.g., bloodstream infections. Given the limited options to tackle 3GC resistant Gram-negative bloodstream infection, 3GC resistance poses a serious public health threat worldwide (21), and an increasing reliance on other agents, e.g. carbapenems, will accelerate the emergence of resistance to these agents.

Previously, 17 European countries out of 22 have reported an increasing incidence (> 85% increase) of 3GC resistant, ESBL positive *E. coli* isolates from human patients. Furthermore, 11

countries in Asia recorded >28% of all *Enterobacteriaceae* – isolated from urinary tract infections – were resistant to 3GCs due to ESBL production between 2009-2010 (120). Recently, *E. coli* isolates resistant to 3GCs (using cefotaxime as selection) have been obtained from human urinary tract infections and from faecally-contaminated sites on dairy farms in South West England. Resistance in both types was predominantly due to CTX-M ESBL carriage (121,122).

Carbapenems are generally considered the last line therapy for patients infected with 3GC-resistant *E. coli*. These antibacterials cannot be used in animals in the UK, and in Europe and the USA, animals are only very rarely colonised with carbapenem resistant *E. coli*. Worryingly, various healthcare organisations have recorded growing numbers of carbapenem-resistant *Enterobacteriaceae* infections in humans, mainly in developed countries but also in medium-to-low-income countries. Strikingly, in 2012, 11% of *K. pneumoniae* and 2 % of all *E. coli* isolated from various hospitals located in the USA were resistant to carbapenems. In India, the amount of carbapenem-resistance in *E. coli* increased up to 13 % in 2013 (120).

An alternative choice for the treatment of ESBL-producing 3GC resistant *E. coli* infection is a β-lactam/β-lactamase inhibitor combination such as piperacillin/tazobactam or amoxicillin/clavulanate, since these inhibitors block the activity of ESBLs (123). However, AmpC β-lactamases are weakly inhibited by clavulanic acid and tazobactam, and so 3GCR strains producing AmpC-type enzymes will be amoxicillin/clavulanate resistant, though unlike ESBL producers they are rarely 4GC resistant (64).

1.7.3 The use of sequence types to classify *E. coli* strains

Bacterial isolates of the same species can differ markedly in terms of their genomic sequence and composition of genes. Often, only a subset of genes, termed the "core genome", is shared across all strains within a given species. In *E. coli*, this core genome consists of approximately half of the total number of genes found in any one isolate. That genetic diversity within the species has a significant impact on the key metabolic processes and biological functions of the bacterium; an area of research that remains poorly understood (124). The advances in genomic and genetic methods of analysis allowed developing a number of tools that can be used to classify and differentiate bacterial isolates using genetic information.

The species *E. coli* comprises an exceptionally diverse group of strains. The strains within the group range from the commensal, probiotic intestinal isolates that make up the normal intestinal microbiota of humans and other warm-blooded animals (125), to the intestinal disease-causing strains and the obligate extraintestinal pathogenic *E. coli* strains (126). While research has revealed numerous recombination events playing a role in the genetic diversity of *E. coli*, the population within the species has a largely clonal structure (127). That characteristic allows the use of genetic data to differentiate and classify phylogenetic groups within the species (125).

Accurate characterisation and classification of *E. coli* strains are key in predicting bacterial virulence, as well as the role of different strains as beneficial probiotics (128). Additionally, it can help to understand, how commensal bacteria can become pathogenic through adaptation to the environment when habitats and niches they occupy undergo change (129). In the past, classification of bacterial strains was done using phenotypic information, differentiating between the isolates based on observable traits, such as pathogenicity, virulence or metabolic

activity or the presence of antigens (126). However, phenotypic classification methods have some significant drawbacks, including being time-consuming and complex. Most importantly, phenotypic methods are often not appropriate to phylogenetically differentiate between strains sharing phenotypic traits due to horizontal gene transfer across the population, resulting in artefactual grouping together of distantly related strains (130). Thus, genetic typing (phylogenetic analysis) allows a more accurate classification of the diverse strains of *E. coli* (125).

Phylogenetic classification of *E. coli* strains can be accomplished via a number of techniques that can include DNA fingerprinting methods, DNA hybridisation techniques, PCR-based assays, multi-locus sequence typing (MLST) (131), and genome sequencing methods (132). Additionally, 16S rRNA gene sequencing and ribotyping methods can be used (130). In DNA hybridisation a fragmented bacterial genome is hybridised to a microarray of oligonucleotide probes and use fluorescent labelling for signal detection to generate the unique identifier for each isolate although the resolution of the method is largely dependent on the number of unique oligonucleotide probes (133). The fingerprinting techniques classify the isolates based on the "fingerprint" created via enzymatic cleavage of bacterial DNA and electrophoretic separation of the resulting DNA fragments, allowing to compare the resulting patterns with known strains (124). Fingerprinting methods might also involve the use of PCR-based assays, such as the random amplified polymorphic DNA PCR method (RAPD). RAPD involves amplification of the bacterial genomic DNA using a set of non-specific fragments and electrophoretic separation of the DNA fragments generated, in the hope of producing a unique pattern (134). As with the previous method, the uniqueness of the fingerprint is largely determined by the specific design on the assay and might fail to differentiate between the *E.*

coli isolates that share some genetic background due to the horizontal gene transfer in the population (125).

Among the most widely used phylogenetic methods is a PCR-based assay developed by Clermont *et al.* (132). The method allows fast and accurate classification of *E. coli* strains, classifying the strains into the phylogroups A, B1, B2, C, D, E, F and an additional Clade I. Initially, the Clermont triplex PCR phylogroup method classify bacterial isolates based on the amplification of three *E. coli genes* (*yjaA*, *chuA* and *TspE4.C2*) and was used effectively in *E. coli* from humans and other animals and in environmental samples (130). However, since the triplex PCR method could accurately validate only 80-85% of *E. coli* phylogroups, it was modified to include the *arpA* gene, found in phylogroups A, B1, C, D and E but not in B2, F and Clade I (135). Consequently, the quadruplex PCR method allows *E. coli* classification into eight phylogroups, A, B1, B2, C, D, E, F and I (136, 137). The quadruple PCR technique is not only fast but also economical and accurate and is thus broadly used (130).

The direct sequencing methods, including targeted gene amplification and whole-genome sequencing, offer the highest potential for accurate phylogenetic classification of the isolates; however, those methods rely on the availability of genomic data for the *E. coli* strains belonging to different phylogenetic groups and isolated from different environmental niches to allow comparison (126). However, as the availability of the whole genome data generated from the broadly sampled strains grows, phylogrouping using direct sequencing approaches is increasingly feasible for new *E. coli* strains, allowing the classification of new strains using alignment-based methods (138). Consequently, with the use of sequence alignment-based genetic methods utilising selected gene sets or the whole genome data, the accuracy of

differentiation and classification of the *E. coli* isolates into the phylogenetic groups can be greatly enhanced (126).

1.7.4 The association of the specific sequence types with colonisation of human and animal hosts and with disease

Phylogenetic analyses conducted on *E. coli* strains isolated worldwide from a range of animal species and environmental niches help to shed light on the associations between phylogenetic relationships and the commensal or pathogenic natures of bacterial strains (137, 139). For example, to date, such analyses have revealed that most of the pathogenic extraintestinal *E. coli* strains can be classified as belonging to the phylogroup B2 and a smaller subset of virulent extraintestinal *E. coli* belong to group D (140-142). On the other hand, most of the commensal strains studied to date are classified as a group A or B1 (143-145).

Sequence types (STs) are used as a more "fine detail" measure of phylogenetic relationship. They are defined based on the sequences of seven genes, extracted from the whole genome sequence (or determined by PCR sequencing) from an isolate, and categorised into allele numbers. Each array or allele numbers defines a unique ST. It is therefore possible to associate specific *E. coli* STs with pathogenicity or commensalism in humans and other animal hosts and to examine ecological and evolutionary histories of the isolates (137). For example, in one recent analysis of the *E. coli* isolates belonging to the STs associated with extraintestinal infections in humans, the researchers analysed isolates retrieved from humans, dogs and cats and revealed the highest representation of the STs from phylogroups A, B1 and B2 among the studied animals, with STs from phylogroup A overrepresented in dogs and B2 in cats (146). Furthermore, there were differences in relative phylogroup abundance identified between the animals kept as pets and those in animal shelters, with B1 more prevalent among animals in

shelters. The phylogroup B2 and D STs associated with extraintestinal infections in humans (ST 69, ST 73, ST95, ST131 and ST 127) were widespread in pets (146). Such studies help to understand how the specific STs associated with colonisation and/or disease in humans can spread to or from other animals, or between humans.

1.8 Aims of the project

The present study aims to identify chromosomal mutations in *E. coli* that reduce its susceptibility to cephalosporins. Also, in *E. coli* strains that carry plasmid-mediated cephalosporin resistance, to identify chromosomal mutations that reduce carbapenem susceptibility.

We first considered the mechanisms of resistance, or pre-resistance (reduced susceptibility but not to the point of clinically relevant resistance) to cephalosporins currently being prescribed for dairy cows and/or humans, in isolates that do not carry mobile β-lactamases. Two overlapping approaches were taken. First, selection of reduced susceptibility up to full, clinically relevant resistance in *E. coli* derived from cattle or humans in the laboratory by sequential exposure to gradually increasing concentrations of 1st or 3rd generation cephalosporins: cephalexin (1st generation cephalosporin used in humans and animals), cefalonium (veterinary-specific 1st generation cephalosporin) and ceftazidime (3GC only used in humans). Second, analysis of isolates from cattle and human urinary tract infections already resistant to cefalexin or 3GCs or 4GCs but without the presence of plasmid mediated β-lactamases, according to whole genome sequencing.

We next considered further mutations giving resistance to the carbapenem ertapenem in strains that were already 3GCR or 4GCR due to AmpC-type or CTX-M type ESBL production.

Learning more about mutational cephalosporin resistance is important for the prediction of resistance, and cross-resistance, directly from whole genome sequencing, and may be of value when considering the concentrations of antibacterial drugs capable of selecting resistance, perhaps suggesting maximal residue concentrations to be allowed in various environments.

The specific objectives of this study were:

1. Characterise 3GCR and 4GCR mechanisms among dairy cattle-derived, and human urinary tract infection *E. coli* isolates that were believed to hyper-produce the chromosomally-encoded AmpC β-lactamase.
2. Check for evidence of zoonotic transmission of confirmed AmpC hyper-producers between dairy farms and humans using phylogenetic analysis.
3. Select mutants in vitro by gradually increasing the concentration of cephalosporin drugs including cephalexin, cefalonium, ceftazidime and ertapenem. The resistance mechanisms identified to be compared between farmed animal and human *E. coli* strains to explore any commonality present.

Chapter 2
Materials and Methods

2.1 Reagents and chemicals

All chemicals and reagents (except those listed in Table 2.1) were from Sigma-Aldrich.

Table 2.1 List of chemicals and reagents used in this study

Sl. No.	Reagent	Manufacturer
1	HyperLadder™ 1kb	Bioline, Cat.No.BIO-33053
2	HyperLadder™ 100 bp	Bioline, Cat.No.BIO-33030
3	2xMyTaq™ Red mix	Bioline, Cat. No. BIO-25044
4	Nitrocefin	Bioscience, Cat. No. 2388-5
5	Bio-Rad protein assay reagent	Bio-Rad, Cat. No 5000006
6	Antibacterial discs	Oxoid Co Ltd (UK)
7	ExoSAP-IT™ PCR Product Clean up Reagent	Fischer Scientific UK Ltd, Cat. No. 78201
8	Phusion High-Fidelity PCR Kit	New England BioLabs, Cat. No. M0530S
9	Restriction enzymes and buffers	Fischer Scientific UK Ltd
10	Rapid DNA Ligation Kit	Fischer Scientific UK Ltd, Cat. No. K1422
11	QIAquick PCR Purification Kit	Qiagen, Cat. No 28104
12	QIAquick Gel Extraction Kit	Qiagen, Cat. No 28704
13	QIAprep Spin Miniprep Kit	Qiagen, Cat. No 27104
14	InstantBlue® Protein Stain	Expedeon, Cat. No. SKU: ISB1L

2.2. Primers

The primers used in this study are given in Table 2.2.

Table 2.2 Details of primers used in the study and their target regions F – Forward primer: R – Reverse prime

Primers	Sequence (5' to 3')	Annealing temperature	Time	Target	Reference
OmpF-1 F	AATCTATTTGCAACCCCGCC	57.3	40s	PCR amplification of *ompF* gene including the promoter region, with a slight overlap to allow full length sequencing of the gene	Present study
OmpF-1 R	GCCCTCAGAGTTGTTACCCT	59.4	40s		
OmpF-2 F	CCGGTTATGGTCAGTGGGAA	59.4	40s		
OmpF-2 R	CTGATGAAAACGCAGGCTGT	59.4	40s		
AmpC-1 F	GCGGGCAAATGGGTTTTCTA	57.3	40s	PCR amplification of *ampC* gene including the promoter and attenuator region, with a slight overlap to allow full length sequencing of the gene.	Present study
AmpC-1 R	AACCGATACTGGAGTTGGCA	57.3	40s		
AmpC-2 F	GGTGCCGGATGAAGTGAAAT	57.3	40s		
AmpC-2 R	GCGCAGAAAAGGTCCGAAAA	57.3	40s		
RAPD1283	GCGATCCCCA	36	5m	to randomly amplify many bands in DNA templates via RAPD-PCR and so identify the relationships between isolates	(147)
ampC cloning F	CCGGAATTCCGGGCGGGCAAATGGGTTTTCTA	59.69	40s	PCR amplification for cloning purpose. Amplicon digested using EcoRI/ BamHI and directly ligated into pk18.	Present study
ampC cloning R	CGCGGATCCGCGGAAAAGCGCAGAAAAGGTCCG	59.11	40s		
Pk-18 up	CTTCCGGCTCGTATGTTGTG	59.00	40s	for PCR Sequencing to check if desired gene was cloned successfully to the vector	Present study
Pk-18 down	GTTGTAAAACGACGGCCAGT	59.06	40s		
rseA cloning F	CGCGGATCCTGCAGAAAACCAGGGAAAGC	58.96	40s	PCR amplification for cloning purpose. Amplicon digested using BamHI/PstI and directly ligated into pKnock vector	Present study
rseA cloning R	TGCACTGCAGCCATTTGGGTAAGCTGTGCC	59.47	40s		
BT 87,543 F	CACTTAACGGCTGACATG	58	40s	for PCR Sequencing to check if desired gene was cloned successfully to the pKnock vector	Present study
BT 87,543 R	TGACGCGTCCTCGGTAC	58.5	40s		
FloR-F	GCATTGATCGGCGAGTTCTT	57.3	40s	to amplify *floR* gene in recipient cells	Present study
FloR-R	TTTAAAAGTGCCACCGCCAA	55.3	40s		
CTX-M-G1F	AAAAATCACTGCGCCAGTTC	52	40s	to check if plasmids were mobilised to the desired recipient through conjugation	(148)
CTX-M-G1R	AGCTTATTCATCGCCACGTT	52	40s		
CTX-M-G9 F	CAAAGAGAGTGCAACGGATG	52	40s	to check if plasmids were mobilised to the desired recipient through conjugation	
CTX-M-G9 R	ATTGGAAAGCGTTCATCACC	52	40s		

underlined sequences show the restriction recognition sites

2.3 Bacterial isolates and growth conditions

In total, forty-five 3GCR *E. coli* isolates including 25 dairy farm isolates and 20 human urinary isolates were used in this study. An additional six *E. coli* isolates were included: Farm-WT (3GCR), and Farm**-WT (cephalosporin/carbapenem susceptible), plus four cefalexin resistant but 3GC-S isolates: two dairy farms isolates (CL-R Farm-1, CL-R Farm-2) and two human urinary isolates (CL-R UTI-1, CL-R UTI-2). *E. coli* fully susceptible clinical isolate ATCC 25922, *E. coli* 17 (provided by Dr. Mandy Wootton, Public Health Wales), *E. coli* DH5α and *E. coli* BW 20767 were used throughout for quality control and/or transformation. All *E. coli* strains used in this study are summarised in Table 2.3. Strains were stored at -70°C in glycerol bead vials (Protect Bacterial Preserves).

Dairy farm *E. coli* isolates were collected between January 2017 and December 2018 from farms located within a 50 x 50 km region of the Southwest of England (149, 121). This region was chosen because it also included the locations of 146 GP practices that submitted urine samples for processing at the Severn Pathology laboratory, as described in a recently published survey of human urinary *E. coli* (122), which was also the source of the human urinary isolates used in the present study.

Cattle-associated isolates were obtained, variously, from faecally contaminated sites around calves, adult heifers, cows, and the near-farm environment. The collected samples were initially processed by selecting for cefalexin resistant isolates, using TBX *E. coli* indicator agar containing 16 mg/L cefalexin. Cefalexin resistant *E. coli* isolates were then sub-cultured onto plates containing cefotaxime (CTX, 3GC, 2 mg/L). The resistant isolates were then tested for mobile β-lactamase genes using a series of multiplex PCR reactions. Following this, only CTX resistant

(3GCR), mobile β-lactamase negative isolates were selected for further investigation as potential chromosomally AmpC-hyper-producing isolates.

Table 2.3 List of all *E. coli* strains used in this study

Isolate ID	Description	Isolate ID	Description
Farm-1	3GC-R cattle isolate	UTI-1	3GC-R human urinary isolates
Farm-2	3GC-R cattle isolate	UTI-2	3GC-R human urinary isolates
Farm-3	3GC-R cattle isolate	UTI-3	3GC-R human urinary isolates
Farm-4	3GC-R cattle isolate	UTI-4	3GC-R human urinary isolates
Farm-5	3GC-R cattle isolate	UTI-5	3GC-R human urinary isolates
Farm-6	3GC-R cattle isolate	UTI-6	3GC-R human urinary isolates
Farm-7	3GC-R cattle isolate	UTI-7	3GC-R human urinary isolates
Farm-8	3GC-R cattle isolate	UTI-8	3GC-R human urinary isolates
Farm-9	3GC-R cattle isolate	UTI-9	3GC-R human urinary isolates
Farm-10	3GC-R cattle isolate	UTI-10	3GC-R human urinary isolates
Farm-11	3GC-R cattle isolate	UTI-11	3GC-R human urinary isolates
Farm-12	3GC-R cattle isolate	UTI-12	3GC-R human urinary isolates
Farm-13	3GC-R cattle isolate	UTI-13	3GC-R human urinary isolates
Farm-14	3GC-R cattle isolate	UTI-14	3GC-R human urinary isolates
Farm-15	3GC-R cattle isolate	UTI-15	3GC-R human urinary isolates
Farm-16	3GC-R cattle isolate	UTI-16	3GC-R human urinary isolates
Farm-17	3GC-R cattle isolate	UTI-17	3GC-R human urinary isolates
Farm-18	3GC-R cattle isolate	UTI-18	3GC-R human urinary isolates
Farm-19	3GC-R cattle isolate	UTI-19	3GC-R human urinary isolates
Farm-20	3GC-R cattle isolate	UTI-20	3GC-R human urinary isolates
Farm-21	3GC-R cattle isolate	CL-R UTI-1	Cefalexin resistant, 3GC-S human urinary isolate
Farm-22	3GC-R cattle isolate	CL-R UTI-2	Cefalexin resistant, 3GC-S human urinary isolate
Farm-23	3GC-R cattle isolate	*E. coli* -17	Fully susceptible human urinary isolate
Farm-24	3GC-R cattle isolate	*E. coli* ATCC 25922	Fully susceptible type strain
Farm-25	3GC-R cattle isolate	*E. coli* DH5α	Fully susceptible laboratory strain
Farm-WT	3GC-R cattle isolate	BW 20767	Fully susceptible laboratory strain
Farm**WT	cephalosporin/carbapenem susceptible cattle isolate	CL-R Farm-1	Cefalexin resistant, 3GC-S cattle isolate
CL-R Farm-2	Cefalexin resistant ,3GC-S cattle isolate		

2.4 DNA extraction, amplification, and sequencing

2.4.1. DNA extraction

Crude DNA template was obtained by suspending one bacterial colony in 100 µL distilled water and boiling at 95 ˚C for 10 min. Bacteria were grown overnight on fresh LB agar at 37 ˚C prior to DNA extraction.

2.4.2 Polymerase chain reaction (PCR)

PCR reactions were carried out using 10 pmol of each desired primer to amplify target sequence. PCR reactions were performed in 25 µL reaction mixtures, prepared as follows: 12 µL of 2× My Taq™ Red, 1 µL of both F and R primers, 1 µL DNA template and sterile H_2O up to final volume 25 µL. The reactions were performed in a 96-well PCR plate, using (PCRmax Alpha Cycler). The thermocycling conditions for all genes were the same except for the annealing temperature which was primer specific. The thermocycling conditions consisted of an initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 45 -70°C for 1 min, extension at 72°C for 1 min, and a final extension for 10 min.

High fidelity Phusion kit was utilised to amplify target genes prior to cloning process following the manufacturer's instruction. PCR reactions were performed in 25 µL reaction mixtures, prepared as follows: 4 µL of 5xPhusion GC buffer,0.2 µL Phusion polymerase, 1 µL (10 pmol) of both F and R primers, 0.4 µL (10 mM dNTPs), 1 µL DNA template and sterile H_2O up to final volume 25 µL. The reactions were performed in a 96-well PCR plate, using (PCRmax Alpha Cycler). The thermocycling conditions consisted of an initial denaturation at 98 °C for 10 min followed by 30 cycles of denaturation at 98°C for 1 min, annealing at 45 -70°C for 1 min, extension at 72°C for 1

min, and a final extension for 10 min. PCR products were subsequently analysed on the agarose gel, as described in the following section.

2.4.3 Agarose gel electrophoresis

1.5% agarose gel was prepared to visualize the DNA size. 1.5 g of agarose (Bioline) was dissolved in 100 mL of 1× TAE buffer and supplemented with ethidium bromide 0.1 µg/mL. 5 µL of the PCR products from each sample was loaded into the wells in the gel, alongside the 5 µL of the 1 kb Hyper Ladder or 100 bp DNA ladder. The samples were run for 30 min at 100 V. The agarose gel was later visualised under UV light using the Bio-Rad GelDoc 1000 system (Bio-Rad, UK).

2.4.4 DNA sequencing

DNA samples were sent to Eurofins Ltd for sequencing. Samples were prepared by adding a final concentration of 25-50 ng/µL of sample (plasmid or PCR product, respectively) plus 1 µL (10 pmol) of the relevant primer.

2.5 DNA purification from agarose gel or PCR reactions

DNA was purified from agarose gel after cutting out bands-containing DNA utilising QIAquick® Gel Extraction Kit or from PCR products by QIAquick® PCR Purification Kit following instruction in accordance with manufacturer's protocol. DNA was eluted in 30 µL of elution buffer and concentration of DNA in these samples was quantified by Nanodrop system.

2.6 Cloning and transformation

2.6.1 Vectors

Two vectors - pK18 and pKnock - were used to clone different genes at different conditions as described below (Figure 2.1 and Figure 2.2), pKnock being used for insertional inactivation.

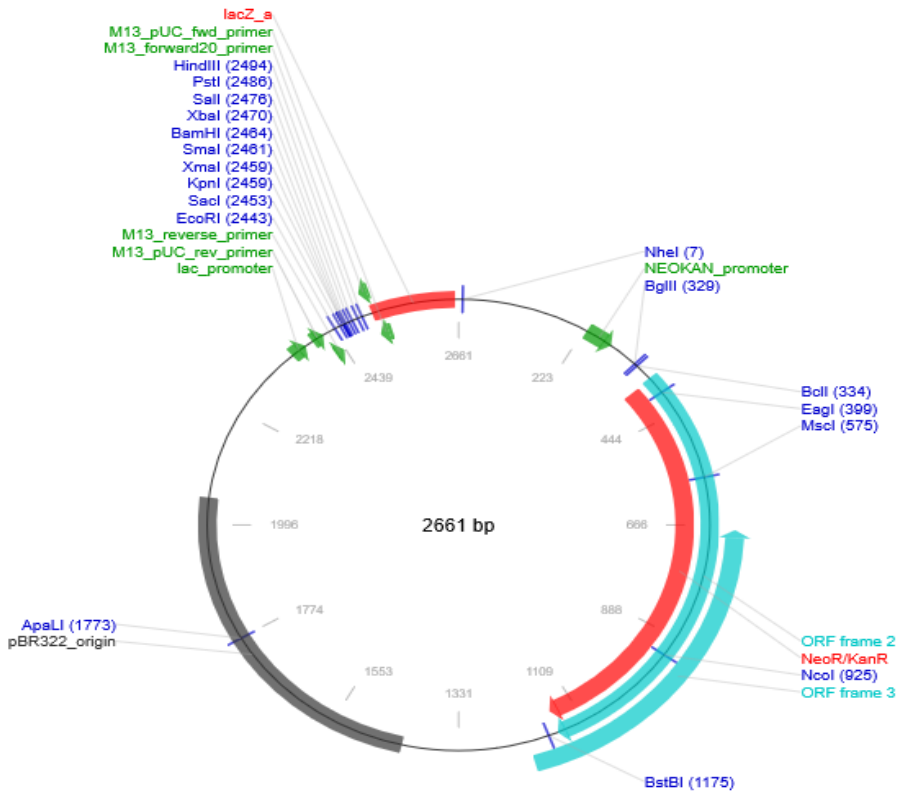


Figure 2.1. pK18 vector is a multi-copy cloning vector that confers kanamycin resistance. PCR products containing overhang restriction sites can be ligated to the multiple cloning site (Source: http://www.addgene.org/vector-database/3300/).

