Beta-Lactamase Resistance Harboured by *Escherichia coli* isolated from a Dairy Farm

Charlotte Gray-Hammerton BSc. (Hons)

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Abstract

CTX-M beta-lactamases have become one of the most prevalent extended spectrum beta-lactamases (ESBL) globally. Their association with mobile elements such as IS*Ecp1*, has allowed for their capture and mobilisation within both human and animal environments. The EVAL farms research programme generated a collection of 1,000 *Escherichia coli* bovine faecal samples isolated from the environment of a dairy farm on selective media and characterised phenotypically for antibiotic resistance via the disc diffusion method. This current study further characterised the antibiotic resistances of 86 *E. coli* from the EVAL farms collection, chosen for their beta-lactamase type resistance phenotype. These 86 isolates were grouped according to their resistance phenotypes, which included those suspected of encoding $bla_{\text{CTX-M}}$ (reduced susceptibility to ampicillin (AMP), cefotaxime (CTX) and aztreonam (ATM) but susceptibility to amoxicillin-clavulanic acid (AMC) and cefoxitin (FOX)) and those suspected of overexpressing *ampC* (reduced susceptibility to AMP, AMC, FOX, CTX and ATM. Confirmation of the presence of $bla_{\text{CTX-M}}$ by PCR resulted in 39 bla_{CTX} strains being identified and the remaining 47 isolates categorised as putative *ampC* strains. The presence of an IS*Ecp1* element was also shown in all bla_{CTX} PCR-positive isolates. The 86 isolates were further characterised phenotypically using minimum inhibitory concentration (MIC) assays via the agar dilution method, with an extended panel of 25 antibiotics which included cefquinome (CFQ). The extended panel included any classes of antibiotics that could cover different resistance mechanisms such as carbapenemases, ESBLs as well as aminoglycoside, tetracycline and colistin resistance. High levels of resistance were seen to AMP, CTX, cefpodoxime

(CPD) and CFQ along with resistance to ceftazidime (CAZ), ATM and tetracycline (TET) in the *bla*$_{CTX}$ isolates.

Genotypic characterisation via whole genome sequencing (WGS) was conducted via both short read Illumina and long read MinION Oxford Nanopore Technologies (ONT), with hybrid assembly on all isolates. This WGS was able to show that all the *bla*$_{CTX-M}$ were of *bla*$_{CTX-M-15}$ type, were chromosomally encoded and in association with IS*Ecp1*. WGS also revealed the IS*Ecp1* element additionally encoded *qnrS1* in all isolates and *tetAR* in 34 of the isolates, with 4 found to contain no *tetAR* genes and one that had the *tetAR* genes located separately from the IS*Ecp1* element, in a different region of the genome.

Subsequent *in silico* multi-locus sequence typing (MLST) using the MLST finder from the Centre for Genomic Epidemiology (CGE), showed all within the *bla*$_{CTX}$ group to be ST2325. Comparison of the 39 isolates in the *bla*$_{CTX}$ group by single nucleotide polymorphism (SNP) analysis with 105 ST2325 isolates from the Enterobase database, produced a maximum likelihood tree, showing that the 39 *bla*$_{CTX}$ EVAL farms isolates were part of their own clonal branch of the tree and within 1-5 SNPs of each other, demonstrating that spread of *bla*$_{CTX-M-15}$ on this dairy farm was likely as a result of clonal expansion. Other ST2325 isolates from Enterobase were found not to be closely related to the 39 *bla*$_{CTX}$ isolates, with the closest an *E. coli* bovine isolate from Spain which was within 36-45 SNPs of the 39 *bla*$_{CTX}$ isolates. It was also noted that ST2325 isolates from other published studies analysed from Enterobase appeared to form separate clonal study-associated clusters, with clonal groups of ST2325 within 0-6 SNPs of each other.

The use of WGS allowed resistance genes to be identified and compared to the phenotypic data, and plasmids and other mobile elements including the IS*Ecp1* elements to be characterised. The potential for IS*Ecp1* to mobilise a chromosomally-encoded $bla_{\text{CTX-M-15}}$ to a resident plasmid and transpose to another strain and whether sub-lethal levels of antibiotics used in dairy farming (including AMP, cloxacillin (CLOX) and CAZ) might enhance this transposition of IS*Ecp1*, was addressed through transposition experiments with four isolates from the bla_{CTX} group. The sub-lethal levels of these antibiotics used, looked to mimic the concentrations that might be encountered by bacteria of treated animals or within the environment of the dairy farm.

Transposition of the IS*Ecp1* element in association with $bla_{\text{CTX-M-15}}$, was successful with all concentrations of AMP, CLOX and CAZ and enhanced transposition with an increased rate of transfer (when compared to the baseline rate of transfer in non-selective media) was successful with some concentrations of AMP, CLOX and CAZ. Levels of enhancement varied from 1.07 fold to 45 fold the baseline rate. The characterisation of subsequent transconjugants encoding IS*Ecp1* elements using WGS via both Illumina short and MinION (ONT) long read with hybrid assembly showed that the IS*Ecp1* elements could either lose or gain downstream genes, through the recognition of a new imperfect IR_R site. This revealed a possible mechanism for the loss or gain of a phenotype within the dairy farm *E. coli* isolates.

Isolates of the second group of *E. coli* displaying a beta-lactamase type phenotype, the *ampC* group, when further characterised through MIC assays showed that many phenotypic resistances indicated by the disc assay were lost and only four of the 47 isolates in the *ampC* group were resistant to

streptomycin (STREP), six were resistant to TET and only one isolate was resistant to trimethoprim/sulfamethoxazole combination (SXT). Only 22 isolates were showing likely overexpression of *ampC* according to the MIC assay results, with high level resistance to AMP along with resistance to CAZ, CPD and ATM with intermediate resistance to CTX.

Through a combination of both PCR and Sanger sequencing, and WGS, 22 isolates were confirmed as overexpressing *ampC* by locating mutations in the promoter regions of *ampC*. WGS of the 47 isolates in the *ampC* group identified additional resistance genes including beta-lactamase type resistance genes $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ in one isolate each respectively, mobile genetic elements (MGEs) and a small number of virulence genes. MLST typing showed, 21 isolates were ST1308 with 20 of these overexpressing *ampC*. These 21 ST1308 were subject to SNP analysis via snippy and a maximum likelihood tree was constructed. From these data it appeared the earliest sampled isolate, which was not overexpressing *ampC*, was within 431-561 SNPs of the remaining 20 ST1308 isolates and therefore was not closely related but did appear to share a common ancestor them. Within the remaining 20, 19 were within 5-1 SNPs of each other and one was within 8-16 SNPs of those 19. Therefore, it appeared the 19 were likely clonal and the 1 within 8-16 SNPs appeared to be very closely related to those 19. This suggested that in this particular dairy farm environment, there had been a small clonal expansion of isolates of the same ST, with the majority also encoding overexpression of *ampC.*

The results of this study showed a potential mechanism for mobility of $bla_{\text{CTX-M-15}}$ within the environment of a dairy farm, demonstrated there had been spread of $bla_{\text{CTX-M-15}}$ as a result of the clonal expansion of ST2325 and also

revealed several different mechanisms in place for beta-lactamase type resistance including both *bla*$_{\text{CTX-M-15}}$ and overexpression of *ampC*. The study also showed the benefits of utilising both phenotypic and genotypic methods together for the identification of resistance mechanisms within *E. coli*.

CONFERENCES AND PUBLICATIONS

SfAM ECS Research Symposium "*Do large low copy number plasmids play a significant role in the carriage of multi-drug resistance within E. coli from commercial animals?"* University of Westminster, London on 19th April 2017, oral presentation

SfAM AMR Looking beyond the microbiological conference "*Dissemination of an ESBL bla$_{CTX-M-15}$ determinant associated with the mobile element ISEcp1 within Escherichia coli isolated from a dairy farm."* London on 14th November 2018, poster presentation

SfAM ECS Research Symposium *"Antibiotic Resistance: What's on your dinner table?"* Manchester Conference Centre on 13th March 2019, oral presentation