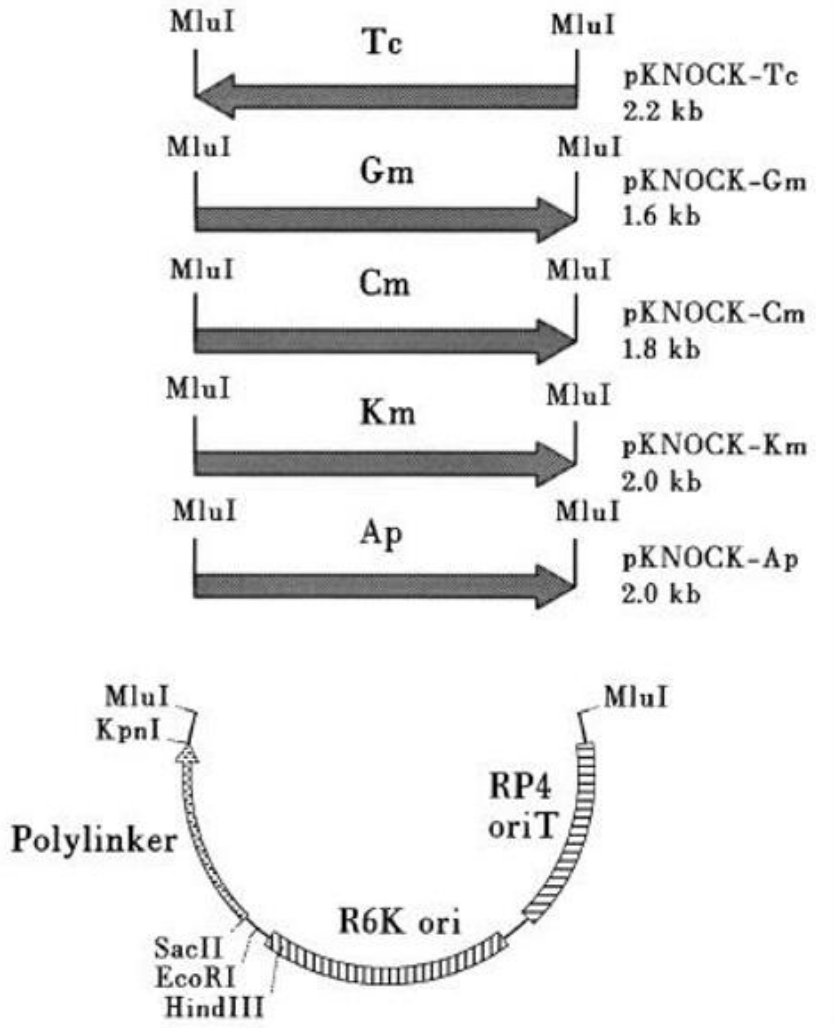


Figure 2.2. pKnock suicide vector obtained from Chulabhorn Research Institute, Thailand used for gene knockout by ligating a fragment of the desired gene at the polylinker site (150).

2.6.2 Plasmid preparation

Plasmids were extracted from overnight culture supplemented by appropriate antibacterial using the QIAprep Spin Miniprep Kit following the manufacturer's protocol. DNA concentration was quantified using Nanodrop system.

2.6.3 Gene cloning

The desired DNA sequence was amplified using suitable primers pairs (see Table 2.2). Each PCR reaction was carried out in 25 µL volume with a high-fidelity PCR kit following the manufacturer's instructions. 3 µL of each amplified product was resolved using 1.5% agarose gel, and following confirmation of the optimal size, the remaining 22 µL of each PCR product was purified using a QIAquick PCR Purification Kit following the manufacturer's instructions.

Vectors were cleaved by appropriate restriction enzymes, (EcoRI/ BamHI in case of pk18) and (BamHI/PstI in case of pKnock), and were visualized on 1% agarose gel for 30 min at 100 V. An uncut vector was used as a control. The desired band was purified from the gel using a Gel purification kit according to the manufacturer's protocol. Subsequently, amplicons were ligated into the linearized vector using rapid ligation kit following the manufacturer's instruction. Recombinant plasmids used to transform into *E. coli* chemically competent cells as detailed below. Transformants were selected on Luria-Bertani (LB) agar supplemented with kanamycin (50 µg/mL) or gentamycin (10 µg/mL). The molecular size of the inserts was estimated by the PCR reaction. 3 µl of the resulting products were run on 1.5% agarose gel. PCR products that revealed successful

cloning were cleaned up with QIAquick PCR Purification Kit and sent to Eurofins for sequencing to confirm the sequence of the cloned gene.

2.6.4 Transformation

2.6.4.1 Chemically competent cell generation

Chemically competent cells were made by sub-culturing 1 mL of overnight culture, which was prepared by inoculating one colony from fresh plate into 10 mL of LB broth, into 50 mL of fresh LB broth and incubating in a shaking incubator 180 rpm, 37°C until OD_{600} 5 (mid-log phase). The cell cultures were aliquoted (25 mL in 50 mL falcon tubes) and pelleted by centrifugation at 4000 x g for 13 min at 4°C, followed by resuspending cells with 25 mL ice chilled 100 mM $CaCl_2$ and incubation in ice for 30 min. Cells were then harvested by centrifugation at 4000 x g for 13 min at 4°C. Pellet was gently resuspended in 200 µL of 100 mM $CaCl_2$ and a 50 µL of 50% (v/v) glycerol was added. Aliquots of 50 µL were stored at -70°C for later use.

2.6.4.2 Transformation via heat shock

Cells were transformed via the heat-shock method. On ice, 50 µL of competent cells were incubated with 10 µL of the ligation reaction for 30 min. Subsequently, cells were incubated at -42°C in a heat block (Grant QBT2) for 45 s, then returned to ice for 5 min incubation. Each aliquot of cells was incubated in a rotating incubator (New Brunswick Scientific) at 180 rpm for one hour at 37°C after adding 500 µL of (pre-warmed at 37°C) Super Optimal Broth (SOB medium). Cells were pelleted by centrifugation at 4000 x g for 10 min and supernatant was discarded. 60 µL of

SOB medium was added to resuspend the cells. 30 µL aliquots of the cells were spread on LB agar plates with appropriate selection antibacterials and grown overnight at 37°C. Colonies were selected for colony PCR, and PCR products were sequenced and analysed to confirm successful insertion into the vector.

2.7 Gene Knockout

Gene knockouts were made via a homologous recombination method using the pKnock suicide vector (Figure 2.2). An amplicon (~ 200 bp) of the gene to be knockout (gene X) was ligated into to pKnock vector utilising appropriate restriction enzymes following the same steps illustrated in section (2.6.3). The molecular size of the insert was estimated by PCR and gel electrophoresis. The construct of (pKnock-gene X) was then transformed into *E. coli* BW 20767 (donor). The recombinant plasmid then was extracted using QIAprep Spin Miniprep Kit and sent to Eurofins Ltd for sequencing to confirm the sequence of the cloned gene fragment. Subsequently, the construct was conjugated from the donor cell *E. coli* BW 20767 to the desired recipient cells following the same procedure explained in section 2.8. 20 µL of the donor and recipient mixture was spread onto a selective plate including 10 µg/mL gentamycin (to select the transferred plasmid) and 4 µg/mL ciprofloxacin or 50 µg/mL kanamycin (depending on the marker being used to counter-select against the donor), and incubated overnight at 37°C. Homologous recombination between the desired gene (gene X) located in bacterial chromosome and in the pKnock plasmid results in insertional inactivation of gene X on the chromosome (Figure 2.3).

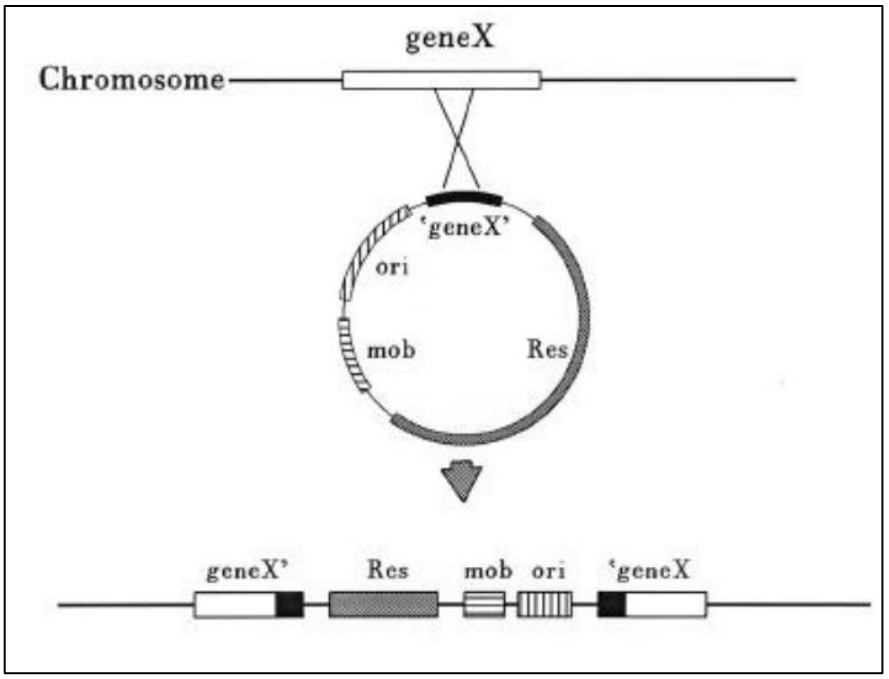


Figure 2.3. Illustration of insertional inactivation (knocking out) of gene x through homologous recombination of gene X carried in the bacterial chromosome and pKNOCK vector (150).

2.8 Conjugation

Donor and recipient strains were grown overnight on LB agar plates supplemented with appropriate antibacterials. A loopful of each culture was scraped and resuspended separately in to 1 mL of phosphate-buffered saline PBS and centrifuged at 12000 x g for 1 min. The pellet was then resuspended in 1 mL 100 mM $CaCl_2$ and incubated on ice for 30 min. A 3:1 ratio of the recipient cell was mixed with the donor and around 4 µL of the mixture was spotted onto non-selective LB agar and incubated for 4-5 h. Spots were scraped up into a micro centrifuge tube containing 500 µL PBS (samples were further diluted when necessary). 30 µL of the mixture was spread in a selective plate relevant for the particular conjugation experiment and incubated

overnight at 37°C. Plasmids introduced to the recipient strains during the conjugation experiment are illustrated in table 2.4.

Table 2.4 List of plamids introduced into the recipient strains via conjugation

Plasmids transferred via conjugation	Source of bacterium
CTX-M 1	Cattle isolate (YYZ 16-1)
CTX-M 14	Cattle isolate (PSA 37-1)
CTX-M 15	Cattle isolate (YYZ 16-1)
CTX-M 32 (pMoo 32)	Cattle isolate (DUK 14-2)

2.9 Minimal Inhibitory Concentration (MIC) determination

The MIC of each actibacterial was determined by broth micro-dilution in 96 wells plate (Corning, Costar) as described in the CLSI standard protocol (151,152). 180 µL per well of 2-fold serial dilution of the desired antibacterial were dispensed starting from the highest concentration in Cation Adjusted Muller Hinton Broth CAMHB (Sigma). 20 µL of the bacterial suspension, prepared by dispersal of bacterial colonies from a 24-h culture on agar into 3 mL PBS to yield a turbidity equal to an OD_{600} 0.1., was dispensed in each well containing a gradient of antibacterial concentration to give a final OD_{600} of 0.001 in a final volume in each well of 200 µL. Bacterial growth was measured after 18-20 h of incubation at 37 °C using a Fluostar Optima (Aylesbury, UK) plate reader. MIC values were defined as the lowest antibacterial concentration to prevent any visible growth.

2.10 Antibacterial susceptibility testing by disc diffusion

Disc susceptibility tests were performed utilising Mueller-Hinton agar MHA (sigma) following the CLSI guidelines, and the results were interpreted using CLSI performance standards (151,153).

2.11 Selection of mutants

Mutants were obtained on MHA (sigma) via in vitro exposure to different concentrations of various antibacterials, as set out in Results. Mutants selected by spreading 50 µL of overnight culture on to MHA plates supplemented with different concentrations of the antibacterial. One representative mutant colony that grew at highest concentration was picked for further analysis.

2.12 Fluorescent Hoescht (H) 33342 dye accumulation assays

Envelope permeability in living bacteria was tested using a standard dye accumulation assay protocol (154) where the dye only fluoresces if it crosses the entire envelope and interacts with DNA. Overnight cultures in CAMHB at 37°C were used to prepare CAMHB subcultures, which were incubated at 37°C until a 0.6-0.8 OD_{600} was reached. Cells were pelleted by centrifugation (4000 x g, 10 min; ALC, PK121R) and resuspended in 1 mL of phosphate-buffered saline. The optical densities of all suspensions were adjusted to 0.1 OD_{600}. Aliquots of 180 µL of cell suspension were transferred to a black flat-bottomed 96-well plate (Greiner Bio-one, Stonehouse, UK). Eight technical replicates for each strain tested were in each column of the plate. The plate was transferred to a POLARstar spectrophotometer (BMG Labtech) and incubated at 37°C. Hoescht dye (H33342, 25 µM in water, (Sigma)) was added to bacterial suspension of the plate using the

plate-reader's auto-injector to give a final concentration of 2.5 µM per well. Excitation and emission filters were set at 355 nm and 460 nm, respectively. Readings were taken in intervals (cycles) separated by 150 s. Thirty-one cycles were run in total. A gain multiplier of 1300 was used. Results were expressed as absolute values of fluorescence versus time.

2.13 Whole genome sequencing (WGS) and analyses

WGS was performed by MicrobesNG (https://microbesng.uk/) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2x250 bp paired end reads. Reads were trimmed using Trimmomatic (155) and assembled into contigs using SPAdes 3.13.0 (156) (https:// http://cab.spbu.ru/software/spades/).

Resistance genes, plasmid replicon types and sequence types (according to the Achtman scheme (157)) were assigned using the ResFinder (158), Plasmid Finder (159), and MLST 2.0 on the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) platform. Contigs were annotated using Prokka 1.2 (160). Pairwise contig alignments to identify mutations versus parent isolate, which were sequenced in parallel, was with EMBOSS Stretcher (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/) using default perameters.

2.14 Phylogenetic analysis

Sequence alignment and phylogenetic analysis was carried out on the Bioconda software package (161) on the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) (162). Sequences were first aligned to a closed read reference sequence and analysed for SNP differences, whilst omitting insertion and deletion elements, using the 'Snippy' alignment program. Alignment was then focused on regions of the genome found across all isolates, using the Snippy-core program, thus eliminating the complicating factors of insertions and deletions (163). Aligned sequences were then used to construct a maximum likelihood phylogenetic tree using RAxML, utilising the GTRCAT model of rate heterogeneity and the software's autoMR and rapid bootstrap to find the best-scoring maximum likelihood tree and including tree branch lengths, defined as the number of base substitutions per site compared (164,165). Finally, phylogenetic trees were illustrated using the web based Microreact program (166).

2.15 Protein studies

2.15.1 Preparation of cell extracts

1 mL of a 10 mL overnight CAMHB culture was transferred to 50 mL CAMHB and cells were grown at 37°C to 0.6-0.8 OD_{600}. Cells were pelleted by centrifugation (10 min, 4,000 × g, 4°C) and resuspended in 35 mL of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1 sec on, 0.5 s off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at 4973 x g (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4°C to pellet intact cells and large cell debris. The

supernatant retained as a source of crude cell protein. Protein concentrations in all supernatants were quantified using Biorad Protein Assay Dye Reagent Concentrate according to manufacturer's instructions.

2.15.2 Separation of proteins using SDS-polyacrylamide gel electrophoresis (PAGE) and proteomics analysis

2.15.2.1 SDS-PAGE

Crude cell extracts, made as described above, were mixed with SDS-PAGE loading buffer (100 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 5% β-mercaptoethanol) and heated at 95°C for 5 min then centrifuged for 5 min. 1 µg/lane of total protein from each sample was separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. Separating gels were obtained by combining 4.2 mL of 30% acrylamide/bisacrylamide solution (37.5:1 Bio-Rad), 2.5 mL of separating buffer (1.5M Tris-Base pH 8.8, 4% SDS), 2.3 mL of Elgastat water, 100µL of 10% w/v ammonium persulphate, and 20 µL of 10% TEMED. The stacking gel solution was made up of 2 mL of 30% acrylamide/bisacrylamide solution (37.5:1, Bio-Rad), 2.5 mL of the stacking buffer (0.5M Tris-Base pH 6.8, 0.4% SDS), 4.5mL of water, 100 µL of 10% w/v ammonium persulphate, and 20 µL of 10% TEMED. Samples were run on the gel at 200 V until the dye front had moved approximately 1 cm into the separating gel. Proteins in all gels were stained with Instant Blue stain for 5 min to be visualised and de-stained in Elgastat water.

2.15.2.2 Proteomic analyses

Proteomic analyses were repeated three times for each strain, using a separate batch of cells each time. The gel lanes including the proteins were cut out and proteins were tryptic digested (in-gel) using a DigestPro automated digestion unit (Intavis Ltd). The resulting peptides from each gel fragment were separately fractionated with an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (v/v) acetonitrile plus 0.1% (v/v) formic acid, peptides were resolved on a 250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min., 6-15% B over 58 min., 15-32% B over 58 min., 32-40% B over 3 min., 40-90% B over 1 min., held at 90% B for 6 min and then reduced to 1% B over 1 min.) with a flow rate of 300 nl.min−1. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.1 kV using a stainless-steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.1 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyse the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 2000 and the top twenty multiply charged ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. Charge state filtering, where unassigned precursor ions were not selected for 60 fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion list size,

500) were used. Fragmentation conditions in the LTQ were as follows: normalized collision energy, 40%; activation q, 0.25; activation time 10 ms; and minimum ion selection intensity, 500 counts.

The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific) and searched against the UniProt *E. coli* K12 using the SEQUEST (Ver. 28 Rev. 13) algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled, and all peptide data was filtered to satisfy false discovery rate (FDR) of 5 %. The Proteome Discoverer software generates a reverse "decoy" database from the same protein database used for the analysis and any peptides passing the initial filtering parameters that were derived from this decoy database is defined as false positive identifications. The minimum cross-correlation factor filter was readjusted for each individual charge state separately to optimally meet the predetermined target FDR of 5 % based on the number of random false positive matches from the reverse decoy database. Thus, each data set has its own passing parameters. Protein Area measurements were calculated from peptide peak areas using the Top 3 method (167) and proteins with fewer than three peptides identified were excluded.

Raw data of three repetitions were uploaded into Microsoft Excel. Raw abundance data of each protein was normalised for the loading variation between samples by dividing each abundance value by the average abundance of 30S and 50S ribosomal proteins in the same sample. A one

tailed, unpaired T-Test was applied to calculate significance of any difference in normalised protein abundance data in the three sets of replicates from the two test conditions. A p-value of less than 0.05 were considered significant. Fold change was calculated using the averages of normalised protein abundance in the three biological replicates for the two-test condition (168).

2.15.3 β-Lactamase assay

To measure the levels of β-lactamase activity in the crude cell extracts, 20 µL of each sonicated sample prepared as detailed in section 2.15.1 above, was loaded into a 96-well plate (Greiner Bio-one, Stonehouse, UK), and 180 µL of 100 µM nitrocefin solution, prepared in filtered (0.2 µm) assay buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$ pH 7.0, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$), was loaded into each well as a substrate. Changes in absorbance over time were measured over 60 cycles at a wavelength of 482 nm (25°C) using a Fluostar Optima (Aylesbury, UK) plate reader. Total protein concentration in each sample was quantified using the Bio-Rad protein assay reagent, according to the manufacturer's instructions, and then utilised to calculate relative specific enzyme activity (nmol nitrocefin hydrolysed per minute per microgram of total protein). Linear gradients (ΔAU/min) were extrapolated, and calculation was based upon the molar extinction coefficient of 17,400 AU/M (169).

Chapter 3

Characterisation of Cephalosporin resistance due to AmpC hyperproduction in Farm *E. coli* isolates

3.1 Introduction

E. coli typically produces a class 1 cephalosporinase, encoded by the *ampC* gene, which is chromosomally located. Expression of *ampC* in wild-type (WT) cells is low and not enough to confer clinically relevant resistance to β-lactam antibacterials (64). Many mutations, insertions and gene duplication events have been shown to cause *ampC* hyperexpression leading to varying β-lactam resistance depending on the actual amount of AmpC produced (64). AmpC hyperproduction was first seen in *E. coli*, in 1979 (170) from human clinical samples, and before the emergence of plasmid-mediated ESBLs. AmpC hyperproduction was the dominant mechanism for resistance in (3GCs) resistant *E. coli* from humans (64). This is, however, no longer the case. For example, in a recent survey of cefotaxime-resistant (CTX-R) *E. coli,* from urine samples collected from Southwest England population, only 24/626 isolates (3.8%) were presumed to be AmpC hyperproducers because of their lack of horizontally acquired β-lactamase genes. Whole Genome Sequencing (WGS) confirmed that 13/13 sequenced isolates had *ampC* promoter mutations typical of AmpC hyperproducers (122).

AmpC is typical class 1 β-lactamase where it does not confer resistance to (4GCs) (64). However, AmpC structural variants in *E. coli* that expand AmpC activity to include cefepime, for example, have been identified in humans (72,73,171,172) and cattle (74). These are dominated by isolates from relatively less pathogenic phylogroup A and particularly ST88(172,74). This is probably because expanded-spectrum activity evolves from existing AmpC hyperproducers among which ST88 isolates are particularly common (173). Members of the research group in which this PhD project was based, recently conducted a survey of 4594 samples collected, from faecal contaminated sites, in 53 dairy farms in Southwest England. Out of these, 384 samples, from 47

farms, were found to be positive for CTX-R *E. coli* isolates (149). Among these samples, Dr Jacqueline Findlay found that 566/1226 of CTX-R *E. coli* isolates studied (from 186 samples from 38 farms) were found to be PCR negative for mobile cephalosporinases and so we presumed these to be chromosomal AmpC hyperproducers (121). If this presumption was proven to be correct, AmpC hyperproduction must be the mechanism of resistance in 46.2% of CTX-R *E. coli* from dairy cattle in this region of the UK. This figure is comparable with the 42.9% presumed AmpC hyperproducers seen in CTX-R *E. coli* from dairy cattle in a recent nationwide Dutch study (174) but is in contrast to 3.8% of AmpC hyperproducers seen in CTX-R isolates in a recent study on human urinary *E. coli* carried out by Dr Jacqueline Findlay within our group (122). Therefore, the main aim of the work carried out as part of this PhD project and reported here was to characterize putative AmpC-hyperproducing *E. coli* from the group's recent survey of dairy farms (149,121) and confirm their phenotype. This was done as part of a wider study that also aimed to identify the risk factors for the presence of AmpC hyperproducers on dairy farms, which was performed by Dr Hannah Schubert and will briefly be mentioned here. This work also allowed a comparative genomics analysis of human and cattle AmpC hyperproducing *E. coli* within the study region, which will be discussed in Chapter 4.

This work has been published as my contribution to the below paper, in which I am first author. The risk factor analysis discussed in this chapter, and also included in the paper was performed by Dr Hannah Schubert. Dr Jacqueline Findlay initially identified the putative AmpC hyper-producing isolates that were studied by me in this chapter. Dr Kate Heesom generated the proteomics data, which I analysed. Other authors in this paper were either project supervisors or not involved in the work reported in this chapter.

Maryam Alzayn, Jacqueline Findlay, Hannah Schubert, Oliver Mounsey, Virginia C. Gould, Kate J. Heesom, Katy M. Turner, David C. Barrett, Kristen K. Reyher and Matthew B. Avison. 2020. Characterization of AmpC-hyperproducing *Escherichia coli* from humans and dairy farms collected in parallel in the same geographical region. *Journal of Antimicrobial Chemotherapy* https://doi.org/10.1093/jac/dkaa207

3.2 Results and discussion

3.2.1 Cephalosporin resistant in *E. coli* from dairy farms

The first aim was to investigate putative AmpC-hyperproducing *E. coli* isolates from dairy farms identified in the Avison group's recent surveillance study of CTX-R *E. coli* (149,121). A decision was made to focus on a 50 x 50-km subregion of the overall study area where 25 farms were found to be positive for putative AmpC hyperproducers, defined as CTX-R *E. coli* isolates that were PCR negative for known mobile cephalosporinase genes (identified by Dr Jacqueline Findlay). The reason for this focus was to allow later comparisons with human isolates collected within this same 50 x 50 km region.

In order to characterise the putative AmpC-hyperproducing isolates, first, antibiograms for β-lactams and chloramphenicol (see below) were determined for one putative AmpC-hyperproducing isolate from each of the 25 farms (Table 3.1). All isolates exhibited resistance or reduced suitability to at least three or more β-lactams, including ampicillin, cephalexin, cefotaxime, and-or ceftazidime. Five isolates – from Farm 1, Farm 15, Farm 21, Farm 22, and Farm 23 - were found to be resistant to cefepime, which is not expected for an AmpC hyperproducing *E. coli*. This will be discussed later in this chapter. None of the isolates analysed were found to be

resistant to the three carbapenems tested: imipenem, ertapenem or meropenem (only data shown for meropenem).

Table 3.1: β -Lactam susceptibility of putative AmpC-hyperproducing *E. coli* isolates from dairy farms.

Isolates	Aztreonam	Cefepime	Cefotaxime	Ceftazidime	Ceftriaxone	Cefotetan	Meropenem	Cefoperazone	Cefalexin	Ampicillin
Farm-1	22	22	22	6	18	17	33	16	6	6
Farm-2	21	33	23	17	22	18	33	21	6	6
Farm-3	21	33	24	19	25	22	32	22	6	6
Farm-4	21	30	23	16	23	16	34	24	6	6
Farm-5	20	31	22	16	23	18	33	21	6	6
Farm-6	23	32	25	21	25	20	33	21	6	6
Farm-7	21	31	24	16	24	18	31	19	6	6
Farm-8	22	33	21	18	22	19	32	21	6	6
Farm-9	18	32	22	16	24	12	33	13	6	6
Farm-10	20	33	22	15	21	18	32	20	6	6
Farm-11	24	34	25	21	27	20	33	20	6	6
Farm-12	24	31	24	23	23	22	31	27	6	6
Farm-13	20	32	22	17	25	17	30	19	6	6
Farm-14	22	32	24	19	25	21	31	22	6	6
Farm-15	20	22	20	16	24	17	33	21	6	6
Farm-16	23	30	25	23	23	21	31	24	6	6
Farm-17	26	33	26	26	27	25	32	26	6	6
Farm-18	21	30	22	21	23	24	33	27	6	6
Farm-19	21	32	24	17	23	19	32	21	6	6
Farm-20	22	33	25	21	23	17	32	19	6	6
Farm-21	22	18	19	17	23	21	32	19	6	6
Farm-22	22	23	21	6	17	19	31	14	6	6
Farm-23	22	23	20	18	24	20	33	21	6	6
Farm-24	19	33	23	18	26	19	32	19	6	6
Farm-25	18	31	21	13	22	16	31	18	6	6

Values shaded in blue represent intermediate (I), grey resistant (R) based on CLSI breakpoints, otherwise susceptible.

Four isolates were selected for further investigation (from Farms 1 to 4). These isolates presented a typical AmpC hyperproducing phenotype: resistance to ampicillin and cefalexin and non-susceptibility/resistance to cefotaxime and ceftazidime. The isolate from Farm 1 was clearly different from the others: resistant to ceftazidime, cefotaxime and ceftriaxone and non-susceptible to cefoperazone and cefepime, based on disc testing (Table 3.1). MIC testing confirmed this difference for ceftazidime and cefepime and extended it to 3GCs/4GCs which is licensed for use in cattle in the UK (Table3.2). Compared to the non-AmpC-hyperproducing control sample, human urinary *E. coli* 17, the four putative AmpC hyperproducers were found to be non-susceptible to ceftazidime and ceftiofur (a 3GC used on several farms during the period of sample collection) but not generally cefoperazone, cefepime or cefquinome (a 4GC used on some study farms during the period of sample collection). The MICs of the 4GCs cefepime and cefquinome were, respectively, six and seven doublings higher in Farm 1 isolates, when compared to control sample *E. coli* 17, and five doublings higher for each drug than against the isolate from Farm 2 (Table 3.2).

Table 3.2: MICs of 3GCs/4GCs against putative AmpC-hyperproducing *E. coli* isolates from dairy farm

Isolate	MIC µg/mL				
	Ceftazidime[h]	Ceftiofur[c]	Cefepime[h]	Cefquinome[c]	Cefoperazone[h,c]
EC17	0.25	0.5	0.125	0.03	0.25
Farm-1	256	16	8	4	64
Farm-2	16	4	0.25	0.125	4
Farm-3	16	4	0.125	0.125	4
Farm-4	32	4	0.5	0.5	32

Values shaded in grey represent resistant (R) according to CLSI breakpoints. EC17, control *E. coli* strain 17. h These cephalosporins are used in humans. c These cephalosporins are licensed for use in cattle in the UK

3.2.2 Confirmation of AmpC hyperproduction among CTX-R *E. coli* from dairy farms and identification of porin loss and *marR* mutation

Using LC-MS/MS proteomics, AmpC β-lactamase hyperproduction was confirmed in the four-representative presumed AmpC hyperproducer CTX-R isolates from Farm 1-4 (Table 3.3). β-lactamase activity was also measured spectrophotometrically in cell extracts of the selected isolates by hydrolysis of nitrocefin. Nitrocefin is a β-lactam-containing compound that changes colour when hydrolysed by a β-lactamase enzyme. As predicted, nitrocefinase activity was found to be enhanced in the four putative AmpC hyperproducers compared to two control isolates *E. coli* 17 and *E. coli* ATCC 25922 (Figure 3.1).

Table 3.3: Abundance of key resistance proteins in putative AmpC-hyperproducing *E. coli* from dairy farms

Accession	Description	EC17	Farm-1	Farm-2	Farm-3	Farm-4
P02931	**OmpF**	0.69± 0.36	0.02±0.03	0.99±0.36	1.03±0.34	0.12±0.08
P00811	**AmpC**	ND	0.79±0.19	0.86±0.20	0.89±0.16	0.96±0.20
P0AE06	**AcrA**	0.10±0.04	0.13±0.05	0.18±0.15	0.11±0.03	0.20±0.01
P31224	**AcrB**	0.07±0.01	0.07±0.06	0.14±0.03	0.08±0.08	0.11±0.02
P02930	**TolC**	0.12±0.06	0.08±0.07	0.13±0.02	0.12±0.02	0.39±0.09

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, (n = 3). Proteins whose abundance is significantly ($P < 0.05$) up or downregulated at least 2-fold relative to the *E. coli* strain 17 (EC17) control (see Materials and methods) are shaded in green and red, respectively. ND not detected.

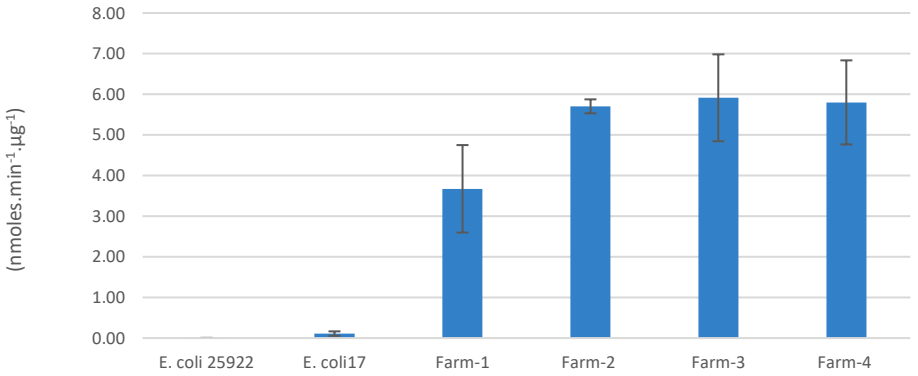


Figure 3.1: Specific AmpC enzyme activity of putative AmpC-hyperproducing *E. coli* isolates from dairy farms and two controls. *E. coli* 17 and *E. coli* ATCC 25922. Samples were incubated with nitrocefin at 25 °C as stated in Materials and Methods. Error bars show SEM, n=3.

Sequencing the *ampC* promoter region revealed that all four AmpC hyperproducers had the same promoter mutations (Figure 3.2), which has previously been shown to cause *ampC* hyperexpression (175). These mutations resulted in the creation of alternate -35 box (**T**TGACA), by a substitution of C to T at position -42. The alternate -35 box was separated by 17bp from the alternate -10 box (T**A**TCGT), which resulted from G to A change at position -18 as well as C to T changes at positions -88 and-1, T to C at -82 and C to T at +58 (Figure 3.2). Further WGS analysis revealed that all 25 putative AmpC hyperproducing CTX-R isolates studied carry these same promoter mutations, confirming their status as AmpC hyperproducers.

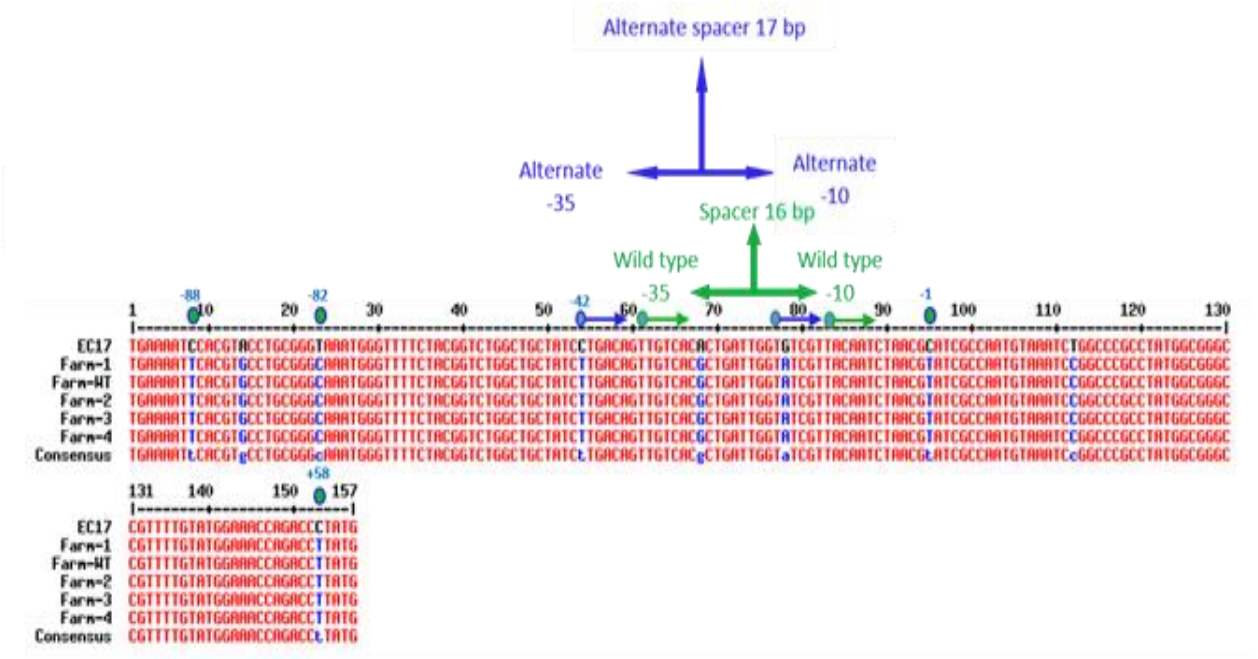


Figure 3.2: Sequence alignment of *ampC* promoter/attenuator from putative AmpC-hyperproducing *E. coli* isolates from dairy farms with a reference sequence of *E. coli* 17 strain. All 25 analysed samples carried the same mutation at positions: -42, -18, -1 and +58. Substitution at (-42) and (-18) resulted in the creation of an alternate -35 box and alternate -10 box, respectively. The two alternate boxes were separated by 17 bp, compared to the 16 bp-long separator that is found in the wildtype sample (*E. coli* 17 strain).

Proteomics showed that, unlike the other three AmpC hyperproducers subjected to detailed analysis, the cefepime-resistant isolate from Farm 1 produced OmpF porin at very low levels (Table 3.3). WGS revealed a loss-of-function mutation in *ompF* caused by the insertion of IS*4* at nt 625 explaining this reduced signal for OmpF production (Figure 3.3). However, OmpF porin loss

did not noticeably affect envelope permeability in the Farm 1 isolate when compared to other three isolates or the control (Figure 3.4). Indeed, only the isolate from Farm 4 had significantly reduced permeability, which was reminiscent of an efflux hyperproduction phenotype (constant reduced accumulation of the fluorescent dye; Figure 3.4).

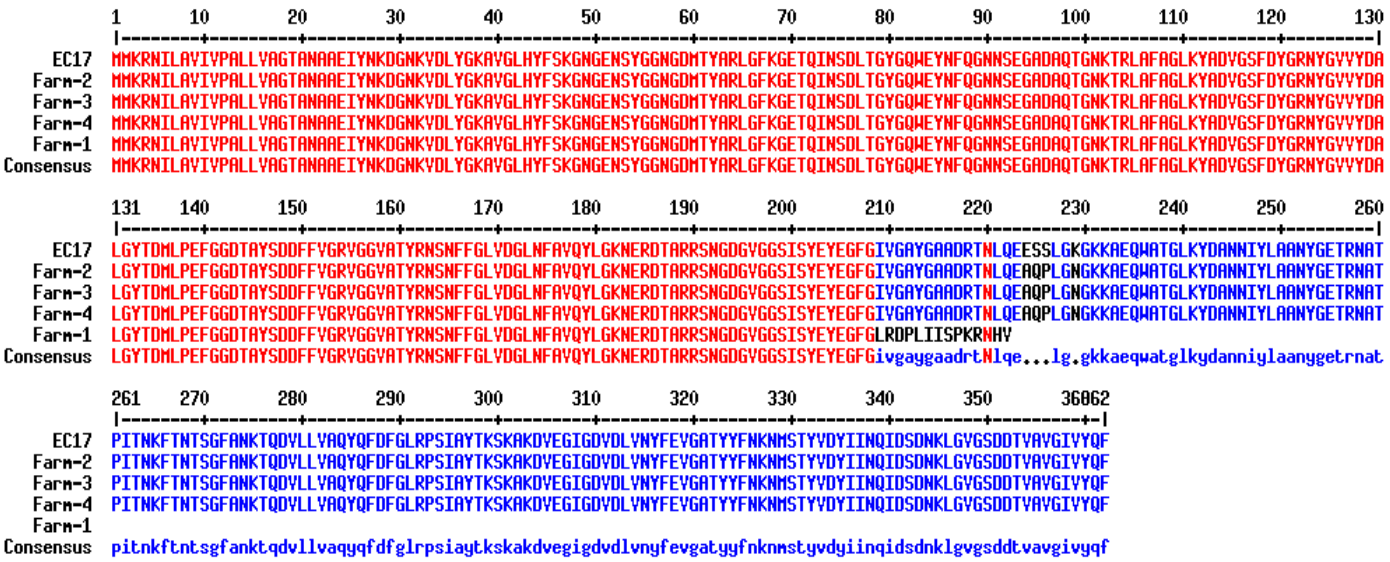


Figure 3.3: Amino acid sequence alignment of the translated *ompF* gene from 4 putative AmpC-hyperproducing *E. coli* isolates from dairy farms with reference sequence from *E. coli* 17. A disruption in the protein translation was detected in Farm-1 due to an insertion mutation in *ompF* gene, identified as belonging to the IS*4* family of insertion sequences. The remaining farm isolates had intact *ompF.*

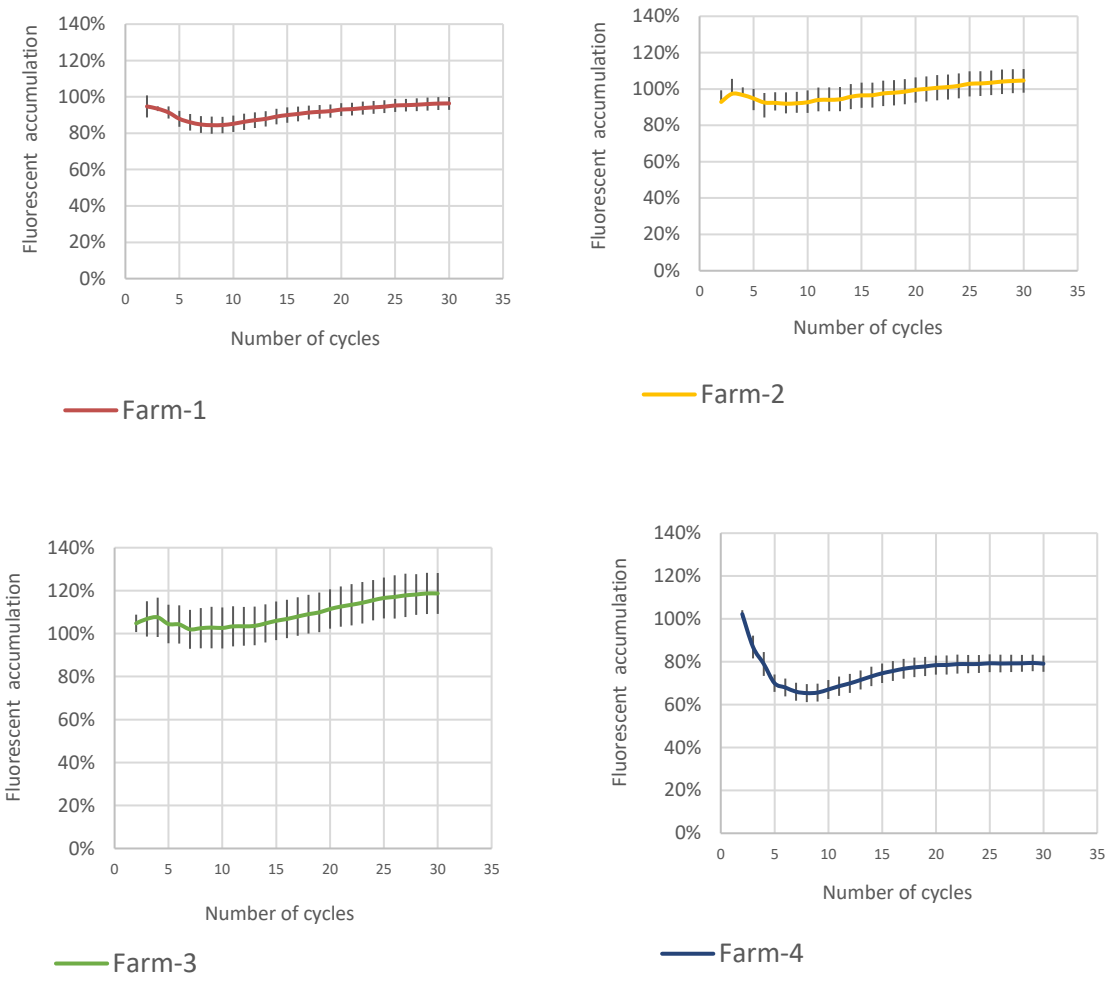


Figure 3.4: Envelope permeability of AmpC hyper-producing *E. coli* determined using fluorescent dye accumulation assays. In each case, fluorescence of an AmpC hyper-producing isolates (Farm-1, -2, etc.) incubated with the dye is presented relative to that in the control *E. coli* strain EC17 after each cycle. Each line shows mean data for 3 biological replicates with 8 technical replicates in each. Error bars define the standard error of the mean.

Proteomics confirmed hyperproduction of AcrAB-TolC in the Farm 4 isolate and down-regulation of the OmpF porin (Table 3.3). This was reminiscent of a Mar phenotype which was confirmed by WGS (*marR* mutation), suspected loss-of-function mutation causing a Pro57Thr change in MarR. As expected of a Mar isolate, the Farm 4 isolate was non-susceptible, according to disc testing, to chloramphenicol, which is known as AcrAB-TolC substrates, but according to WGS the isolate did not carry any relevant mobile resistance genes compared to the other chloramphenicol resistant isolates that were harbouring a *floR* gene, Table 3.4.

Table 3.4: Chloramphenicol susceptibility of putative AmpC-hyperproducing *E. coli* isolates from dairy farms

Isolates	Resistance	Chloramphenicol
Farm-1	-	26
Farm-2	-	25
Farm-3	-	26
Farm-4	-	13
Farm-5	-	28
Farm-6	-	29
Farm-7	-	29
Farm-8	-	30
Farm-9	-	30
Farm-10	-	28
Farm-11	*floR*	6
Farm-12	-	25
Farm-13	-	27
Farm-14	-	28
Farm-15	*floR*	6
Farm-16	-	28
Farm-17	-	29
Farm-18	-	25
Farm-19	*floR*	6
Farm-20	-	20
Farm-21	*floR*	6
Farm-22	-	27
Farm-23	*floR*	6
Farm-24	-	27
Farm-25	*floR*	6

Values shaded in blue represent intermediate (I), grey resistant (R) based on CLSI breakpoints, otherwise susceptible.

Interestingly, the Farm 4 isolate was cefoperazone-resistant (Table 3.2). It would seem, therefore, that a combination of AmpC plus AcrAB-TolC hyperproduction and/or OmpF down-regulation leads to cefoperazone resistance in *E. coli*. Cefoperazone has been used, albeit rarely, as a therapy for mastitis in dairy cows in the UK. The *ompF* mutant AmpC hyperproducing isolate from Farm 1 was also cefoperazone resistant, but additionally was cefepime resistant (Table 3.2) and so an alternative mechanism was suspected.

3.2.3 First identification of expanded-spectrum AmpC variants in *E. coli* from UK dairy farms

As mentioned above, among 25 AmpC hyperproducing *E. coli* isolates tested five (Farm 1, Farm 15, Farm 21, Farm 22, and Farm 23) were found to be cefepime resistant (Table 3.1), which is not normally associated with AmpC hyperproduction. WGS analysis revealed that three out of these five isolates (Farm 15, Farm 21, Farm 23) harbour a bla_{OXA-1} gene along with AmpC hyper production. Carrying bla_{OXA-1} has previously been associated with cefepime resistance in *P. aeruginosa* (176) and *E. coli* (177). We ruled out additional AmpC hyperproduction as the cause of cefepime and cefoperazone resistance in the isolate from Farm 1. Since, its production in this isolate was not more than the other three confirmed AmpC-hyperproducing farm isolates subjected to LC-MS/MS proteomics analysis (Table 3.3). So, we next looked at the *ampC* gene sequence. There were several nucleotide sequence polymorphisms, from one *ampC* gene to the next amongst our four representative isolates (Farm 1 to Farm 4), but only one in the Farm 1 isolate stands out. This mutation is predicted to cause a His312Pro change (His296Pro when considering the mature AmpC protein following removal of the signal peptide), which has been

shown to enhance the hydrolytic activity of AmpC (68). Analysis of WGS of AmpC -hyperproducing isolates from other dairy farms in the Southwest of England, not included in the analysis above, identified another isolate with identical *ampC* open reading frame and promoter sequence to the Farm 1 isolate. However, this additional isolate did not carry the single mutation predicted to be required for enhanced spectrum AmpC activity. We named this additional isolate Farm-WT. A mutant named Farm-WT-M1 was selected using the 3GC ceftazidime at CLSI agar dilution breakpoint MIC (8 mg/L) using Mueller-Hinton agar. The mutant did not have altered production of key resistance proteins relative to its parent, Farm-WT (Table 3.5).

Table 3.5: Abundance of key resistance proteins in cefepime resistant mutant and its parent isolate

Accession	Description	Farm-WT	Farm-WT-M1
P02931	**OmpF**	1.54±1.34	0.81±0.24
P00811	**AmpC**	1.13±0.77	0.76±0.24
P0AE06	**AcrA**	0.13±0.07	0.12±0.02
P31224	**AcrB**	0.04±0.01	0.05±0.01
P02930	**TolC**	0.19±0.10	0.19±0.05

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, (n = 3).

Sequencing of the *ampC* gene from Farm-WT-M1 revealed a mutation predicted to cause identical His296Pro change in AmpC, as seen in the isolate from Farm 1 (Figure 3.5) and the mutant had the same expanded-spectrum antibiogram as the isolate from Farm 1 (Table 3.6). Since Farm-WT-M1, like its parent, had a wild type *ompF* sequence according to WGS, and expression according to proteomics (Table 3.5), this confirmed that the insertional inactivation of *ompF* seen in Farm 1 isolate had little impact on the MICs of 3GCs and 4GCs in the presence of an expanded-spectrum AmpC variant (Table 3.6). Interestingly, we selected mutants from two other farm isolates using ceftazidime in same concentration (8 mg/L) using Mueller– Hinton Agar but His296Pro mutation was not detected, and the selected mutants were susceptible to cefepime based on disc testing, suggesting the importance of this amino acid substitution.

Based on analysis of *ampC* sequence from WGS data, only one other AmpC hyper-producer isolate - Farm 22 - was found to carry a known expanded-spectrum AmpC variant. Again, this was the same His296Pro mutation (Figure 3.5) and the same expanded-spectrum antibiogram as seen in Farm 1 isolate was observed (Table 3.6). Interestingly, the *ompF* porin gene was intact in the isolate from Farm 22. Measurement of MICs for the isolates provided further evidence that loss of OmpF was not important for 3GC/4GC resistance conferred by the expanded-spectrum AmpC (Table 3.6).

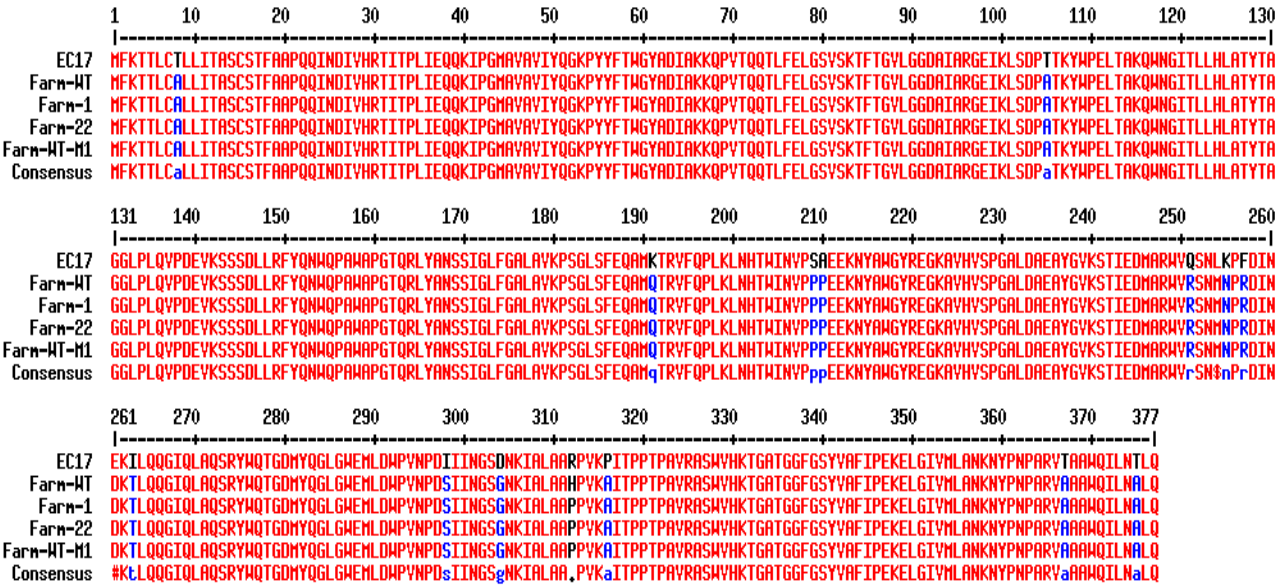


Figure 3.5: Sequence alignment of AmpC amino acid from CTX-R *E. coli* dairy isolates with a reference sequence of *E. coli* 17 strain. Amino acid sequences of Farm-1 and Farm-22 samples showed a single amino acid substitution His296Pro, compared to *E. coli* isolate from other farms. Same amino acid substitution was detected in selected mutant (Farm-WT-M1) compared to its parent isolate (Farm-WT). This mutant was selected using Mueller– Hinton Agar supplemented by (8 mg/L) ceftazidime.

Table 3.6: MICs of 3GCs/4GCs against putative expanded AmpC-hyperproducing *E. coli* isolates and mutants

Isolate	MIC µg/mL				
	Ceftazidime	Ceftiofur	Cefepime	Cefquinome	Cefoperazone
EC17	0.25	0.5	0.125	0.03	0.25
Farm-1	256	16	8	4	64
Farm-WT	8	8	1	2	8
Farm-WT-M1	128	8	8	8	32
Farm-22	128	4	8	4	32

Values shaded in grey represent resistant (R) according to CLSI breakpoints. EC17, control *E. coli* strain 17

3.2.4 Identification of genetic variation of farm *E. coli* isolates by Random Amplification of Polymorphic DNA (RAPD-PCR) and WGS

To identify clonality between the various AmpC hyperproducers in farm *E. coli* isolates, RAPD PCR was carried out. This method was used prior to WGS data acquisition to test the variability of isolates. This included 6 isolates collected from Farm 1 each from separate samples over a 12-month period, 2 isolates from separate samples from Farm 2 and one from each of Farm 3 and Farm 4. Five types of banding patterns were seen with PCR products of varying sizes when compared to the markers from two molecular ladders: the 100 bp and the 1kb ladder (Figure 3.6). Seemingly identical RAPD PCR patterns were observed for *E. coli* isolates from Farm 1 (isolates 1-4), suggesting clonality. A similar – but not identical - profile was also seen in Farm 1 (isolates 5 and 6). Farm 1 (isolate 5) is the is the isolate named Farm 1 and used for all above analysis. Another similar pattern was seen in Farm 2 (isolate 1), and this was identical to Farm 2 (isolate 2), again suggesting clonality on this farm. The Farm 3 and Farm 4 isolates had unique RAPD PCR patterns and all the farm isolates showed different banding patterns when compared to human *E. coli* 17 control. Interestingly, the four seemingly clonal Farm 1 isolates were collected from different parts of the same farm on different dates, which is in line with previous studies, showing clonality amongst *E. coli* strains isolated from the same farm (178).

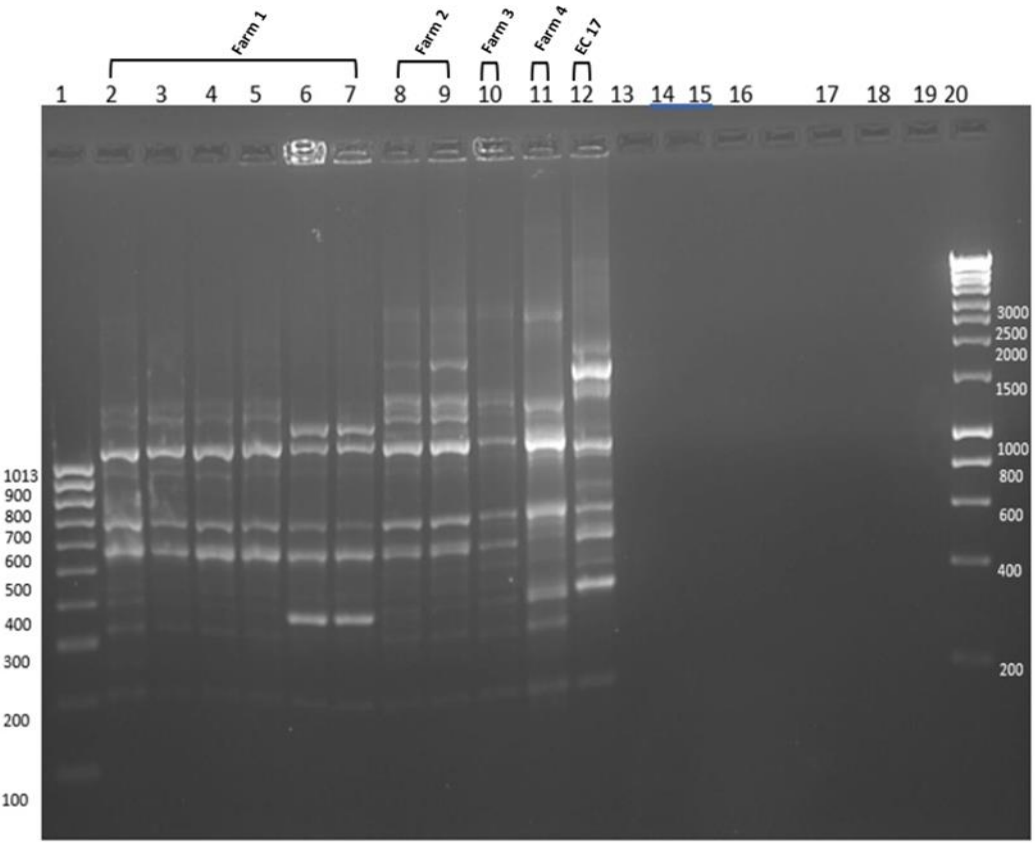


Figure 3.6: RAPD pattern profile of *E. coli* collected from dairy farms. RAPD PCR was performed using primer 1283, and the products were separated in 2% agarose gel at 100V for 1 h. Lanes 1 and 20 are the 100 bp and 1 kb molecular ladders, respectively. Lanes from 2 to 12 are ordered as following: Farm1 (isolate 1), Farm 1 (2), Farm 1 (3), Farm 1 (4), Farm 1 (5), Farm 1 (6), Farm 2 (isolate 1), Farm 2 (2), (from left to right the date of collection is later), Farm 3, Farm 4, *E. coli* 17, and a negative (blank) control for the RAPD PCR reaction. Five groups of farm isolate RAPD pattern can be identified as the following: group 1 (lanes 2-5), group 2 (6,7), group 3 (8,9), group 4 (lane10) and group 5 (lane 11).

Based on the WGS data, the 25 AmpC hyperproducer farm isolates were identified as belonging to 9 different sequence types where ST88 was dominant (10/25 isolates). This finding is similar to data collected from a cattle study in France (173). Table 3.7 shows the spread of *E. coli* STs amongst the 25 study isolates. Both Farm 2 and Farm 3 isolates belong to ST88, and they are only 15 SNPs apart in the core genome, based on phylogenetic analysis, performed as described in Materials and Methods and discussed in more detail in Chapter 4. *E. coli* isolates from Farm 1 and Farm 4 belong to different sequence types (ST641) and (ST388) respectively. Interestingly, there were 1–13 SNPs across six sequenced isolates collected from Farm 1 which showed only two different banding patterns, suggesting mobile genetic differences.

This can be compared with isolates from Farm 22 that appeared to be the only isolate other than Farm 1 carrying a known expanded-spectrum AmpC variant, the same His296Pro mutation as seen in the isolate from Farm 1 and had the same sequence type as Farm-1 (ST641). These two isolates, from farms 40 km apart, were both only 64 SNPs apart. As mentioned above, the *ompF* porin gene was intact in the isolate from Farm 22, so OmpF disruption must have occurred following separation of the isolates. Interestingly, another ST641 isolate, from Farm 7 (which is 7 km from Farm 1), had 1520 SNPs different from the isolate from Farm 1, and did not have the expanded-spectrum AmpC mutation or an *ompF* mutation. These features were also seen in Farm 14 isolates with only 35 SNPs but 45 km away from Farm 7 (Phylogenetic analysis is discussed in detail in chapter 4).

Table 3.7: STs of AmpC-hyperproducing *E. coli* isolates representing 25 dairy farms

Farm isolates	ST	Farm isolates	ST
Farm-1	641	Farm-14	641
Farm-2	88	Farm-15	88
Farm-3	88	Farm-16	278
Farm-4	388	Farm-17	661
Farm-5	88	Farm-18	88
Farm-6	75	Farm-19	88
Farm-7	641	Farm-20	278
Farm-8	23	Farm-21	345
Farm-9	162	Farm-22	641
Farm-10	88	Farm-23	88
Farm-11	2522	Farm-24	278
Farm-12	88	Farm-25	88
Farm-13	278		

3.3 Conclusions

All the 25 proposed AmpC hyperproducer *E. coli* characterized in this study were confirmed to be AmpC hyperproducers, based on the identification of promoter mutations, and proteomic analysis of a sample of isolates. Therefore, we conclude that all CTX-R *E. coli* identified in the Avison group's previous study (121) to be PCR negative for mobile cephalosporinase genes are highly likely to be AmpC hyperproducers. This means that 46.2% of CTX-R *E. coli* from dairy cattle across the 53 farms enrolled in that study were AmpC hyperproducers. As published separately, 52.9% were CTX-M producers the remainder being plasmid AmpC producers (121). Findings from this study in relation to the proportion of AmpC hyperproducers among CTX-R *E. coli* are similar to that of a national survey in the Netherlands (174). Therefore, attempts to

reduce the prevalence of CTX-R, and 3GC resistance in general on dairy farms must address the specific factors that trigger the accumulation of AmpC hyperproducers. In parallel to the work reported here, Dr Hannah Schubert performed a risk factor analysis to identify factors associated with the increased odds of finding AmpC hyperproducing *E. coli* (confirmed as described above) in a faecal sample on a dairy farm in this study. Three farm-level fixed effects and two sample-level fixed effects were identified as important (Table 3.8) and full information about the analysis that was performed can be found in the publication associated with this chapter (179). Importantly, there was no association between cephalosporin use (including 3GC use) and increased risk of finding AmpC hyperproducers. However, the total usage of amoxicillin/clavulanate was associated with a higher risk of finding AmpC-hyperproducing *E. coli* on a farm (p = 0.009). This association can be explained by direct selection since AmpC hyperproduction confers amoxicillin/clavulanate resistance in *E. coli* (64). This finding is important because amoxicillin/clavulanate is currently not identified as a highest priority critically important antimicrobial (HP-CIA) by the WHO (180). While great strides have been made within the UK farming industry to reduce antibacterials use (181), there is a particular focus on reducing HP-CIA, e.g., 3GC use. The associations identified in our risk factor analysis suggest that reducing HP-CIAs without also reducing amoxicillin/clavulanate use may not have an impact on the prevalence of CTX-R, AmpC-hyperproducing *E. coli* on farms. Indeed, a bigger

concern is that reducing 3GC use on farms may drive up amoxicillin/clavulanate use providing additional co-selective pressure for 3GC-resistant *E. coli*.

Table 3.8: Significant associations (P < 0.05) with AmpC-hyperproducing *E. coli* from dairy farms from the multilevel, multivariable logistic regression model (work done by Dr Hannah Schubert).

Risk factor	OR (95% CI)	P value
Sample taken from the environment of pre-weaned heifers	3.92(2.72-5.67)	<0.001
Total usage of amoxicillin/clavulanate on the farm	1.41(1.08-1.84)	0.009
Routine use of vaccination against respiratory disease in calves	2.58(1.22-5.47)	0.012
Samples taken from pastureland	0.33(0.15-0.73)	0.005
Calving all year round as opposed to in seasonal blocks	4.2(1.49-11.8)	0.005

Chapter 4
Characterisation of Cephalosporin resistance due to AmpC hyperproduction in urinary human *E. coli* isolates and the possibility of zoonotic transmission between farm-human *E. coli*

4.1 Introduction

Since the introduction of (3GC), an enormous number of *E. coli* resistant strains, producing either (ESBL) or AmpC β-lactamase (chromosomally or plasmid-mediated), have emerged (182,183). Resistance to 3GC have been reported in *E. coli* from both animal and human populations (184,185) and treating such infections has since become a challenge for clinicians (184). Some of the 3GC are considered to be HP-CIAs by WHO due to their importance for human health (180).

Antibacterial utility in food animal production might lead to selective pressure for resistance mutations/plasmids which could in-turn potentially transmit to humans (186). In dairy farms, antibacterials are used to treat infections such as mastitis (therapeutically), or to prevent infection, for example dry cow therapy is an antibacterial preparation inserted into the teats of cows between lactations (107). The (4GC) cefquinome which is listed as a HP-CIA was the most utilised dry cow therapy active ingredient reported in a survey of dairy farms in England and Wales in 2012 (108). However, in 2017, only 5.3% of the total amount of active ingredients used as dry cow therapy comprises HP-CIAs. Since June 2018, the use of HP-CIAs on dairy farms has further significantly declined in the UK (181).

The potential routes of transmission for ESBL/AmpC producing *E. coli* are complicated. Transmission may occur directly or indirectly through different pathways from environmental or animal sources to humans and vice versa (187). 3GCR *E.coli* have been reported in farms, food and the environment (188), suggesting a global prevalence of these strains, which can facilitate further transmission to humans through the faecal-oral route (183).

In this transmission paradigm, human infection by 3GCR *E. coli* could occur via two pathways. Direct transmission of the resistant harbouring bacterium to humans, where a bacterium from a food producer animal source is ingested, which might cause colonisation and possibly opportunistic extraintestinal infection with minimal genetic changes, indicative of its origin. Alternatively, via the horizontal transfer of resistance genes, mediating 3GCR of animal origin to a human colonizer or pathogenic *E. coli* strain. Transfer of mobile genetic elements could occur at any stage of the transmission process, and within the human gastrointestinal tract when 3GCR *E. coli* of animal origin interacts with susceptible *E. coli* that are established colonisers in the human gut (187). In which case, close sequence identity is confined to the horizontally-acquired gene, and the original animal-associated 3GCR *E. coli* host strain may be lost.

Horizontal gene transfer is widely reported as the method for ESBL gene transmission, such as the transmission of $bla_{CTX\text{-}M}$ genes, frequently through conjugative IncF plasmids, which have been reported as the dominant vehicle carrying these genes (189,190). Evidence of transmission of both 3GCR *E. coli* strains and ESBL plasmids, across animal and human populations via the food chain, have been reported using typing methods involving WGS (187,191). However, there is limited knowledge available on the possibility of transmission occurring during interaction of people with farm environments (192).

The work reported here aimed to characterize putative AmpC-hyperproducing *E. coli* from a recent survey of human urinary 3GCR *E. coli* isolates collected in Bristol and surrounding regions. In this survey, putative AmpC hyper-production (defined as 3GCR but PCR negative for a known acquired 3GCR gene) was low prevalence - only 24/626 isolates (3.8%) (122). The work reported

here also investigated potential zoonotic transmission of AmpC hyperproducers found on dairy farms (as characterised in chapter 3) to humans by using WGS-based phylogenetic analysis. Importantly, the human urinary *E. coli* and dairy farm isolates being compared here were collected in parallel from the same 50 x 50 km region (121,122,193).

This work has been published as my contribution to the below publication, in which I am first author. Dr Jacqueline Findlay initially identified the putative AmpC hyper-producing isolates that were studied by me in this chapter. Dr Kate Heesom generated the proteomics data, which I analysed. Dr Oliver Mounsey taught me to do the phylogenetic analysis. Other authors in this paper were either project supervisors or not involved in the work reported in this chapter.

Maryam Alzayn, Jacqueline Findlay, Hannah Schubert, Oliver Mounsey, Virginia C. Gould, Kate J. Heesom, Katy M. Turner, David C. Barrett, Kristen K. Reyher and Matthew B. Avison.2020. Characterization of AmpC-hyperproducing *Escherichia coli* from humans and dairy farms collected in parallel in the same geographical region. *Journal of Antimicrobial Chemotherapy* https://doi.org/10.1093/jac/dkaa207.

4.2 Results and discussion

4.2.1 Confirmation of AmpC hyperproduction in human urinary *E. coli*

We investigated 20 human urinary *E. coli* presumed to hyperproduce AmpC, collected during the same time frame, from the Severn Pathology Laboratory at Southmead Hospital, Bristol and initially identified by Dr Jacqueline Findlay. Samples were collected from people living in the same geographical range as the 25 farms for which WGS data of AmpC-hyperproducing *E. coli* reported in chapter 3, had been obtained (122). First, antibiograms were determined for all human putative AmpC-hyperproducing isolates against β-lactam and non β-lactam antibacterials (Table 4.1). Most of the UTI isolates presented typical AmpC-hyperproducing phenotype: resistance to ampicillin, cefalexin and cefoxitin and non-susceptibility to cefotaxime and/or ceftazidime but not generally cefoperazone or cefepime. All isolates were susceptible to the carpabenem ertapenem, and also to chloramphenicol and minocycline. Five out of 20 isolates (UTI-18, UTI-16, UTI-12, UTI-9 and UTI-1) showed susceptibility to all tested antibacterials except ampicillin, cefalexin and cefoxitin based on disc testing. MIC testing is a more accurate method of defining susceptibility/resistance, and this confirmed cefotaxime non-susceptibility (the phenotype originally used for selection (122) and that there were differences in MICs of this and other 3GCs and cefepime (4GC) relevant for human medicine among eight representative isolates (UTI-1, UTI-8, UTI-9, UTI-10, UTI-12, UTI-16, UTI-17 and UTI-18) which were selected for further analysis based on their pattern of resistance (Table 4.2) and the broad spectrum of *ampC* promoter mutations observed (see below).

Table 4.1: β-Lactam and non β-Lactam susceptibility of putative AmpC-hyperproducing urinary *E. coli* isolates from humans

Sample	Cefotetan	Cefotaxime	Cefriaxone	Cefoperazone	Cefepime	Chloramphenicol	Minocycline	Ertapenem	Azetroname	Ceftazidime	Cefoxitin	Ampicillin	Cefalexin
UTI-1	21	25	26	26	32	27	24	29	23	21	12	6	6
UTI-2	21	25	26	23	31	26	22	29	24	21	18	6	6
UTI-3	17	25	25	22	31	28	23	27	21	19	14	6	6
UTI-4	18	24	25	22	30	26	22	28	22	16	16	6	6
UTI-5	18	23	24	23	32	28	25	27	21	20	18	6	6
UTI-6	17	23	24	23	32	28	26	27	21	20	10	6	6
UTI-7	17	25	25	21	32	28	23	29	23	19	14	6	6
UTI-8	17	22	22	21	31	28	25	28	20	18	12	6	6
UTI-9	20	27	25	24	32	27	23	28	21	19	9	6	6
UTI-10	16	19	22	21	31	28	26	29	17	15	6	6	6
UTI-11	15	20	22	21	32	29	29	27	19	16	6	6	6
UTI-12	20	27	27	28	32	25	19	29	24	20	12	6	6
UTI-13	19	22	22	23	31	26	23	28	21	15	12	6	6
UTI-14	19	24	24	22	33	25	21	28	22	19	17	6	6
UTI-15	22	25	27	24	33	25	23	30	25	22	21	6	6
UTI-16	20	25	27	27	34	29	29	29	23	21	12	6	6
UTI-17	16	23	25	17	29	29	22	28	23	20	12	6	6
UTI-18	25	27	31	28	34	30	27	32	27	20	14	6	6
UTI-19	21	25	24	21	30	26	21	30	20	16	14	6	6
UTI-20	20	24	24	23	31	27	21	28	22	16	12	6	6
EC17	30	32	32	32	34	30	24	31	33	28	28	20	25

Values shaded in blue represent intermediate (I), grey resistant (R) based on CLSI breakpoints, otherwise susceptible.

Table 4.2: MICs of 3GCs/4GCs against putative AmpC-hyperproducing urinary *E. coli* isolates from humans

Samples	MIC (mg/L)			
	Cefepime	Ceftazidime	Cefotaxime	Cefoperazone
EC 17	0.125	0.25	0.25	0.125
UTI-1	0.125	4	2	4
UTI-8	0.25	16	4	64
UTI-9	0.125	4	2	16
UTI-10	0.125	16	4	32
UTI-12	0.125	8	2	4
UTI-16	0.125	4	2	2
UTI-17	0.25	8	2	>128
UTI-18	0.25	8	2	4

Values shaded in grey, and blue represent resistant and intermediate, respectively according to CLSI breakpoints. EC17, control *E. coli* strain 17.

LC-MS/MS proteomics confirmed statistically significant AmpC hyperproduction in these eight isolates relative to the control *E. coli* EC17 (though there were differences in level of production). The highest AmpC abundance was detected in UTI-10 followed by UTI-8 (5.79 ± 0.01 and 2.13 ± 0.21) respectively, and the lowest was in UTI-12 (1.10 ± 0.01). In each case abundance of AmpC is reported relative to ribosomal proteins for each sample. AmpC in the control isolate EC17 was not detectable, and the limit of detection is approximately 100-fold less than the abundance of AmpC detected in UTI-12 (Table 4.3). In addition, β-lactamase assays revealed high enzyme activity in five tested isolates where UTI-12 showed the lowest activity, confirming the proteomics results (Figure 4.1). UTI-10 was excluded as the enzyme activity was too high to accurately measure and UTI-8 had the highest measurable activity, again confirming the relative abundance of AmpC seen in proteomics data. UTI-17 and UTI-18 were also excluded from the β-lactamase assay because they were shown by WGS to carry a mobile β-lactamase gene $bla_{\text{TEM-1}}$, which whilst not

contributing to cephalosporin resistance, does hydrolyse nitrocefin, the substrate used in the AmpC enzyme assays.

Table 4.3: A summary of *ampC* promoter and attenuator mutations in representative AmpC-hyperproducing urinary *E. coli* from humans and the abundance of AmpC and also the OmpC porin

Isolate	Promoter/attenuator mutation	AmpC		OmpC	
		Mean±SEM	t-test, *p* (C vs I)	Mean±SEM	t-test, *p* (C vs I)
EC-17	-	ND	-	2.86± 0.10	-
UTI-12	G insertion -13 (17bp spacer)	1.10±0.01	<0.01	5.86±0.26	<0.01
UTI-18	T insertion at -13 (17bp spacer)	1.14±0.04	<0.01	ND	<0.01
UTI-1	-32 T-A (consensus -35 box), +35 C-A, +58 C-T	1.19±0.02	<0.01	1.11±0.16	<0.01
UTI-9	T insertion at -13 (17bp spacer), +32 G-A,	1.35±0.20	<0.01	0.84±0.25	<0.01
UTI-16	-32 T-A, (consensus -35 box), -11 C-T (consensus -10 box),	1.66±0.09	<0.01	1.28±0.12	<0.01
UTI-17	-42 C - T (alternative displaced consensus -35 box), T-A -18, 17bp spacer,	1.76 ±0.55	0.04	ND	<0.01
UTI-8	-42 C - T (alternative displaced consensus -35 box), T-A -18, 17bp spacer, (G)deletion at +37	2.13±0.21	<0.01	0.45±0.04	<0.01
UTI-10	-11 C- T (Consensus -10 box), T insertion -13 (17bp spacer), +37 G-A, +35 G-A	5.79±0.01	<0.01	0.21±0.0	<0.01

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, (n = 3). Proteins whose abundance is significantly (t-test, $p < 0.05$) up or downregulated at least 2-fold relative to the E. coli strain 17 (EC17) control are shaded in green and red, respectively. ND not detected. C, control. I, Isolate. UTI-17 and UTI-8 both also had four additional substitutions: (C-T -88 and -1), (T-C -82), (C-T +58). Note: pairwise t-test analysis was additionally performed for UTI-8 vs UTI-17 ($p=0.25$, not significantly different), UTI-1 vs UTI-16 ($p=0.01$, significantly different), UTI-9 vs UTI-18 or vs UTI-12 ($p>0.13$, neither significantly different) and UTI-16 vs UTI-18 or UT-12 ($p<0.003$, both significantly different).

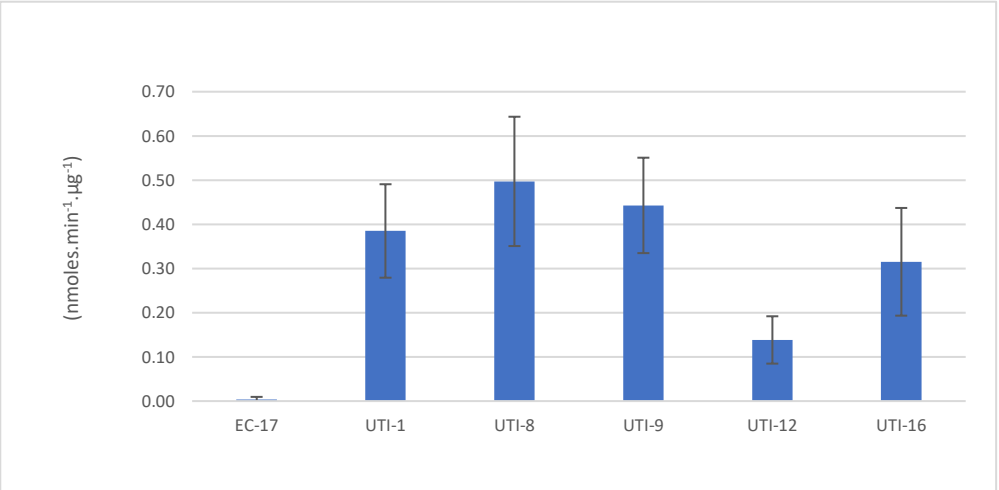


Figure 4.1: AmpC enzyme activity of five representative UTI isolates and the control EC-17. Samples were incubated with nitrocefin at 25 °C for 18 min (30 cycles). Error bars report SEM of three biological replicates.

4.2.2 Sequence analysis of the *ampC* promoter region

According to sequence of the *ampC* promoter/attenuator region, there were nine different *ampC* promoter/attenuator types seen across the 20 AmpC-hyperproducing human isolates (Figure 4.2). Table 4.3 reports promoter types for the 8 representative isolates for which proteomics data are available alongside a report of the abundance of AmpC present in each. The most common mutations (seen in 11/20 UTI isolates) were C to T and T to A substitutions at position -42 and -18 respectively, which resulted in the creation of an alternative displaced consensus -35 box (**T**TGACA) separated by the consensus 17bp from its -10 box T**A**TCGT. These mutatons, therefore,

generate a second active promoter upstream of *ampC*. Additionally, there were substituitions at positions -88, -82, -1 and +58 in these 11 UTI isolates. Alternatively displaced -35 and -10 boxes have previously been associated with these same additional substitutions at positions −88, −82, −1 and +58 (194,195)**.** Furthermore, all 25 AmpC-hyperproducing farm isolates studied in chapter 3 have these same -42 and -18 mutations creating a second promoter, as well as identical mutations at -88, -82, -1 and +58 (Figure 4.2). Given that the 36 isolates studied here represent multiple *E. coli* STs, it seems unlikely that all these mutations have accumulated by chance on numerous occasions in multiple lineages, and perhaps therefore, this is suggestive of the actions of recombination.

UTI-8 was one of the isolates with this collection of mutations including those at -42 and -18 generating a second promoter, but additionally, it carried a one base pair deletion in the attenuator region, at +37. Attenuator mutations are believed to destabilize the mRNA hairpin structure, thus enabling rapid read-through by the ribosome (196,197). Abundance of AmpC in UTI-8 relative to ribosomal proteins was 2.13 ± 0.21, Mean ± SEM, n=3. This can be compared with AmpC production in UTI-17 (1.76 ± 0.55) which had the same -42 and -18 promoter mutations but lacked the attenuator mutation at +37. However, a t-test showed that the apparent difference between AmpC abundance in UTI-8 and UTI-17 was not significant (p=0.25), suggesting that the additional attenuator mutation in UTI-8 does not further increase AmpC production over that provided by the two promoter mutations, Table 4.3.

Another *ampC* promoter mutation observed in two hyperproducer isolates (UTI-1 and UTI-16) was in the -32 position, resulting in a consensus -35 box TTG**A**CA that strengthens the original

ampC promoter. The latter isolate also harbored a C to T change at -11 additionally resulting in a consensus -10 box TA**T**AAT in the original promoter. Notably the abundance of AmpC in UTI-16 was 1.66 ± 0.09 compared with 1.19 ± 0.02 in UTI-1 (t-test, p=0.01) confirming that the -10 promoter mutation enhances AmpC production above that seen with the -35 promoter mutation alone.

Seven isolates had a single base insertion at position -13, shifting the length of the interspace between the -10 and -35 boxes of the original *ampC* promoter to the optimal 17 bp. One representative isolate, UTI-9, also had a +32 attenuator mutation. Again, the abundance of AmpC in UTI-9 (1.35 ± 0.20) was not significantly (p>0.13) more than in UTI-18 1.14 ± 0.04 or UTI-12 1.10 ± 0.01, which have the -13 insertion but no attenuator mutation, suggesting again that the attenuator mutation adds little to the amount of AmpC produced via a promoter mutation.

Three isolates carrying the -13 promoter insertion all had the same three additional mutations: two in the attenuator region (+17 to +37) and one in the promoter (C to T at -11, creating a consensus-10 box of the original *ampC* promoter). Notably, UTI-10 (assayed as representative of these three isolates) had by far the highest AmpC abundance of all assayed strains (5.79 ± 0.01) and its β-lactamase activity was too high to accurately measure. Because we did not see an isolate with the same promoter mutations but without these attenuater mutations, we cannot determine how much these attenuater mutations affect AmpC production.

The final point to make is that generating a -35 plus -10 consensus (UTI-16, AmpC abundance 1.66 ± 0.09) has a significantly greater ($p<0.003$) impact than simply creating a consensus spacing between the wild-type -35 and -10 boxes (i.e. UTI-18, AmpC abundance 1.14 ± 0.04 and UTI-12, AmpC abundance 1.10 ± 0.01).

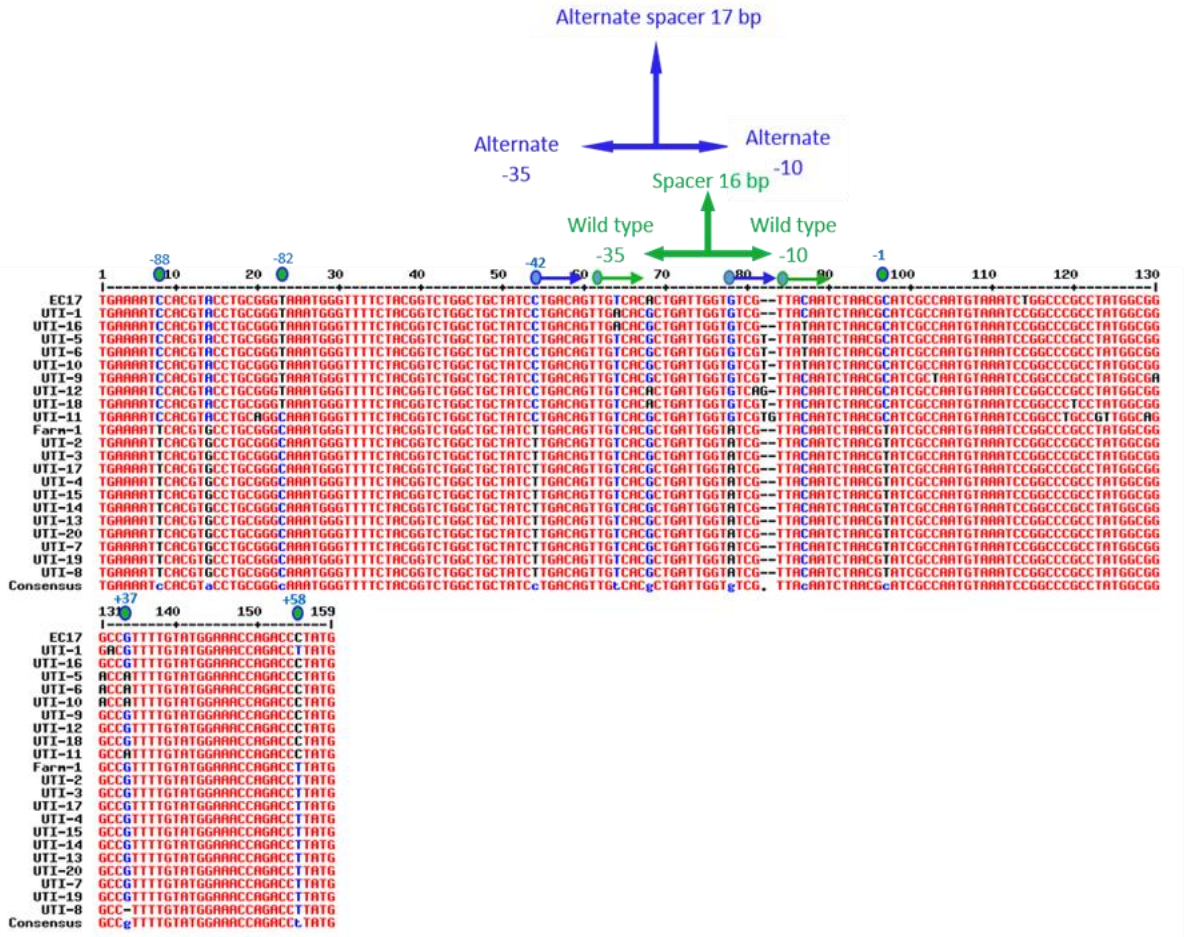


Figure 4.2: Promoter/attenuator sequences for *ampC* from AmpC-hyperproducing *E. coli* isolates in comparison with a WT *E. coli*. Modified residues, relative to the control *E. coli* strain (EC17), seen in AmpC-hyperproducing *E. coli* from farms (Farm-1 to Farm-25) and human urinary *E. coli* (UTI-1 to UTI-20) are noted, with their positions relative to the transcriptional start site. Novel promoter(s) created are annotated. All 25 farm isolates had an identical sequence in this region, represented by the isolate from Farm 1.

Previous studies have shown that mutations that create a consensus -35 box (TTGACA), whether in the original *ampC* promoter, or to create an alternative, second promoter, are the most important factors in increasing AmpC production (194,175,198,199). Studies have also shown that a base pair insertion mutation that increases the distance between the -10 and -35 boxes of the original *ampC* promoter is the second most significant factor in increasing AmpC production. In contrast, mutations strengthening the -10 box of the original promoter appear to be less important, and least important are alterations in the attenuator region (200). Our observations based (in contrast to these earlier studies) on proteomics analysis, generally agree. We did not see -10 box strengthening or attenuator mutations on their own. However, the UTI isolates analysed here were all selected as being cefotaxime (3GC) resistant, so it is possible that such mutations could increase AmpC production and provide elevated MICs, without conferring resistance. The "weakest" mutations seen in isolates producing sufficient AmpC to confer cefotaxime resistance were those which either optimized the spacer in the original promoter or optimized the -35 box in the original promoter. Next in the hierarchy was a double mutation that either optimized the -35/-10 of the original promoter (without optimizing its spacing) or the very common double mutation creating a second promoter. Finally, whilst -10 optimising or attenuator mutations were not seen alone, and the single attenuator mutations seen alongside promoter mutations did not have significant effects on AmpC production, the isolate producing by far the most AmpC had a double mutation in the original promoter, one optimizing the spacing and one optimizing the -10 box, plus two mutations in the attenuator region. Reasons why such very high levels of AmpC production might have been selected will be considered below.

4.2.3 Relationship between AmpC production level and cephalosporin MICs

The variety of *ampC* promoter mutations and therefore AmpC production seems not to have a clear-cut and predictable impact on the MICs of tested cephalosporins against the UTI *E. coli* isolate. For example, MICs were within one doubling against UTI-10 and UTI-8 despite the big difference in their AmpC abundance (5.79 ± 0.01) and (2.13 ± 0.21), respectively. Other evidence can be seen in UTI-16 which had moderately high AmpC abundance (1.66±0.09) and yet showed little difference in MICs against ceftazidime, cefotaxime and cefoperazone compared with UTI-1 (1.19±0.02) and UTI-12 (1.10±0.01), being the two lowest AmpC producers among these isolates selected for cefotaxime resistance (Tables 4.2 and 4.3). Previous studies have found weak correlations between AmpC production and β-lactam MIC (197,175). It seems therefore, that AmpC production has little effect on cephalosporin MIC when going over a certain level or it may be that other factors are at play.

To explain some of these MIC differences, we first looked at the amino acid sequence of AmpC for all 20 isolates that revealed different amino acid changes relative to the control isolate EC-17 (Figure 4.3). Most similar amino acid sequence to EC-17 were sequences of UTI-12, UTI-18 and UTI-1 which showed the lowest cephalosporin MICs, but it is difficult to associate AmpC mutations and enzyme activity without considerable extra work using purified proteins. What we can be certain of is that none of the human isolates had the His296Pro mutation indicative of an expanded-spectrum AmpC variant, which was identified in chapter 3. This was confirmed phenotypically using cefepime disc susceptibility testing; all isolates were susceptible as expected for a wild-type AmpC.

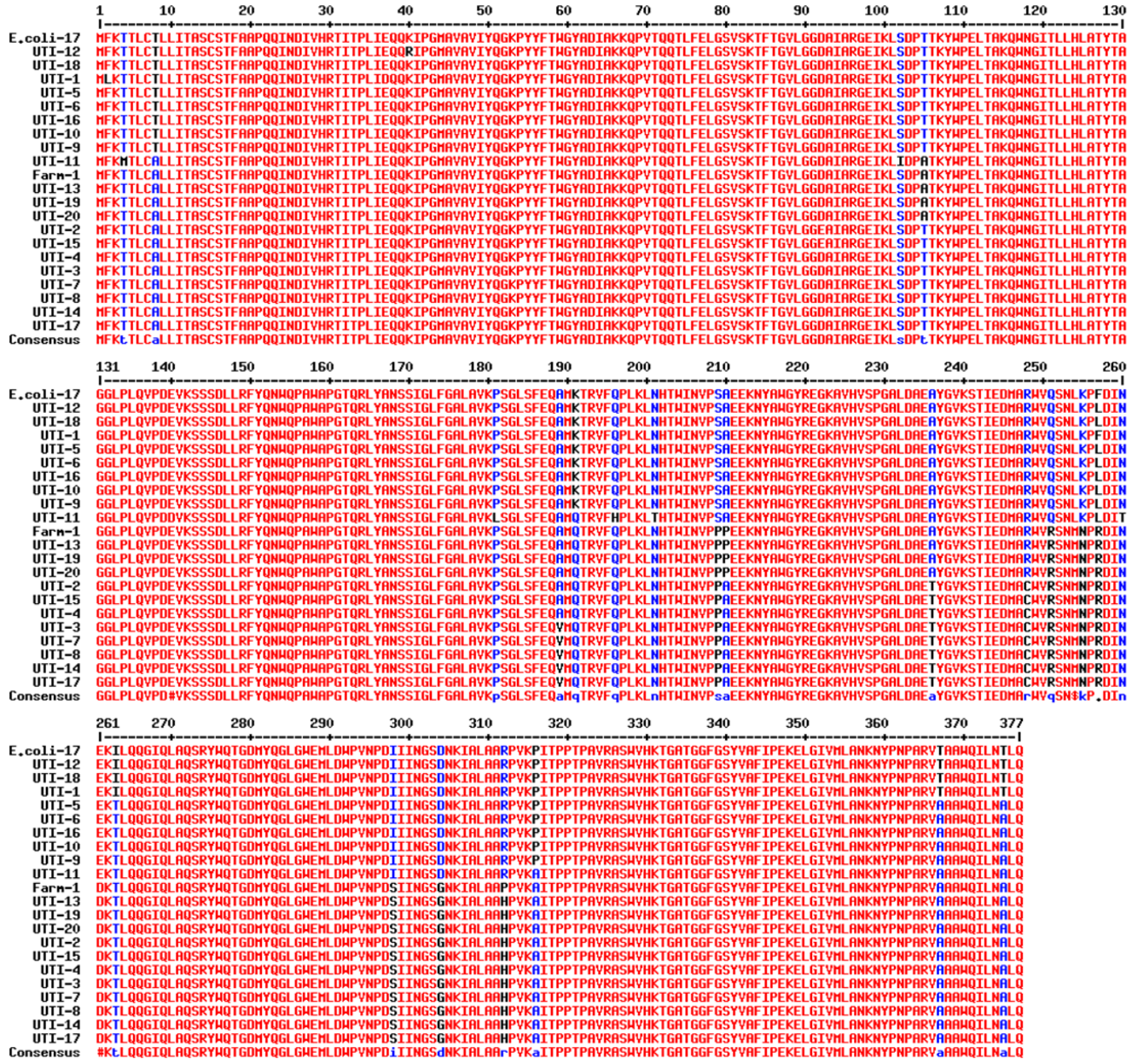


Figure 4.3: Amino acid sequences for AmpC from urinary *E. coli* AmpC-hyperproducing isolates in comparison with a WT *E. coli* 17. None of human isolates had a His296Pro mutation suggestive of an extended-spectrum AmpC variant that had been seen in two farm isolates, represented by the isolate from Farm 1.

Alteration in β-lactam resistance profiles due to variant AmpC proteins has been reported previously (201,69). However, isolate UTI-17 shared an identical amino acid sequence to UTI-14, UTI-8, UTI-7 and UTI-3 but cefoperazone had the highest MIC against UTI-17 than against all other isolates, which makes this isolate clearly different. This suggests another mechanism for causing this phenotype. So, we checked the abundance in the proteomics data, of a number of key proteins associated with antibacterial resistance in *E. coli* such as the porins and efflux pumps but there were no significant changes detected, with the exception of OmpC. Interestingly, OmpC abundance was significantly lower than control EC-17 ($p<0.05$) in 7/8 UTI isolates and was undetectable in UTI-17 and UTI-18 (Table 4.3). Furthermore, the three cefoperazone resistant isolates had the lowest OmpC porin abundance (UTI-8 and UTI-10, although these also had the highest AmpC production levels) and UTI-17 where OmpC abundance was undetectable (Table 4-3). However, OmpC downregulation is not the only reason for high cefaperazone MIC, since UTI-18 also had undetectable OmpC but there was a greater than six doubling lower cefaperazone MIC (4 mg/L) vs (>128 mg/L) against UTI-18 versus UTI-17. We considered that the difference between UTI-17 and UTI-18 could be due to different levels of TEM-1 production. Indeed, UTI-17 (0.49 ±0.15 abundance of TEM-1) was resistant to cefeperazon while UTI-18 (0.07±0.01 abundance of TEM-1) was susceptible. It has been shown before that hyperproduction of TEM-1 β-lactamases is associated with cefoperazone resistance in *E. coli* (202), which suggests an explanation for cefoperazone resistance in UTI-17. Indeed, there was no difference for ceftazidime – having the same MIC against both UTI-17 and UTI-18, and TEM-1 is not associated with increased ceftazidime MIC. Overall, therefore, we conclude that cefoperazone MIC is associated with very high AmpC levels (UTI-8, UTI-10), or high AmpC levels

plus TEM-1 hyper-production (UTI-17). Reduced OmpC levels might be relevant, but this will require significant additional experimentation to confirm.

4.2.4 No evidence for recent human/farm transmission of AmpC hyperproducing *E. coli* isolates collected in parallel in a 50x50 km region

Our final aim was to identify whether there was any evidence of sharing AmpC-hyperproducing *E. coli* between humans and cattle, as dominance of ST88 has previously been reported in humans in Northern Europe (173) and we also found an over-representation of ST88 on our farms. However, ST88 was only found in 1/20 human isolates (Table 4.4).

A phylogenetic tree drawn, based on core genome comparison, showed that the cattle and human isolates were intermixed only to a small extent (Figure 4.4). Importantly, all 10 ST88 cattle isolates were 15 or fewer SNPs apart, suggesting very recent farm-to-farm transmission; the human ST88 isolate (UTI-19) was, at its closest distance, 1279 SNPs different from the cattle isolates. Fewer than 100 SNPs are usually required before transmission is considered likely (203). The two other examples where isolates from the same ST, which were found in farm and human samples, painted the same picture (Figure 4.4) for ST75 - the two human isolates (UTI-2 and UTI-15) were 60 SNPs apart, but the cattle isolate (Farm-6) was 1972 SNPs different at best. For ST23, the human and cattle isolates (UTI-13 and Farm-8, respectively) were 2754 SNPs different. Otherwise, there was no ST sharing and all cattle isolates fell into phylogroups B1 and C, with 8/20 human isolates falling into the highly pathogenic phylogroup B2, including a cluster of ST73 isolates (Table 4.4), of which three were only two SNPs apart (Figure 4.4).

Table 4.4: STs of AmpC-hyperproducing *E. coli* isolates representing 25 dairy farms and 20 human urine samples

Farm isolates	ST	Phylogroup	Human isolates	ST	Phylogroup
Farm-1	641	B1	UTI-1	141	B2
Farm-2	88	C	UTI-2	75	B1
Farm-3	88	C	UTI-3	200	B1
Farm-4	388	B1	UTI-4	155	B1
Farm-5	88	C	UTI-5	73	B2
Farm-6	75	B1	UTI-6	73	B2
Farm-7	641	B1	UTI-7	200	B1
Farm-8	23	C	UTI-8	54	B1
Farm-9	162	B1	UTI-9	73	B2
Farm-10	88	C	UTI-10	73	B2
Farm-11	2522	B1	UTI-11	405	D
Farm-12	88	C	UTI-12	131	B2
Farm-13	278	B1	UTI-13	1499	C
Farm-14	641	B1	UTI-14	200	B1
Farm-15	88	C	UTI-15	75	B1
Farm-16	278	B1	UTI-16	73	B2
Farm-17	661	B1	UTI-17	200	B1
Farm-18	88	C	UTI-18	428	B2
Farm-19	88	C	UTI-19	88	C
Farm-20	278	B1	UTI-20	448	B1
Farm-21	345	B1			
Farm-22	641	B1			
Farm-23	88	C			
Farm-24	278	B1			
Farm-25	88	C			

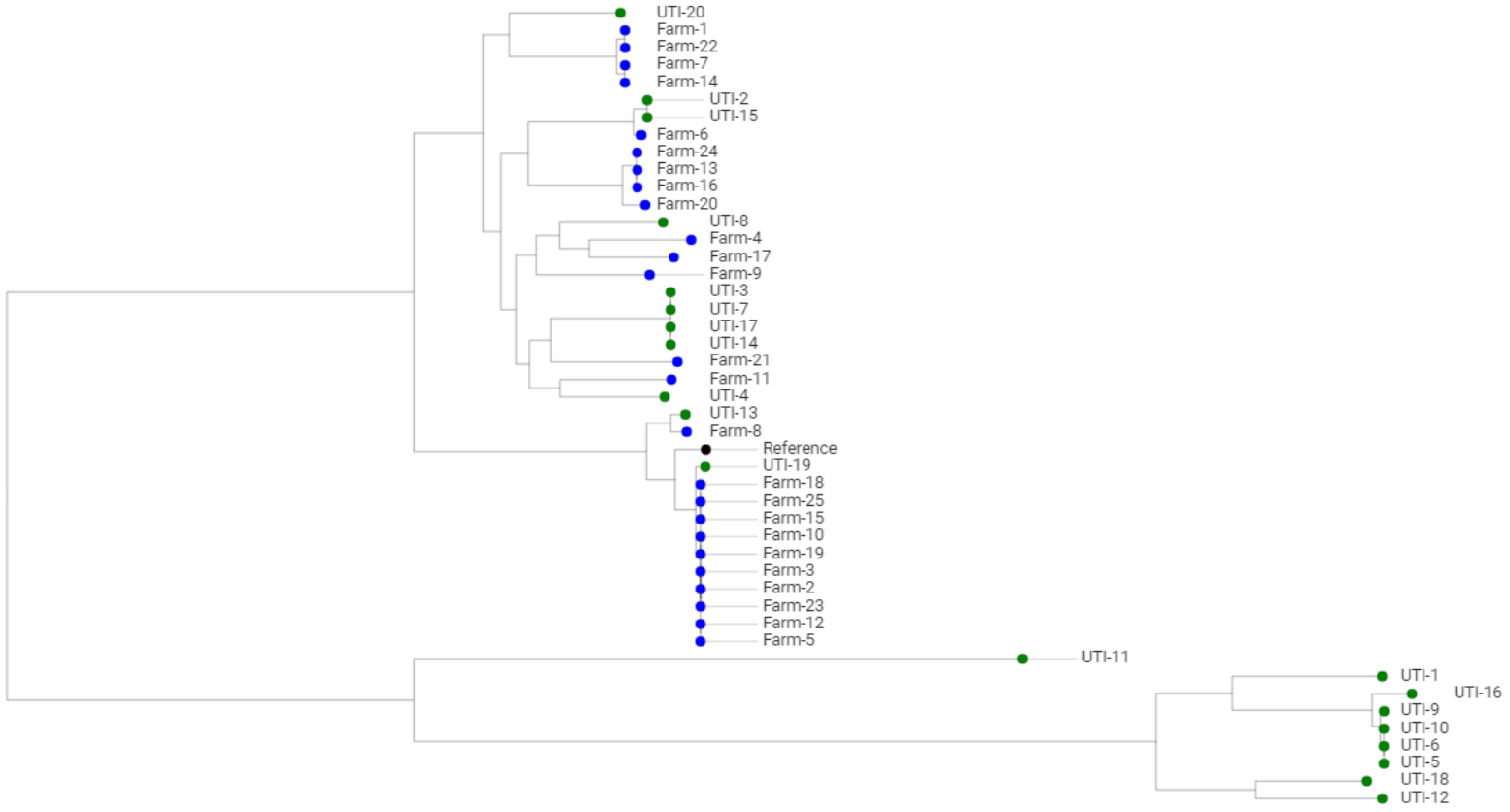


Figure 4.4: Phylogenetic tree of farm and human urinary AmpC-hyperproducing *E. coli*. The phylogenetic tree was illustrated using the Microreact program using a maximum-likelihood tree generated from core genome alignments as described in Materials and methods. Isolates are coloured green (human urinary) and blue (farm). The ST88 finished reference genome (Accession: NZ_CP031546.1) used to generate the alignments is noted.

4.3 Conclusions

Unlike *E. coli* isolates from dairy farms, AmpC hyperproduction was less common among 3GCR human urinary *E. coli* isolates (46.2% versus 3.8%). AmpC hyperproduction was caused by different promoter/attenuator mutations, although, the creation of displaced alternative -35 and -10 boxes is the predominant mechanism used to enhance transcription of the *ampC* gene. It was found in 9 out of 20 human *E. coli* isolates and in all strains collected from dairy farms. This finding is in agreement with previous studies (195).

AmpC hyperproduction level was important for, but was not the sole reason for 3GC MIC. We saw this particularly for cefoperazone. When considering the farm isolates analysed in chapter 3, cefoperazone resistance in AmpC hyperproducers was associated with expanded spectrum AmpC mutations in the isolate Farm-1, and with AcrAB-TolC hyperproduction in Farm-4, TEM-1 hyperproduction in the isolate UTI-17 or extremely high AmpC hyperproduction in the isolates UTI-10, UTI-8, in these last three cases possibly with the involvement of OmpC downregulation, but this has not been confirmed.

Finally, our comparison between AmpC-hyperproducing farm and human urinary *E. coli* in the same region provided no evidence of local sharing of AmpC hyperproducers between farms and the local human population. Accordingly, whilst reducing the on-farm prevalence of AmpC-hyperproducing *E. coli* should be an important aim, the primary reason for achieving this would be to reduce the likelihood of difficult-to-treat infections in cattle rather than because of any direct zoonotic threat.

Chapter 5

OmpF downregulation mediated by Sigma E or OmpR activation confers cefalexin resistance in *E. coli* in the absence of acquired β-Lactamases

5.1 Introduction

Cefalexin is a 1st generation cephalosporin widely used in humans and farmed animals. In 2016, in Bristol, United Kingdom, and surrounding regions, (a population of 1.5 million people) 27.6 cefalexin courses were dispensed per 1000 patient population (2.8% of all dispensed items). Whilst dispensing rates had dropped by 19.5% since 2013, the proportion of *E. coli* from community-origin urine samples resistant to cefalexin in this region rose from 7.06% to 8.82% (204).

E. coli resistance to cefalexin is caused by hyper-production of the chromosomally-encoded class 1 cephalosporinase AmpC, or acquisition of (pAmpC), or (ESBLs). These are also mechanisms of (3GC-R). It was recently reported that among community-origin urinary *E. coli* from Bristol and surrounding regions collected in 2017/18, 69% of cefalexin resistant isolates were 3GC-R, suggesting that cefalexin resistance in the absence of ESBL/AmpC production was common (122). A similar observation was made when analysing faecal samples from dairy cattle in the same region, where only 30% of samples containing cefalexin resistant *E. coli* yielded 3GC-R isolates (193). Hyper-production of commonly acquired penicillinases such as TEM-1 and OXA-1 does not confer cefalexin resistance in *E. coli* (205). Furthermore, the involvement of efflux pump over-production, e.g., AcrAB-TolC in *E. coli* has not been reported, but OmpF porin loss is known to reduce cefalexin susceptibility (206). Indeed, an early publication by Kobayash and co-workers showed that cefalexin more efficiently used OmpF than OmpC porin to enter *E. coli* (207).

Characterisation of cefalexin resistance mechanisms in *E. coli* lacking acquired β-lactamases or AmpC hyper-production, by investigating resistant mutants selected *in vitro* was the major aim of the work reported in this chapter. Another aim was to characterise mechanisms of cefalexin

resistance observed in 3GC-susceptible (3GC-S) human urinary and cattle isolates described in earlier surveillance studies conducted by colleagues (122,193). The final aim was to determine if the cefalexin resistance mechanisms identified by addressing the previous two aims enhance β-lactam resistance mediated by AmpC hyper-production, including the expanded-spectrum AmpC variant identified in chapter 3.

This work has been published as my contribution to the below paper, in which I am first author. Dr Punyawee Dulayangkul assisted with the insertional inactivation mutagenesis. Naphat Satapoomin prepared extracts of isolate UTI-80710 for proteomic analysis. Dr Kate Heesom generated proteomic data, which was analysed by me.

Maryam Alzayn, Punyawee Dulayangkul, Naphat Satapoomin, Kate J. Heesom, and Matthew B. Avison. 2021. OmpF Downregulation Mediated by Sigma E or OmpR Activation Confers Cefalexin Resistance in *Escherichia coli* in the Absence of Acquired β-Lactamases. *Antimicrobial Agents and Chemotherapy*. doi: 10.1128/AAC.01004-21.

5.2 Results and Discussion

5.2.1 Cefalexin resistance in *E. coli* is associated with OmpF/OmpC porin downregulation due to *ompR* mutation.

One spontaneous cefalexin resistant mutant was selected from each of three *E. coli* parent strains: EC17, ATCC25922 and Farm**. Cefalexin MICs against these isolates and their mutant derivatives are summarised in Table 5.1. In each case, to identify the possible cause of cefalexin resistance, LC-MS/MS whole-cell proteomics was performed comparing each mutant with its parent. No mutant over-produced the chromosomally encoded AmpC β-lactamase (Table 5.2), and no promoter/attenuator sequence mutations upstream of *ampC* were identified in any of the mutants, based on WGS (Figure 5.1), so AmpC hyper-production is not the mechanism of cefalexin resistance. The only significant ($p<0.05$; >2 fold) protein abundance change common to all three wildtypes/mutant pairs was downregulation of OmpF porin (Table 5.2). There was no evidence of AcrAB-TolC efflux pump over-production in the proteomics data for any mutant (Table 5.2). Despite OmpF porin downregulation, a comparison of *ompF*-containing WGS contigs from wild-type/mutant pairs revealed no mutations in *ompF* or within 10 kb up- or downstream. Therefore, it is likely that there is a *trans*-regulatory mutation affecting OmpF abundance in each mutant.

Table 5.1: MIC of Cefalexin against *E. coli* isolates and mutant derivatives.

Antibacterial	MIC (µg/mL)					
	Farm**	Farm**(M)	ATCC25922	ATCC25922(M)	EC17	EC17(M)
Cefalexin	16	32	16	32	8	32

Shaded values represent resistance based on CLSI breakpoints, otherwise susceptible.

Table 5.2: LC-MS/MS proteomic comparisons of selected outer membrane proteins and efflux pumps abundance in *E. coli* isolates versus cefalexin resistant mutant derivatives.

Isolates/Mutants	AmpC	OmpF		AcrA		AcrB		TolC		OmpC		OmpA	
	Mean±SEM	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)
Farm**	ND	0.23±0.05		0.13±0.01		0.05±0.003		0.16±0.019		5.93±1.00		3.24±0.32	
Farm**(M)	ND	0.00±0.00	0.005	0.08±0.02	>0.25	0.04±0.006	>0.25	0.13±0.037	>0.25	0.17±0.04	0.002	3.69±0.74	>0.25
ATCC25922	ND	1.69±0.10		0.13±0.01		0.07±0.01		0.14±0.02		1.66±0.26		5.55±0.41	
ATCC25922(M)	ND	0.29±0.14	0.0006	0.13±0.03	>0.25	0.05±0.02	>0.25	0.11±0.02	>0.25	0.24±0.05	0.003	1.00±0.17	0.0003
EC17	ND	1.15±0.15		0.11±0.003		0.07±0.01		0.20±0.03		2.49±0.50		3.79±0.29	
EC17 (M)	ND	0.39±0.16	0.01	0.15±0.03	>0.25	0.08±0.02	>0.25	0.10±0.03	>0.25	2.25±0.66	>0.25	4.47±0.78	>0.25

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, (n = 3). Proteins whose abundance is significantly ($P < 0.05$) downregulated at least 2-fold relative to the parent *E. coli* strains (see Materials and methods) are shaded in red. ND not detected.

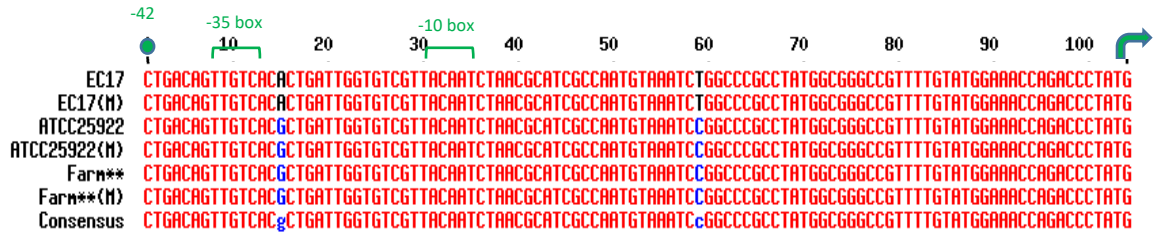


Figure 5.1: *ampC* promoter sequences of *E. coli* isolates and cefalexin resistant mutant derivatives.

Since the two-component system OmpR/EnvZ is known to control porin gene transcription in *E. coli* (208), searching among WGS data for mutations in the genes encoding this regulator was conducted, and a mutation was found in *ompR* in the cefalexin resistant derivative of isolate Farm**, predicted to cause a Gly63Ser change in OmpR. A Gly63Val substitution in OmpR has previously been shown to cause OmpF and OmpC porin downregulation in *E. coli* (209), and proteomics confirmed that OmpC was also downregulated in the Farm**-derived cefalexin resistant mutant relative to Farm**, but the third major porin OmpA was not (Table 5.2). Accordingly, we conclude that OmpR mutation explains cefalexin resistance due to OmpF (and possibly OmpC) downregulation in the mutant derivative of isolate Farm**. However, *ompR* and *envZ* were found to be wild type in the other two cefalexin resistant mutants, suggesting alternative regulatory mutations.

5.2.2 DegP over-production due to RseA anti-Sigma E mutation is associated with OmpF porin downregulation and cefalexin resistance in *E. coli*.

Eight proteins, including OmpF, were significantly differentially regulated in the same direction in the cefalexin resistant mutants, derived from isolates EC17 and ATCC25922, each relative to their parent strain. In particular, three proteins (BamD, DegP and YgiM) were upregulated and five (NmpC, DctA, ArcA, OmpF and YhiL) were downregulated (Tables 5.2 and 5.3). It was interesting to note that one upregulated protein in both mutants was DegP (Table 5.3), which is a protease known to degrade porin proteins (210,211). Interestingly, in the Farm**-derived *ompR* mutant with downregulated OmpF and OmpC, described above, DegP production was 2-fold lower than in the wild-type parent, suggesting a feedback response to porin downregulation (Table 5.3). DegP production was increased 7-fold in the ATCC25922- derived mutant and OmpF was downregulated 5.9-fold, as was OmpC (6.7-fold) and OmpA (5.6-fold) (Tables 5.2and 5.3); which is a typical Sigma E response (212). In the EC17- derived mutant, DegP was upregulated a more modest 4.4-fold, and here, OmpF was downregulated 2.9-fold, but OmpC and OmpA were not significantly ($p<0.05$) downregulated, suggesting a weaker Sigma E response (Tables 5.2 and 5.3). The observation led to the suggestion that OmpC downregulation, seen in the Farm**-derived and ATCC25922- derived cefalexin resistant mutants alongside OmpF downregulation (Table 5.2) is not necessary for cefalexin resistance. To confirm this conclusion, *ompF* was disrupted in ATCC25922 and found this to be sufficient for cefalexin resistance (Table 5.4). Consequently, additional downregulation of OmpC is not necessary.

Analysis of WGS data identified that the ATCC25922-derived mutant expressing a phenotype typical of a strong Sigma E response had a mutation predicted to cause a Trp33Arg change in

RseA, which is a known Sigma E anti-sigma factor (213,214). Loss of RseA is expected to release Sigma E so that it can bind, among others, to the *degP* promoter, increasing transcription, leading to porin degradation and cefalexin resistance (212). *rseA* was disrupted in ATCC25922 and confirmed that this mutation does cause cefalexin resistance (Table 5.4).

Table 5.3: LC-MS/MS proteomic comparisons of proteins and DegP abundance in *E. coli* isolates versus cefalexin resistant mutant derivitives.

Isolates/Mutants	BamD		DegP		YgiM		NmpC		DctA		ArcA		Yhil	
	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)	Mean±SEM	P(WT/M)
Farm**	0.06±0.02		0.06±0.01		0.00±0.00		0.00±0.00		0.04±0.0004		0.16±0.08		0.00±0.00	
Farm (M)**	0.04±0.01		0.03±0.01	0.03	0.00±0.00		0.00±0.00		0.03±0.003		0.30±0.03	>0.25	0.00±0.00	
ATCC25922	0.05±0.01		0.08±0.01		0.00±0.00		2.60±0.19		0.13±0.01		0.64±0.1		0.05±0.01	
ATCC25922(M)	0.11±0.01	0.01	0.54±0.10	0.005	0.07±0.01	0.0005	0.20±0.03	0.0005	0.00±0.00	0.0005	0.07±0.01	<0.01	0.01±0.01	0.04
EC17	0.06±0.006		0.17±0.03		0.00±0.00		2.97±0.18		0.09±0.020		0.68±0.13		0.04±0.004	
EC17 (M)	0.13±0.02	0.01	0.74±0.10	0.003	0.09±0.01	0.0005	0.81±0.13	0.0005	0.00±0.00	0.01	0.22±0.11	0.03	0.01±0.01	0.03

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, (n = 3). Proteins whose abundance is significantly ($P < 0.05$) up or downregulated at least 2-fold relative to the parent *E. coli* strains (see Materials and methods) are shaded in green and red, respectively.

Table 5.4: MIC of Cefalexin against *E. coli* isolates and derivatives where different genes had been insertionally inactivated.

Antibacterial	MIC (µg/mL)			
	ATCC25922	ATCC25922 *ompF*	ATCC25922 *rseA*	ATCC25922 *gmhB*
Cefalexin	16	32	64	64

Shaded values represent resistance based on CLSI breakpoints, otherwise susceptible.

5.2.3 Perturbation of Lipopolysaccharide heptosylation due to *gmhB* mutation causes cefalexin resistance in *E. coli*.

The EC17-derived cefalexin resistant mutant which appears to have a weaker Sigma E response was shown through WGS analysis to have a mutation predicted to cause a frameshift in *gmhB* affecting the encoded protein beyond amino acid 117. This gene encodes the enzyme D-alpha, beta-D-heptose-1,7-bisphosphate phosphatase, which is part of a pathway responsible for producing heptose for lipopolysaccharide biosynthesis (215). Loss of enzymes involved in this system are associated with increased outer membrane permeability, but interestingly, deletion of *gmhB* does not disrupt full length LPS production or compromise the outer membrane permeability barrier (215,216). The obvious conclusion is that this perturbation in envelope structure activates the Sigma E regulon, resulting in OmpF degradation by DegP. We disrupted *gmhB* in ATCC25922 and found that this mutation causes cefalexin resistance (Table 5.4). In the ATCC25922 background, the *rseA* and *gmhB* mutants were similar, in MIC terms, to the *ompF* mutant (Table 5.4). This further supports the conclusion that despite other porin production changes caused by *rseA* mutation and *ompR* mutation, as identified above, it is OmpF downregulation that is driving the cefalexin resistance phenotype observed in these three in vitro selected mutants.

5.2.4 Loss and downregulation of OmpF in cefalexin resistant *E. coli* from cattle and humans and further associations with lipopolysaccharide modification

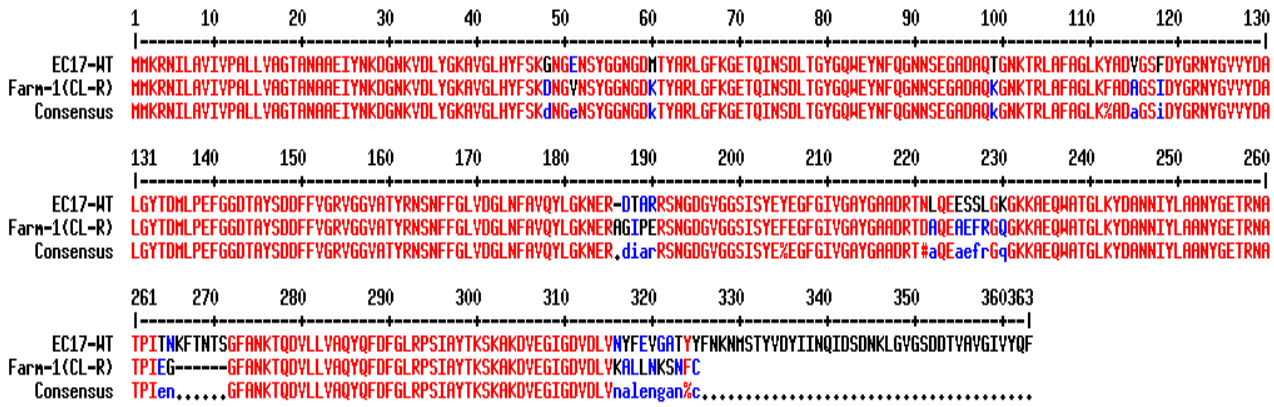
Two cefalexin resistant 3GC-S *E. coli* isolates (therefore without acquired cephalosporinases and with wild-type AmpC production levels) were chosen at random from a previous survey on dairy farms (193), and two from a survey on human urinary *E. coli* (122). Cefalexin resistance was confirmed for each isolate by measuring its MIC against them (Table 5.5). WGS revealed disruption of *ompF* in both farm isolates: in CL-R Farm-1, a Tn*5* insertion disrupted *ompF*, truncating OmpF after amino acid 316. In CL-R Farm-2 a frameshift mutation disrupted OmpF after amino acid 96 (Figure 5.2).

Table 5.5: MIC of Cefalexin against cefalexin resistant, 3GC-S *E. coli* isolates.

Antibacterial	MIC (μg/mL)			
	CL-R Farm-1	**CL-R Farm-2**	**CL-R UTI-1**	**CL-R UTI-2**
Cefalexin	64	64	32	32

Shaded values represent resistance based on CLSI breakpoints, otherwise susceptible.

A



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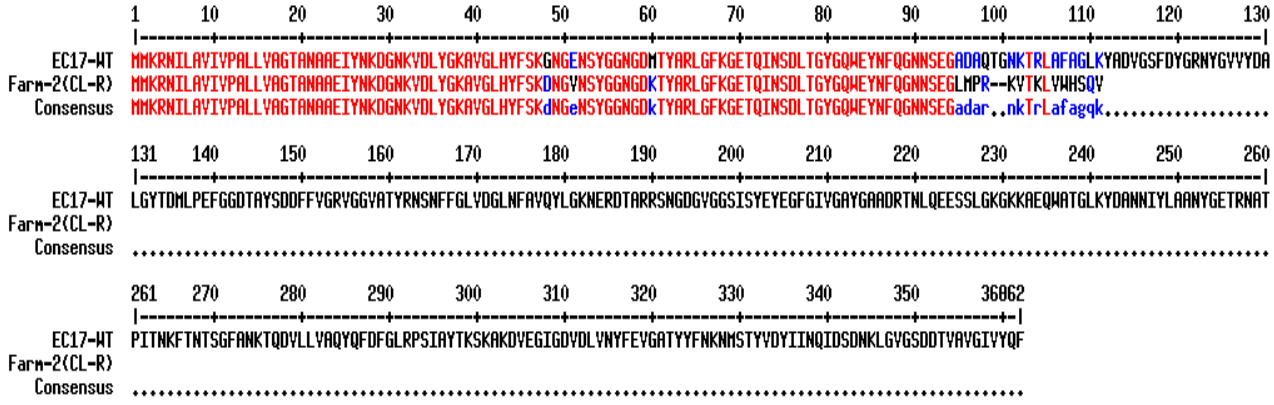

Figure 5.2: Type of mutations detected in the outer membrane protein (OmpF) of cefalexin resistant farm isolates. A) Tn*5* insertion mutation in cefalexin resistant (CL-R) Farm-1 isolate. B) frame shift mutation in cefalexin resistant (CL-R) Farm-2 isolate.

The *ompF* gene was intact in both human urinary isolates, which were identified by WGS as ST131, the most common ST found in urinary *E. coli* in the previous survey (122). Proteomics showed, however, significant (p<0.05) downregulation of OmpF abundance relative to ribosomal proteins in both cefalexin resistant urinary isolates compared with the control human isolate EC17 (1.43 +/- 0.16, Mean +/- SEM, n=3) and a very closely phylogenetically related control ST131 urinary isolate, collected in parallel (122) UTI-80710 (1.15 +/- 0.09, n=3). In CL-R UTI-1, OmpF downregulation was approximately 2-fold relative to both controls (OmpF abundance: 0.70 +/- 0.12, n=3), but in CL-R UTI-2, OmpF was approximately 10-fold downregulated relative to both controls (OmpF abundance: 0.13 +/- 0.03, n=3). Notably, CL-R UTI-1 also had a nonsense mutation at codon 82 in *ompC*. As expected, therefore, OmpC was undetectable by proteomics in CL-R UTI-1, but OmpC abundance relative to ribosomal proteins in CL-R UTI-2 (2.64 +/- 0.84, n=3) was not significantly lower (p>0.25) than in control isolates UTI-80710 (3.14 +/- 0.31, n=3) and EC17 (2.55 +/- 0.61, n=3). Most interestingly, CL-R UTI-2 produced >2-fold elevated (p<0.05) levels of DegP (abundance relative to ribosomal proteins: 0.42 +/- 0.04, n=3) compared to control isolates EC17 (0.20 +/- 0.04, n=3) and UTI-80710 (0.13 +/- 0.02, n=3), suggestive of a phenotype like that of the *gmhB* mutant, described above. CL-R UTI-1 did not produce DegP at levels significantly different from control (p>0.25).

According to WGS, ST131 isolate CL-R UTI-2 did not have a mutation in *gmhB*, *rseA*, *ompR*, or *ompF* relative to the ST131 control isolate UTI-80710. However, there was a mutation in *lpxB* predicted to cause R138I in the protein. LpxB is an essential and highly conserved protein in the early stages of lipopolysaccharide biosynthesis (217). Using protein blast, we found that only five *E. coli* NCBI database entries have this R138I mutation. Furthermore, this non-conservative change is adjacent to residues making key interactions essential for the tertiary structure of the enzyme: W128, R129, R132(218). Accordingly, it seems highly likely that this

R138I mutation alters the activity of LpxB such that lipopolysaccharide biosynthesis is affected, a known signal for Sigma E activation (217). This therefore provides an explanation for elevated DegP leading to reduced OmpF levels, and cefalexin resistance in UTI-2.

Moreover, given the complexity of Sigma E activation signals and the impact that many different changes in envelope structure can have on it (217), clinical *E. coli* isolates could carry multiple different mutations that activate this regulon. Indeed, searches of the NCBI database identified carbapenem-resistant human *E. coli* isolate E300, identified in Japan (219), which had an 8-nucleotide insertion, leading to a frameshift in *rseA* after nucleotide 34 (Accession Number AP022360). Furthermore, two human clinical isolates were found to have a single nucleotide insertion leading to a frameshift in *gmhB* after nucleotide 126; one from China (Accession Number CP008697) and one from the USA (Accession Number CP072911); and three commensal *E. coli* from the USA (220) were found to have frameshift mutations at various positions in *gmhB* (Accession Numbers CP051692, CP054319, and CP054319). Accordingly, we conclude that mutations likely to cause the same phenotypes found in our laboratory-selected cefalexin-resistant mutants are also found in clinical and commensal *E. coli* samples across the world.

5.2.5 Anti-Sigma E response is associated with OmpF porin downregulation and cefalonium resistance in *E. coli*.

After identifying OmpF downregulation as the main cause for cefalexin resistance, it was interesting to test if this mechanism can also lead to cefalonium resistance (cefalonium is a veterinary-specific 1st generation cephalosporin used on many of the farms studied in chapter 3). One spontaneous cefalonium resistant mutant was selected in vitro at 4 µg/mL cefalonium

using Muller Hinton agar from the farm** *E. coli* parent isolate (also used above to select cefalexin resistant mutant that was found to have an *ompR* mutation). LC-MS/MS proteomics showed that the cefalonium resistant mutant did not hyper-produce AmpC relative to its parent (Table5.6). Interestingly, DegP was found to be significantly (p<0.05) 10-fold higher in abundance in the cefalonium resistant mutant than in the parent isolate Farm**(Table5.6). Furthermore, the two major porins OmpF and OmpC were significantly (p<0.05) downregulated, 7.6 fold and 197.6 fold, respectively. OmpA was also significantly (p<0.05) 1.7-fold downregulated relative to the parent isolate. All this was suggestive of a phenotype similar to the ATCC25922-derived cefalexin resistant mutant, described above. Notably, there was a 31 amino acids deletion in OmpC which may explain the almost total loss of OmpC in the proteomics data (Table 5.6). However, according to WGS the cefalonium resistant mutant did not have a mutation in *gmhB*, *rseA*, *ompR*, *lpxB* or *ompF* relative to its parent isolate. Therefore, the regulatory mutation leading to elevated DegP levels, reduction in OmpF levels, and cefalonium resistance in this mutant have not been identified. It is possible that the mutation in OmpC itself is activating Sigma E and DegP production, reducing OmpF levels as well, and if so, this would be an important finding, and requires experimental confirmation.

The resistance profile of the Farm**-derived cefalonium resistant mutant and Farm**-derived cefalexin resistant mutant (*ompR* mutant, discussed above), in both cases involves OmpF and OmpC downregulation was similar. Interestingly, however, a one doubling higher MIC cefalonium against the *ompR* derived mutant was observed than against the cefaonium resistant mutant (Table 5.7). Notably, OmpC abundance in the cefalonium resistant mutant was 5.6 fold lower than against the cefalexin resistant *ompR* mutant, 0.03±0.004 versus 0.17±0.04, Mean +/- SEM, n=3), suggesting that OmpC downregulation, is not necessary for cefalonium resistance. Supporting this, the ATCC25922-*ompF* mutant, both cefalexin resistant

Farm isolates (with *ompF* mutation) and both cefalexin resistant UTI isolates (CL-R UTI-1 harboured a mutation in *ompC* and both having OmpF downregulation) were all cefalonium resistant Table 5.7. This led to a conclusion that OmpF downregulation is sufficient for cefalonium resistance as well as cefalexin resistance as stated above.

Table 5.6: LC-MS/MS proteomic comparisons of outer membrane protein and DegP abundance in *E. coli* isolates versus cefalonium resistant mutant derivatives.

Strains	AmpC	OmpF		OmpC		OmpA		DegP	
	Mean± SEM	Mean± SEM	P (WT/M)	Mean± SEM	P (WT/M)	Mean± SEM	P (WT/M)	Mean± SEM	P (WT/M)
Farm**	ND	0.23±0.05		5.93±1.00		3.24±0.32		0.06±0.01	
Farm (Cefalonium-R M)**	ND	0.03±0.01	0.01	0.03±0.004	0.0005	1.90±0.17	0.0005	0.61±0.05	0.04

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, (n = 3). Proteins whose abundance is significantly (P < 0.05) up or downregulated at least 2-fold relative to the parent *E. coli* strains (see Materials and methods) are shaded in green and red, respectively.

Table 5.7: MIC of Cefalonium against *E. coli* isolates and mutant derivatives.

Antibacterial	MIC (µg/mL)											
	Farm**	Farm** (Cefalonium R-M)	Farm** (*ompR*)	ATCC25922	ATCC25922 *ompF*	ATCC25922 *rseA*	EC17	EC17 (M)	(CL-R) Farm-1	(CL-R) Farm-2	(CL-R) UTI-1	(CL-R) UTI-2
Cefalonium	2	8	16	2	4	8	2	4	16	8	8	4

Shaded values represent resistance based on CLSI breakpoints, otherwise susceptible.

5.2.6 Influence of OmpF porin loss and downregulation on late generation cephalosporin susceptibility in combination with AmpC hyperproduction

The final aim was to test the impact of OmpF loss and downregulation, due to *ompR* mutation, activation of Sigma E, or mutation on the *ompF* gene itself, on late generation cephalosporin MICs against *E. coli* hyper-producing AmpC. To address this aim, two *ampC* genes with identical promoter sequences and identical open reading frames with only one mutation, causing one amino acid difference, His296Pro that resulted in an expanded-spectrum AmpC (68), as seen in the Farm-1 isolate in Chapter 3, were cloned separately into the plasmid pK18 (221) using the method shown in Chapter 2. Both constructs were sequenced, and sequence results revealed that both genes were successfully cloned into pK18 as shown in Figure 5.3. Then, the pK-*ampC*^{WT} and pK-*ampC** (mutant) recombinants were introduced into the strains/mutants with different *ompF* backgrounds: cefalexin resistant farm isolates that had a loss of function mutations in *ompF* (Farm-1 (CL-R) and Farm-2 (CL-R), and the in vitro selected cefalexin resistant mutants with mutations in *rseA, ghmB* and *ompR (*ATCC25922 (M), EC17(M) and Farm**(M), respectively) and their wild type parents.

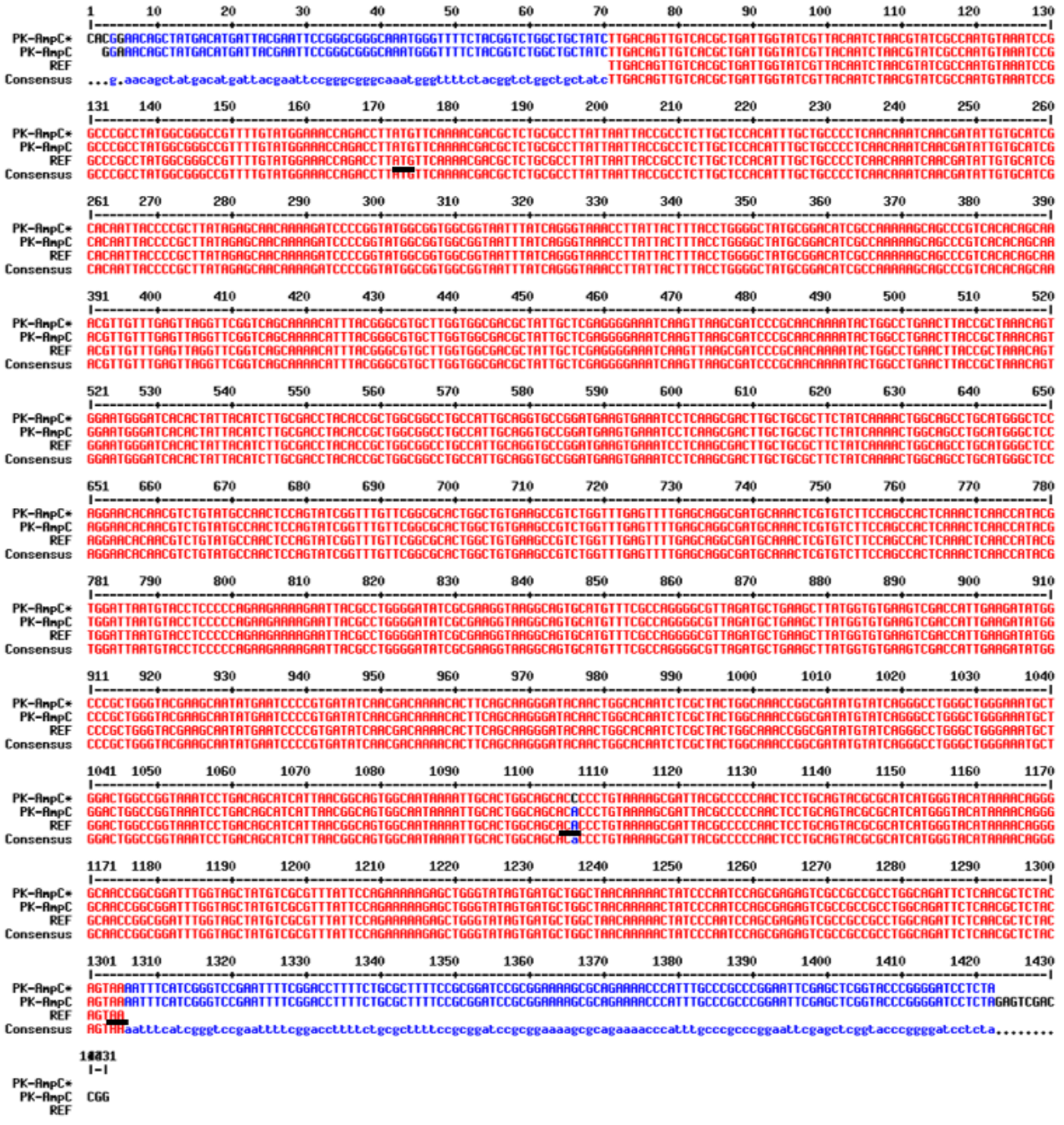

Figure 5.3: Sequencing alignment of the *ampC* genes in the pk18 vector. Underline sequences are the ATG start codon, A-C amino acid change in the expanded spectrum AmpC, and stop codon for the cloned gene, respectively. *ampC expanded spectrum AmpC.**

MICs of 3GCs and 4GCs used in humans (ceftazidime, cefepime) or cattle (ceftiofur, cefquinome) were measured against all pK-*ampC* transformants (Table 5.8). In the transformants of two CL-R Farm isolates, hyper-production of expanded spectrum AmpC (AmpC*) conferred resistance to all five cephalosporins tested, wild type AmpC did not confer cefepime or cefquinome resistance in these isolates, as expected given the impact of the AmpC mutation of 4GC resistance described in chapter 3. Similar MIC pattern were seen against wild-type parent isolates ATCC25922 and EC17 producing this cloned, expanded spectrum AmpC*. Activation of Sigma E through *rseA* mutation (cefalexin resistant ATCC25922 mutant) and *gmhB* mutation (cefalexin resistant EC17 mutant) caused a one doubling increase in cefepime MIC against both mutants and a two doubling MIC increase of ceftiofur and cefquinome against EC17 mutant compared to their parent strains also producing the cloned AmpC*. Hence there is some synergy between expanded-spectrum AmpC* production and porin downregulation.

Producing AmpC* gave the highest cefepime MIC against the Farm**(M) *ompR* mutant, however, with the cefepime MIC being two doublings higher than against AmpC* transformants of the ATCC25922 *rseA* mutant derivative (Table 5.8). The greater impact of *ompR* mutation versus *rseA* mutation on reducing OmpC levels as demonstrated above likely explains this increase in cefepime MIC as OmpC downregulation has been shown to raise MICs of 4GCs in CMY-2 β-lactamase producing *E. coli* (222).

Table 5.8: MICs of cefalexin resistant isolates/mutant transformants. ampC*: expanded spectrum AmpC, ampC^{WT}: wild type AmpC.

Strains	MIC µg/mL				
	Cefepime	Ceftiofur	Cefiquinome	Caftazedime	Cefaperazone
Farm-1(CL-R) PK18	0.25	0.5	0.125	0.5	0.5
Farm-1(CL-R)-ampC*	64	64	64	>256	>256
Farm-1(CL-R)-ampC^{WT}	4	32	4	256	256
Farm-2(CL-R) PK18	0.25	0.5	0.125	0.5	0.5
Farm-2(CL-R)-ampC*	64	32	32	>256	>256
Farm-2(CL-R)-ampC^{WT}	2	16	2	256	128
EC17- PK18	0.25	0.125	0.125	0.25	0.25
EC17- ampC*	32	16	8	128	>256
EC 17 ampC^{WT}	2	16	2	64	256
EC17 (M) PK18	0.25	0.25	0.125	0.5	0.25
EC17 (M) ampC*	64	64	32	>256	>256
EC17 (M) ampC^{WT}	8	32	8	256	>256
FarmPK18**	0.25	0.25	0.125	0.25	0.25
Farm ampC***	32	32	16	>256	>256
Farm ampC^{WT}**	2	32	1	128	256
Farm (M) PK18**	0.25	0.5	0.25	0.25	0.25
Farm(M) ampC***	128	32	64	>256	>256
Farm(M) ampC^{WT}**	4	32	4	128	>256
EC25922 PK18	0.25	0.25	0.125	0.25	0.25
EC25922 ampC*	32	32	32	>256	>256
EC25922 ampC^{WT}	2	16	2	128	>256
EC25922 (M) PK18	0.25	0.25	0.125	0.25	0.25
EC25922 (M)ampC*	64	32	32	>256	>256
EC25922(M)ampC^{WT}	2	16	2	128	256

Shaded values represent resistance based on CLSI breakpoints, otherwise susceptible. Off-white shaded cells show the control transformants of each strain/mutant (with empty pK18).

5.7 Conclusions

Cefalexin is a widely used antibacterial drug in human and veterinary medicine and for this reason resistance to cefalexin is of considerable clinical importance. Despite this, mechanisms of resistance have not been given very much attention, particularly in the post-genomic age. It was surprising to find that, in recent surveys, on human and cattle cefalexin resistant isolates, acquired cephalosporinase (pAmpC or ESBL) or chromosomal AmpC hyper-production were not the cause of cefalexin resistance in a large proportion of isolates (122,193). A piece of strong evidence has been shown here that OmpF loss or downregulation is a key mechanism of cefalexin (and the veterinary specific 1st generation cephalosporin cefalonium) resistance in *E. coli* in the absence of β-lactamase production. Whilst OmpF loss contributes to resistance to a wide range of antibacterials (206), our findings show that cefalexin/cefalonium resistance is unusual in being caused solely by OmpF loss. Furthermore, we show that OmpF downregulation can also confer this phenotype. This may explain why *ompF* loss of function mutations are found among *E. coli* from clinical samples, but our work suggests there may also be numerous different regulatory mutations found among clinical isolates, each downregulating OmpF.

Such is the wide range of regulatory systems controlling OmpF production, both at transcriptional, translational, and post-translational levels (208,217,223), it is not surprising that cefalexin resistance mutations arise in many different genes in the laboratory, as shown in the discussed above publications. The other regulatory mutations affecting OmpF levels found in the laboratory-selected mutants reported here work through Sigma E mediated DegP over-production. It is well known that DegP degrades porins (211,217), but it has not previously been reported that DegP-mediated degradation of OmpF is sufficient to cause resistance to any antibacterial drugs.

These findings are also potentially important because they suggest that OmpF is more susceptible to DegP mediated proteolysis in vivo in *E. coli* than the other two main porins, OmpC and OmpA. In the *rseA* mutant with "maximal" Sigma E activation and DegP upregulation, OmpF, OmpC and OmpA levels all fell, but in the gmhB mutant overproducing DegP to a lesser extent, only OmpF levels significantly fell, and this was still sufficient to cause cefalexin resistance.

It is known that mutations affecting the outer membrane and lipopolysaccharide structure activate Sigma E because they affect envelope integrity (217). It has not previously been shown, however, that mutations disrupting gmhB can do this, and further, that this can cause cefalexin resistance. We considered that despite such mutations arising in the laboratory, this perhaps overstates their clinical relevance because disruption of the Gmh system causes significant attenuation and increased susceptibility to envelop stresses, though significantly, loss of GmhB has the mildest effect in this regard (216). Accordingly, it was very interesting to find an LpxB mutation in a human urinary ST131 cefalexin resistant isolate, having a non-conservative mutation close to key region of the protein (218). LpxB is an essential and highly conserved protein in *E. coli* that sits at the start of lipopolysaccharide biosynthesis (217). Importantly, our observation of a LpxB mutant in a clinical isolate as well as the clear evidence of *rseA* and *gmhB* loss-of-function mutations among clinical and commensal *E. coli* from secondary analysis of WGS data. This provides strong evidence that mutations constitutively activating Sigma E, including those which do this by altering lipopolysaccharide structure, can be tolerated by *E. coli* in a clinical setting. These mutations, and possibly others yet to be identified, cause clinically relevant cefalexin resistance in the absence of β-lactamase production through DegP-mediated OmpF proteolysis.

Chapter 6

Understanding the mechanisms of Ertapenem resistance in *E. coli* porin mutants producing AmpC or ESBLs

6.1 Introduction

Carbapenems, antibacterial drugs that belong to β-lactam class, are effective against many Gram-positive and Gram-negative pathogens, having a broad spectrum of antibacterial activity due to their unique molecular structure that confers remarkable stability against most β-lactamases including the ESBLs (224). Carbapenems, including meropenem and ertapenem, are often used as the last resort to treat severe infections caused by multidrug resistance Enterobacteriaceae isolates, especially infection caused by ESBL producers (225). Accordingly, the emergence and rapid expansion of carbapenem resistance globally, predominantly among Gram-negative bacteria, is a global public-healthcare problem of major concern since carbapenem resistance makes antibacterial treatment options extremely restricted (224).

Resistance to carbapenems in the Enterobacteriaceae is caused mainly by carbapenemases, a group of β-lactamases that can hydrolyse carbapenems, as well as, in many cases, conferring resistance to cephalosporins and penicillins. Carbapenem resistance via this mechanism can be transmitted among different bacterial strains and species as genes that encode carbapenemases are primarily carried by conjugative plasmids that facilitate the horizontal transfer of these genes (226). Another carbapenem resistance mechanism can occur by a combination of AmpC overexpression and/or production of an ESBL (both very weak carbamenemases) alongside mutations that reduce outer membrane permeability (thus slowing down the entry of carbapenems). Various non-carbapenemase β-lactamase producer strains with an absence or reduced production of the two major porins in *K. pneumoniae* (OmpK35 and OmpK36) (227,228), *E. coli* and *E. cloacae* (OmpF and OmpC) or *E. aerogenes* (Omp35 and Omp36) (229-231) have been reported to be resistant to carbapenems. Ertapenem is the most affected carbapenem when plasmids encoding an ESBL or a plasmid

mediated AmpC were introduced into a porin-deficient strain of *K. pneumoniae* (232). In contrast to carbapenemase production, carbapenem resistance mechanisms involving porin loss or downregulation is not able to spread via horizontal transfer but may disseminate through clonal expansion (233).

The work reported here aimed to test the mechanism of carbapenem (ertapenem) resistance in expanded spectrum AmpC *E. coli* producers by selecting ertapenem resistant mutant *in vitro*. The work reported here also investigated the influence of porin defect or downregulation in combination with production of (CTX-M type) ESBLs on ertapenem susceptibility.

Part of this work has been published as my contribution to the below paper, in which I am first author. Dr Kate Heesom generated proteomic data, which was analysed by me. Otherwise, none of the other authors contributed to the work presented in this chapter:

Maryam Alzayn, Punyawee Dulayangkul, Naphat Satapoomin, Kate J. Heesom, and Matthew B. Avison. 2021. OmpF Downregulation Mediated by Sigma E or OmpR Activation Confers Cefalexin Resistance in Escherichia coli in the Absence of Acquired β-Lactamases. Antimicrobial Agents and Chemotherapy. doi: 10.1128/AAC.01004-21.

6.2 Result and discussion

6.2.1 Ertapenem resistant mutants selected In vitro and identification of *ompC* porin loss

To understand how carbapenem resistance might evolve from cephalosporin-resistant isolates, Ertapenem was utilised to select mutants from the farm isolate that produced an expanded spectrum AmpC (Farm-1), and lacked OmpF due to a frameshift mutation, as described in chapter 3. As this isolate was already 4GC resistant, due to actions of the mutant AmpC, we were interested to see how ertapenem resistance might evolve. A spontaneous ertapenem resistant mutant (Farm-1 ERT-M) was selected at (1 mg/L) using Mueller-Hinton Agar. MICs assays revealed a four doubling higher MIC of ertapenem against Farm-1 ERT-M mutant comparing to its parent isolate, and a two doubling higher MIC of cefepime and cefquinome. While there was no difference detected in ceftazidime and ceftiofur MIC in the parent/mutant pair (Table 6.1).

Table 6.1: MICs of 3GCs/4GCs and ertapenem against ertapenem resistant *E. coli* mutant and its parent isolate

Antibacterial	MIC µg/mL	
	Fam-1	Farm -1 ERT -M
Cefepime	8	32
Cefquinome	4	16
Ceftazidime	256	256
Ceftiofur	16	16
Ertapenem	0.125	>2

Values shaded in grey, and blue represents resistance and intermediate, respectively according to CLSI breakpoints.

According to LC-MS/MS proteomics, the mutant Farm-1 ERT-M did not have altered production of key resistance proteins relative to its parent, Farm-1 except the production of the OmpC, which was significantly reduced ($p<0.05$); indeed, it became undetectable (Table 6.2). Sequencing of the *ompC* gene from Farm-1 ERT-M revealed a frameshift mutation due to a deletion of T nucleotide at position 830, Figure 6.1. This mutation is likely to cause ertapenem resistance in the selected mutant because its parent isolates Farm-1 had a combination of expanded spectrum AmpC hyperproduction, and an *ompF* frameshift mutation but remained susceptible to ertapenem, Table 6.1. Loss of the OmpF and OmpC porins did not noticeably affect envelope permeability in the Farm 1 ERT-M isolate relative to its parent isolate based on fluorescent dye accumulation (Figure 6.2). The contribution of OmpC loss in carbapenem and cefepime resistance in *E. coli* clinical isolates that produce the CMY-2 (AmpC-type) enzyme or CTX-M-type ESBLs has been reported previously (229,222).

Table 6.2: Abundance of key resistance proteins in ertapenem resistant *E. coli* in vitro-selected mutant relative to its parent isolate

Accession	Description	Farm-1	Farm-1-ERT M	
		Mean±SEM	Mean±SEM	P(WT/M)
P06996	**OmpC**	3.14 ±0.42	0.00±0.00	<0.01
P02931	**OmpF**	0.02±0.02	0.05±0.03	0.25
P00811	**AmpC**	0.82±0.12	0.76±0.06	0.33
P0AE06	**AcrA**	0.13±0.03	2.13±2.01	0.19
P31224	**AcrB**	0.07±0.03	0.02±0.01	0.16
P02930	**TolC**	0.08±0.04	0.14±0.01	0.11

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, (n = 3). Proteins whose abundance is significantly ($P < 0.05$) downregulated at least 2-fold relative to the *E. coli* parent strain (Farm-1) as control (see Materials and methods) are shaded in red.

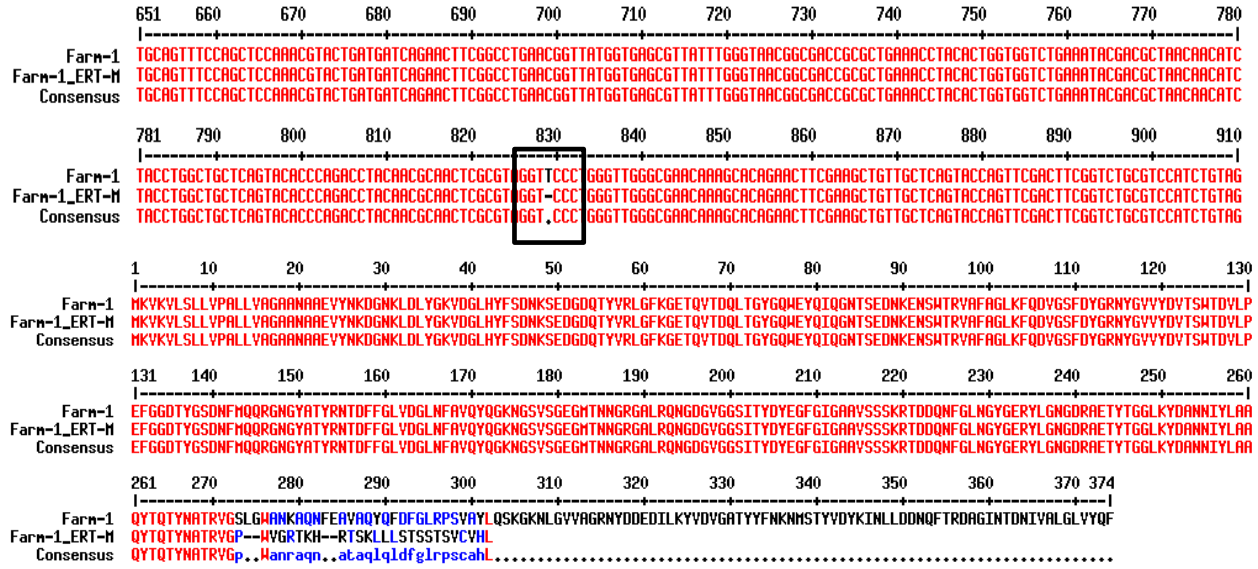

Figure 6.1: Sequence alignment of *ompC* gene from ertapenem resistant mutant (Farm-ERT-M) with a reference sequence of Farm-1 parent strain. Frameshift mutation due to Thymine (T) deletion at position 830 was detected in ertapenem resistant mutant. Top panel: nucleotide sequence. Bottom panel: amino acid sequence.

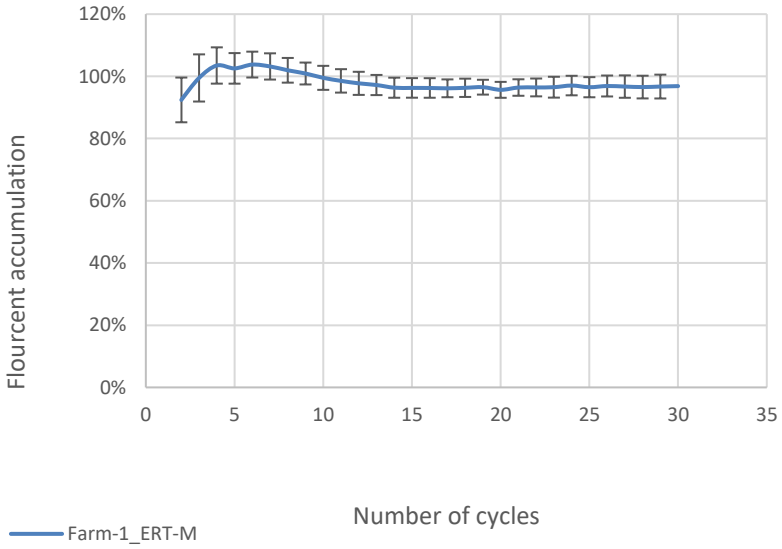

Figure 6.2: Envelope permeability of ertapenem resistant *E. coli* mutant determined using fluorescent dye accumulation assays. Fluorescence of an ertapenem resistant *E. coli* mutant incubated with the dye is presented relative to that in the control *E. coli* parent strain (Farm-1) after each cycle. Each line shows mean data for three biological replicates with eight technical replicates in each. Error bars define the SEM.

6.2.2 Influence of *ompF* porin loss and downregulation on late generation cephalosporin and carbapenem susceptibility in the presence of various CTX-M β-lactamases

Having identified an interaction between expanded spectrum AmpC hyperproduction and OmpC loss as a possible cause of ertapenem resistance in *E. coli*, the final aim was to investigate the impact of OmpF loss and downregulation, due to OmpR mutation, or activation of Sigma E, on late generation cephalosporin or carbapenem MIC in *E. coli* producing CTX-M β-lactamases (mutations have been discussed in chapter 5). To do this, natural plasmids carrying various $bla_{\text{CTX-M}}$ variants commonly identified in human and cattle 3GC-R *E. coli* in Southwest England: encoding CTX-M-1, CTX-M-14, CTX-M-15 and CTX-M-32 (pMoo-32 plasmid found in farm isolates) (122,192) were introduced, using conjugation, to cefalexin resistant, cefotaxime susceptible recipient *E. coli* isolates and mutants previously characterized in chapter 5, Table 6.3.

In the last step of conjugation, the donor-recipient mixture was spread on a selective media that includes 4 µg/mL cefotaxime and 2 µg/mL ciprofloxacin or 50 µg/mL kanamycin as stated in the Material and Method chapter. Ciprofloxacin or kanamycin were used, depending on the resistance profile of the recipients, to kill donor strains and ensure the growth of only recipient cells including desired plasmids. One recipient strain (Farm**) was resistant to ciprofloxacin and had a *floR* gene (chloramphenicol resistant), ciprofloxacin was used for selection, while *floR* gene was used as a PCR resistance marker. Recipient strains that lack a marker (ATCC 25922) were first transformed to kanamycin resistance with pk18 and so kanamycin was utilised during the conjugation. However, conjugation with CTX-M-32 (pMoo32) was conducted to the *floR* recipient strain only because this plasmid harbours the Aminoglycoside-3'-phosphotransferase *aph(3')-IIa* gene and so the donor is resistant to kanamycin (121).

Transconjugant colonies were screened by PCR to confirm the presence of *floR* gene or pK18 along with the plasmids carrying the various CTX-Ms, Figure 6.3.

Table 6.3: Recipient strains used in the conjugation

Recipients	Porin background
Farm**	A ciprofloxacin resistant farm isolate
Farm**(M)	Cefalexin resistant *in vitro* selected mutant having a mutation in the transcriptional regulator *ompR* which results in OmpF and OmpC downregulation
ATCC25922	Wild type *E. coli* strain
ATCC25922-M	Cefalexin resistant *in vitro* selected mutant having a mutation in the *rseA* gene (Anti-sigma-E factor) which results in OmpF and OmpC downregulation
ATCC25922 - *ompF*	Cefalexin resistant constructed mutant, having an *ompF* insertional inactivation
(CL-R) Farm-1	Cefalexin resistant farm isolate having *ompF* insertional inactivation

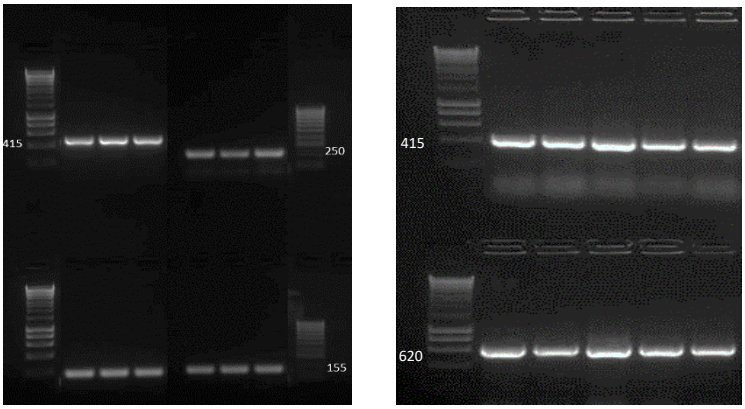

Figure 6.3: An example of agarose gel electrophoresis pattern of colony PCR products to confirm transfer of plasmid DNA to the recipient cells. The bands at 415 and 250 bp illustrate successful conjugation of CTX-M group 1 and CTX-M group 9 respectively. Bands at 155 and 620 bp revealed presence of pk18 and *floR* gene respectively. CTX-M group 1 includes (CTX-M-32, CTX-M-15, and CTX-M-1) while CTX-M group 9 (includes CTX-M-14).

MICs were measured of 3GCs and 4GCs used in humans (ceftazidime, cefepime) or cattle (ceftiofur, cefquinome), and the carbapenem ertapenem against transconjugants of *E. coli* parent strains, their *ompF*, *rseA* or *ompR*, mutant derivatives and a CL-R farm isolate (Table 6.4). Fold changes in MICs were calculated for each transconjugant relative to its parent strain (CTX-M free mutants/isolates), Table 6.5.

In wild-type ATCC25922, as expected, CTX-M-1 and CTX-M-15 conferred resistance to all four cephalosporins, CTX-M-14 did not confer ceftazidime resistance, and none of the enzymes conferred ertapenem resistance. Disruption of *ompF* or *rseA* did not change the susceptibility profile but there were some MIC changes. Disruption of *ompF* caused a two-doubling increase in ertapenem MIC in the presence of CTX-M-1 and CTX-M-15 (16- and 32-fold increase, respectively), but there was no change in the CTX-M-14 transconjugant, Table 6.5. Disruption of *rseA* caused a similar impact on ertapenem MIC against CTX-M-1 or CTX-M-15 producers, but additionally caused a two-doubling (4 fold) increase in MIC against the CTX-M-14 producer. This additional effect is likely due to the downregulation in OmpC additionally seen in the *rseA* mutant, being a key carbapenem porin (234) an already highlighted above in the context of ertapenem resistance in AmpC hyperproducers.

In the wild-type *E. coli* isolate, Farm**, the profiles of resistance seen were identical among CTX-M producers as they were for ATCC25922 transconjugants (Table 6.4). However, the *ompR* point mutation had a stronger effect on MIC change than the *rseA* mutation did in the ATCC25922 background. In this case, both CTX-M-1 and CTX-M-15 conferred ertapenem non-susceptibility in this *ompR* mutant, the MIC being one doubling higher than against the *rseA* mutant producing these enzymes. Additionally, the ertapenem MIC was four doublings higher (4 fold higher) against the *ompR* mutant producing CTX-M-32 comparing to its wildtype parent

isolate (Farm**), though remaining susceptible (Tables 6.4 and 6.5). The greater impact of OmpR mutation rather than RseA loss on reducing OmpC and OmpF levels (Table 6.6) likely explains this difference, and *ompR* mutation is commonly associated with ertapenem non-susceptibility in ESBL producing *E. coli* (234,209).

Interestingly, CTX-M-1 and CTX-M-15 conferred ertapenem non-susceptibility in cefalexin resistant farm isolate (Farm-1-CL-R)- which has *ompF* insertional inactivation due to a Tn*5* (as shown in chapter 5). the MIC of ertapenem was two and one doublings higher, respectively, against Farm-1 producing CTX-M-1 or CTX-M-15 than against the *ompF* laboratory constructed insertional inactivation mutant (ATCC25922 *ompF)* producing these enzymes. Isolate Farm-1 did not show a significant difference in OmpC production compared to the control isolate *E. coli* 17**,** Table 6.6, however. This suggests that there may be other factors in addition to OmpC loss or downregulation that can confer ertapenem non-susceptibility in *ompF* mutants producing CTX-M-1 and CTX-M-15.

Table 6.4: Influence of *ompF*, *rseA* and *ompR* mutations on late generation cephalosporin and carbapenem MIC against *E. coli* producing CTX-M variants.

Strain Name	MIC µg/mL				
	Cefepime	**Cefquinome**	**Ceftazidime**	**Ceftiofur**	**Ertapenem**
ATCC25922(pK18)	0.25	0.125	0.25	0.25	0.016
ATCC25922(pK18) CTX-M-1	>128	>128	16	>128	0.0625
ATCC25922(pK18) CTX-M-15	>128	>128	32	>128	0.125
ATCC25922(pK18) CTX-M-14	64	>128	2	>128	0.0313
ATCC25922(pK18) *ompF*	0.25	0.125	0.5	0.5	0.016
ATCC25922(pK18) *ompF* -CTX-M-1	>128	>128	32	>128	0.25
ATCC25922(pK18) *ompF* CTX-M-15	>128	>128	64	>128	0.5
ATCC25922(pK18) *ompF* -CTX-M-14	>128	>128	4	>128	0.0313
ATCC25922(pK18) *rseA*	0.25	0.25	0.5	0.5	0.0313
ATCC25922(pK18) *rseA* -CTX-M-1	>128	>128	16	>128	0.5
ATCC25922(pK18) *rseA* -CTX-M-15	>128	>128	32	>128	0.5
ATCC25922(pK18) *rseA*- CTX-M-14	>128	>128	4	>128	0.125
Farm**	0.125	0.125	0.5	0.25	0.016
Farm**CTX-M-1	>128	>128	32	>128	0.125
Farm**CTX-M-15	>128	>128	32	>128	0.25
Farm**CTX-M-14	128	>128	8	>128	0.0313
Farm** CTX-M-32	64	128	32	>128	0.0313
Farm** (M) (*ompR*)	0.125	0.25	0.5	0.5	0.0626
Farm** (M) (*ompR*) -CTX-M-1	>128	>128	16	>128	1
Farm**(M) (*ompR*)- CTX-M-15	>128	>128	16	>128	1
Farm**(M) (*ompR*)- CTX-M-14	>128	>128	4	>128	0.5
Farm**(M) (*ompR*) -CTX-M-32	128	>128	16	>128	0.25
Farm-1(CL-R)	0.25	0.25	0.5	1	0.125
Farm-1(CL-R)- CTXM-1	>256	>128	8	>128	1
Farm-1(CL-R)- CTXM-15	>256	>128	16	>128	1
Farm-1(CL-R)- CTXM-14	256	>128	4	>128	0.25

CTX-M variants were delivered on natural plasmids by conjugation. Plasmid pK18 was added to provide a marker (Kanamycin resistance) to allow selection for recipients in conjugation. Isolate Farm** is fluoroquinolone resistant, so this was not necessary. Values shaded in grey, and blue represent resistant and intermediate, respectively according to CLSI breakpoints.

Table 6.5: Fold change in late generation cephalosporin and carbapenem MICs in *E. coli* producing CTX-M variants comparing to their wild type (CTX-M free) isolate

Strain Name	Fold change in MICs				
	Cefepime	**Cefquinome**	**Ceftazidime**	**Ceftiofur**	**Ertapenem**
ATCC25922(pK18)	-	-	-	-	-
ATCC25922(pK18) CTX-M-1	>512	>1024	64	>512	4
ATCC25922(pK18) CTX-M-15	>512	>1024	128	>512	8
ATCC25922(pK18) CTX-M-14	256	>1024	8	>512	2
ATCC25922(pK18) *ompF*	-	-	-	-	-
ATCC25922(pK18) *ompF* -CTX-M-1	>512	>1024	64	>256	16
ATCC25922(pK18) *ompF* CTX-M-15	>512	>1024	128	>256	32
ATCC25922(pK18) *ompF* -CTX-M-14	>512	>1024	8	>256	2
ATCC25922(pK18) *rseA*	-	-	-	-	-
ATCC25922(pK18) *rseA* -CTX-M-1	>512	>512	32	>256	16
ATCC25922(pK18) *rseA* -CTX-M-15	>512	>512	64	>256	16
ATCC25922(pK18) *rseA*- CTX-M-14	>512	>512	8	>256	4
Farm**	-	-	-	-	-
Farm**CTX-M-1	>1024	>1024	64	>512	8
Farm**CTX-M-15	>1024	>1024	64	>512	16
Farm**CTX-M-14	1024	>1024	16	>512	2
Farm** CTX-M-32	512	1024	64	>512	2
Farm** (M) (*ompR*)	-	-	-	-	-
Farm** (M) (*ompR*) -CTX-M-1	>1024	>512	32	>256	16
Farm**(M) (*ompR*)- CTX-M-15	>1024	>512	32	>256	16
Farm**(M) (*ompR*)- CTX-M-14	>1024	>512	8	>256	8
Farm**(M) (*ompR*) -CTX-M-32	1024	>512	32	>256	4
Farm-1(CL-R)	-	-	-	-	-
Farm-1(CL-R)- CTXM-1	>1024	>512	16	>128	8
Farm-1(CL-R)- CTXM-15	>1024	>512	32	>128	8
Farm-1(CL-R)- CTXM-14	1024	>512	8	>128	2

Table 6.6: LC-MS/MS proteomic comparisons of porin protein abundance in *E. coli* isolates versus cefalexin resistant isolates-mutant derivatives.

Strain name	OmpF	OmpC
	Mean± SEM	Mean± SEM
Farm**	0.23±0.05	5.93±1.00
Farm (M) *ompR***	0.00±0.00	0.17±0.04
ATCC25922	1.69±0.10	1.66±0.26
ATCC25922 (M) *rseA*	0.29±0.14	0.24±0.05
***E. coli* 17**	1.15±0.15	2.49±0.50
Farm-1 CL-R	0.21±0.11	3.35±0.70

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is mean ± SEM, (n = 3). Proteins whose abundance is significantly ($P < 0.05$) downregulated at least 2-fold in the mutants relative to their *E. coli* parent strains or to *E. coli* 17 (see Materials and methods) are shaded in red.

6.3 Conclusion

The main finding of the reported work is the fact that *in vitro* expanded AmpC hyper producer with an OmpF loss of function mutant, can easily mutate due to loss of *ompC* and become resistant to ertapenem. Mutations that affect OmpC levels might also be important to cause ertapenem resistance when combined with other mechanisms. For this reason, it can also be concluded that the lower the OmpC level the more likely an AmpC or ESBLs producer isolate is to be ertapenem resistant. For example, some of these mutations largely affect OmpC levels (*ompC* disruption mutant mentioned above and cause ertapenem resistance) or can cause significant OmpC downregulation (e.g. in the *ompR* mutant mentioned above) that can give rise to carbapenem non-susceptibility if the mutant acquires a common ESBL such as CTX-M-15 or if it was an AmpC hyper producer.

Although mutants producing low levels of OmpC (*ompR* mutant) and harbouring an ESBL were only borderline non-susceptible to ertapenem, genotypically similar non-susceptible mutants have been seen in the clinic derived from patients who have received ertapenem to treat an infection caused by an ESBL-producing *E. coli* (209), suggesting that ertapenem should be used carefully to reduce the risk of selection of ESBL-producing bacteria with reduced carbapenem susceptibility.

Chapter 7

General discussion

Based on the annual report of the European Antimicrobial Resistance Surveillance System, there is an ongoing increase in cephalosporin resistance, and particularly 3GCR in *E. coli*. Among these resistant isolates, ESBL production, AmpC cephalosporinase hyper-production, or production of plasmid-mediated AmpC β-lactamases are known to be important, along with OXA-type penicillinases (113). The overall aim of the present study was to better understand mechanisms of cephalosporin resistance in *E. coli*, particularly the role of different chromosomal mutations (including those not previously identified) that confer cephalosporin resistance in *E. coli* in the absence of mobile β-lactamases. Also, to identify how chromosomal mutations increase the impact of ESBL and/or AmpC enzyme production on cephalosporin and carbapenem susceptiblity.

In this study, *E. coli* were characterised from two sources derived from surveillence studies performed by colleagues within the Avison research group during the time of this project: dairy farms and human urine samples. As well as characterising mechanisms of resistance using a variety of molecular bacteriology and functional genomics approaches, phylogenetic relationships between isolates from the two compartments (human/dairy farm) were investigated, to consider the potential of zoonotic transmission. Important new information was generated about the regulatory mechanisms that control envelope permeability have been identified, and their effect on cephalosporin and carbapenem susceptibility in the presence of AmpC hyperproduction or CTX-M β-lactamases has been confirmed.

Chapter 3 focused on the characterisation of 3GCR *E. coli* from dairy farms which were thought to be AmpC-hyperproducers. The main aim of the work reported was to confirm using proteomics and an assessment of mutations within the *ampC* promoter that AmpC hyperproduction was actually the cause of 3GCR. In doing this, a number of interesting isolates

with 4GC and/or cefoperazone resistance phenotypes were identified and the mechanism(s) of resistance identified. We confirmed that all 25 isolates were AmpC hyper-producers. Interestingly, despite diverse farm origins (confirmed in **chapter 4** to relate to deverse phylogenies) all 25 isoaltes carried the same four point-mutations in the *ampC* promoter region. It is likely that these mutations allow the right balance of AmpC hyper-production required for the spectrum of resistance necessary for success in a farm environment, without conferring a fitness cost. However, this remains to be seen. Interestingly in **chapter 4**, we saw a very diverse range of promoter mutations in AmpC hyperproducer *E. coli* from human urine samples, as discussed below. These findings contributed to the wider group's conclusion that AmpC hyperproduction is common mechanism of 3GCR in *E. coli* from UK dairy farms (almost 50% of isolates) with the rest being almost exclusively CTX-M producers. This also allowed us to confirm that amoxicillin/clavulanate use in dairy farms is a significant risk factor for the presence of AmpC hyper-producers, and this is important because it suggests that farmers simply moving away from using 3GCs and 4GCs and switching to amoxicillin/clavulanate use, will maintain selection for these AmpC hyperproducers that are 3GCR (and in some cases, 4GCR). Indeed, for the first time, this study has also identified an enhanced spectrum AmpC cephalosporinase (caused by a His296Pro substitution in a hyper-produced AmpC) in animal-associated *E. coli* in the UK (179). The importance of this mutation in improving the activity spectrum of AmpC was confirmed by selecting mutants using ceftazidime *in vitro*, where ceftazidime-resistant mutants harbouring identical His296Pro substitution were no longer susceptible to 4GCs and cefaperazone. Whilst such mutants have been seen elsewhere in Northern Europe, the identification of these mutants on two farms among 25 farms studied, suggests that they may be rather common in the UK.

We also identified other mechanisms of cefoperazone resistance among AmpC hyper-producers that have not been previously noticed. In one farm isolate studied, we identified a MarR mutation, which is predicted to cause over-expression of the transcriptional activator MarA (235), and we confirmed that this leads to AcrAB-TolC efflux pump over-production and downregulation of OmpF. Other mechanisms of cefoperazone resistance were identified in AmpC hyperproducer *E. coli*, as discussed in **chapter 4**.

Following the completion of the work reported in **chapter 3**, the focus then moved to the characterisation of 20 putative AmpC-hyperproducing 3GCR *E. coli* from human urinary isolates. Survey data confirmed that unlike the situation in cattle, AmpC hyper-production is a rare (<5%) in 3GCR *E. coli*. The explanation for this is not entirely clear, but it may be that CTX-M (accounding for almost 85% of 3GCR) being plasmid mediated, occurs alongside other resistance genes, and so these plasmids provide a wider spectrum of resistance than AmpC hyper-production, and humans may generally be exposed to a wider range of antibacterial drugs. However, this is speculative.

Importantly, the urine samples were collected from the same geographical range as the 25 farms from which the AmpC-hyperproducing cattle *E. coli* were obtained. As mentioned above, AmpC hyperproduction was due to different *ampC* promoter and attenuator mutations, though the dominance of the promoter mutation was seen in all 25 farm isolates was also seen among human urinary isoaltes (11/20 isolates). Given the range of promoter/attenuator mutations seen, we attempted to measure the impact of each on AmpC production using LC-MS/MS, where the higher AmpC-producing isolates were found to have either the same mutations as that of farm mutations or a combination of different mutations that contribute to the overall level of AmpC production. Unfortunately for the mutant types only appearing in

our collection once, it is possible that the AmpC abundance measured is potentially being affected by some other factor, so it is premature to make any generalised conclusions about the abundance of AmpC and the type of mutation, for example to create rules necessary to allow prediction of the level of AmpC production (and its effect on cephalosporin MICs) directly from WGS data. So further work is needed with a broader collection of isolates.

Indeed, even measuring AmpC abundance levels might not give a single path to predicting cephalosporin MICs. In chapters 3 and 4, it was documented that although AmpC hyperproduction was important for 3GCR, it was not the sole cause of resistance to all 3GCs. For example, as already mentioned, cefoperazone resistance was affected by the combination of AmpC hyperproduction and AcrAB-TolC hyperproduction, or where the overproduced AmpC had an expanded-spectum mutation. In the urinary isoltes, we confirmed that certain levels of AmpC hyperproduction, requiring mutations in addition to those seen among the farm isolates, were associated with cefoperazone resistance, as was the combination of AmpC hyperproduction and TEM-1 hyperproduction. There remains the possibility OmpC downregulation is required here, and this needs to be confirmed by constructing a selected series of mutants. The overall aim of studies such as this is to generate a series of rules that will allow prediction of cephalosporin MIC and the work reported here takes us further down the path necessary to achieve that objective.

Finally in **chapter 4**, the possible transmission of AmpC hyperproducing *E. coli* isolates between dairy farms and humans was examined. WGS and the following core genome SNP analysis confirmed very recent transmission between farm but there was no evidence for the transmission of AmpC hyperproducers between cattle and humans. Hence, controlling the on-

farm prevalence of AmpC-hyperproducing *E. coli* is an important aim to reduce the likelihood of difficult-to-treat infections in cattle rather than any direct zoonotic threat.

Before the identification of amoxicillin/clavulanate as the main driver of the increased prevalence of AmpC hyperproducer *E. coli* on dairy farms (**chapter 3**), several attempts were made to identify possible selective agents *in vitro*. Identifying a large number of 3GCR, AmpC hyperproducer isolates on farms led to the initial hypothesis that this mechanism was driven by cephalosporin usage. Hence, attempts to select resistant *E. coli in vitro* were made using different generations of cephalosporins including ceftiofur, because it is the main 3GC used for the treatment of dairy cows. Our aim was to see if AmpC hyperproducers emerged following this selection. However, it was not possible to select ceftiofur resistance directly from cephalosporin susceptible *E. coli* strains, and those mutants selected where the ceftiofur MIC was elevated did not show an elevation in AmpC production (data not shown). Subsequently, in **chapter 5**, resistant mutants were selected using cefalexin (a 1st generation cephalosporin widely used in veterinary medicine, including on the farms under study, and in humans). Again, we were surprised to find that AmpC hyperproduction was not detected in cefalexin resistant mutants, and we set out to understand the mechanism of cefalexin resistance since cefalexin resistance among *E. coli* was widely detected in the farms under study. Notably, also only 30% of cefalexin resistant isolates found on the farms were also 3GCR, again suggesting that cefalexin resistance is caused by a different mechanism. This finding led to important new understandings about cephalexin-resistance and about the link between envelope stress and permeability to antibacterials, as described in **chapter 5**.

LC-MS/MS proteomics showed that there was no increase in AmpC production in cefalexin resistant mutants and Farm/UTIs isolates, but the OmpF porin was downregulated/disrupted

in all mutants and isolates. The further analysis gave strong evidence that OmpF loss or downregulation is a key mechanism of cefalexin resistance in *E. coli* in the absence of cephalosporinase (hyper)production. OmpF insertional inactivation was shown to cefalexin resistance in mutants constructed in the laboratory. Furthermore, OmpF loss was observed in the two cefalexin resistant farm isolates which was associated with loss-of-function mutations within *ompF*, whereas OmpF downregulation in the three cefalexin resistant mutants and the two UTI isolates did not came from mutations within or close to *ompF*. One cefalexin resistant mutant was found to have an OmpR mutation resulting in OmpF (and OmpC) downregulation. OmpR is a member of OmpR/EnvZ two-component system that controls porin gene transcription in *E. coli* so this finding was not particularly surprising (208). In the other two cefalexin resistant mutants, OmpR was intact, suggesting alternative, novel, regulatory mutations. By a process of whole genome pairwise analysis, comparing parent with mutant sequence, mutations in *rseA* and *gmhB* were identified, respectively in each of the remaining two mutants. Both mutations were associated using proteomics with Sigma E mediated DegP-overproduction, therefore leading to degradation of OmpF, and so cefalexin resistance, and also degradation of OmpC to varying degrees. The roles of these *rseA* and *gmhB* mutations were confirmed by gene knockout *in vitro,* and the generated mutants were cefalexin resistant. Whilst it has previously been shown that loss of *rseA* activates Sigma E in the laboratory (214), we also identified genome sequences in the NCBI database suggesting that *rseA* loss of function mutants are also found in a clinical and environmental setting. Furthermore, whilst *gmhB* mutation has been associated with disruption of lipopolysaccharide and so envelope structure, and it is known that such changes can activate Sigma E, this was the first report that *gmhB* loss of function can induce this phenotype, and again we found evidence of such mutants in clinical and environmental strains.

DegP was also upregulated in one of the cefalexin resistant UTI isolates having OmpF downregulation, suggestive of activation of sigma E response although *rseA* and *gmhB* were intact. Remarkably, a mutation in *lpxB* was found which may alter its activity and affect lipopolysaccharide biosynthesis, therefore activating Sigma E. This still needs to be experimentally confirmed. Because lpxB is an essential gene, it cannot simply be knocked out, as were the other genes where mutations were located in this study. A method needs to be established to allow trans-complementation of *lpxB*, or perhaps we could use CRISPR to make the specific nucleotide sequence changes found in the UTI isolate in a laboratory strain to test the effect. Unfortunately, however, there was no time to perform these experiments, and this part of the work remains incomplete. Nonetheless, this study provides strong evidence that mutations that activate Sigma E can be tolerated by *E. coli* and offer clinically relevant cefalexin resistance in the absence of β-lactamase production through DegP-mediated OmpF proteolysis. This therefore opens up a whole area of research associating mutations in the very many different envelope and lipopolysaccharide biosynthesis genes with porin downregulation and reduced antibacterial penetration. This level of detail will be important for correctly predicting antibacterial resistance from WGS data, which is a clinical aspiration to provide rapid susceptibility data.

It was also shown in **chapter 5** that *ompF* loss or downregulation (through Sigma E activation) can slightly increase MICs of 4GCs againt E. *coli*-hyperproducing AmpC, though mutations within AmpC such as His296Pro, which lead to expanding the activity of AmpC, had a much greater impact on 4GC MIC, and were actually additive to the effect of OmpF loss/downregulation. Hence this led us to consider if OmpF loss/downregulation, including that occurs alongside OmpC downregulation might enhance the activity of CTX-M or AmpC type β-lactamases with respect to carbapenem MICs.

In **chapter 6**, therefore mechanisms causing ertapenem resistance in cephalosporinase producing *E. coli* were investigated. This was achieved by selecting ertapenem-resistant mutants from an expanded spectrum AmpC *E. coli* hyperproducer, and also by introducing CTX-M encoding plasmids via conjugation into cefalexin resistant *E. coli* isolates/ mutants reported in **chapter 5** with downregulated or disrupted OmpF.

The selected ertapenem resistant mutant (hyperproducing the expanded spectrum AmpC) was found to carry a loss of function mutation in *ompC*. Cefalexin resistant mutants/isolates having loss/downregulation of OmpF, engineered to carry CTX-M-15 showed ertapenem non-susceptibility, but only when the mutation they carried reduced OmpC abundance (e.g. in the OmpR mutant mentioned in **chapter 5**). Mutations causing moderate OmpC downregulation (*resA* mutation which activates Sigma E) slightly increased ertapenem MIC but mutants remained susceptible. Therefore, we conclude that OmpF downregulation/loss is not associated with ertapenem resistance in cephalosporinase producers, and that OmpC loss or downregulation is key for this, which fits with previous work (234). Furthermore, we can conclude that this effect was limited to ertapenem, and CTX-M-14 did not give the same effect. Again, these findings will help in the shift towards the routine use of WGS as a tool for antibacterial susceptibility testing, which is receiving some support (236) though it is acknowledge that current complexities in the prediction of genotype-phenotype relationships, particularly when gene expression is the key for the actual phenotype seen, limit the current applicability of WGS analysis as a predictive tool (237). Our use of a combination of WGS and whole-cell proteomics allows for monitoring the effect of different mutations seen in WGS data on the expression of resistance genes. For example, proteomics allows the identification of changes in protein abundance, associated with enhancing resistance either by hyperproduction of AmpC due to different mutations in the promoter region, or by envelope

permeability reduction. In this case, the overproduction of AcrAB and TolC were due to the mutation in MarR as well as the reduction in the OmpF and OmpC porins due to mutations in OmpR or ResA or GmhB. Ultimately, all the mutations that cause these subtle changes in permebility protein abundance will be mapped, and prediction of resistance based on WGS will be more universibly applicable.

In summary, this project revealed clinically relevant and important information about the mechanisms of resistance used by *E. coli* to resist cephalosporins and ertapenem. Understanding these underlying mechanisms, will help us better predict antibacterial susceptibility from WGS, and the identification of a link between lipopolysaccharide biosynthesis and porin abundance, opens significant new ground in our understanding of antibacterial drug resistance in *E. coli*, and likely in other related Gram-negative bacteria.

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