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"***Psalms 37:5 Commit thy way unto the Lord; trust also in him; and he shall bring it to pass***"

"***Psalms 28:7 The Lord is my strength and my shield; my heart trusted in him, and I am helped: therefore my heart greatly rejoiceth; and with my song will I praise him***"

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INDEX OF ABBREVIATIONS

°C	Degrees Centigrade
A. baumannii	*Acinetobacter baumannii*
AAC	Aminoglycoside N-Acetyltransferases
Abbrv.	Abbreviation
AGISAR	Advisory group on Integrated Surveillance of Antimicrobial Resistance
AIEC	Adherent-Invasive *E. coli*
AMC	Amoxicillin Clavulanic Acid
AMP	Ampicillin
AMR	Antimicrobial Resistance
ANT	Aminoglycoside O-Adenyltransferases
APEC	Avian Pathogenic *E. coli*
APH	Aminoglycoside O-Phosphotransferases
ARG	Antimicrobial Resistance Gene
AST	Antibiotic Susceptibility Testing
AST	Antimicrobial Susceptibility Testing
AT	Adenosine Thymine
ATCC	American Type Culture Collection
ATM	Aztreonam
ATP	Adenosine Triphosphate
AUS	Australia
AZM	Aztreonam
B. fragilis	*Bacteroides fragilis*

B. subtilis	*Bacillus subtilis*
BC	Bactericidal
BHSSC	Bulling Heifer Shed Scraper Channel
bla	beta-lactamase
BLAST	Basic Local Alignment Search Tool
blastn	BLAST nucleotide
blastp	BLAST protein
BMRG	Biocide/Metal Resistance Genes
bp	base pair
BS	Bacteriostatic
C. freundii	*Citrobacter freundii*
CA	CHROMagar ESBL
CAN	Canada
CAZ	Ceftazidime
CCFA	Ceftiofur Crystalline Free Acid
CDC	Centres for Disease Control
CDS	Coding Sequence
CFQ	Cefquinome
CFU	Colony Forming Units
CGE	Centre for Genomic Epidemiology
CHLOR	Chloramphenicol
CIA	Critically Important Antimicrobials
CIP	Ciprofloxacin
CL	Containment Level
CLOX	Cloxacillin

CLSI	Clinical and Laboratory Standards Institute
CM	Cell Membrane
COL	Colistin
Concn	Concentration
CO_{WT}	Wild Type Cut Off
CPD	Cefpodoxime
CPE	Carbapenemase Producing Enterobacteriaceae
CRE	Carbapenem Resistant Enterobacteriaceae
CTC	Chlortetracycline
CTX	Cefotaxime
CVM	Center for Veterinary Medicine
DAEC	Diffusely-Adherent *E. coli*
DEC	Diarrheagenic *E. coli*
DEFRA	Department for Environment, Food and Rural Affairs
DLO	Dairy Lane Outside
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
DR	Direct Repeat
DSSC	Dairy Shed Scraper Channel
DT90	Degradation Time for 90%
E. aerogenes	*Enterobacter aerogenes*
E. casseliflavus	*Enterococcus casseliflavus*
E. casseliflavus	*Enterococcus casseliflavus*

E. cloacae	*Enterobacter cloacae*
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
E. durans	*Enterococcus durans*
E. faecalis	*Enterococcus faecalis*
E. gallinarum	*Enterococcus gallinarum*
E. hirae	*Enterococcus hirae*
EAEC	Enteroaggregative *Escherichia coli*
EAST1	EAEC Heat-Stable Enterotoxin 1
ECOFF	Epidemiological Cut Off
EDTA	Ethylenediaminetetraacetic Acid
EFT	Ceftiofur
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ENR	Enrofloxacin
EPEC	Enteropathogenic *E. coli*
Eq	Equation
ESACs	Extended Spectrum AmpC Beta-Lactamases
ESBL	Extended Spectrum Beta-Lactamase
ETEC	Enterotoxigenic *E. coli*
ETOH	Ethanol
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing

EVAL-Farms	Evaluating the Threat of Antimicrobial Resistance in Agricultural Manures and Slurries
ExPEC	Extraintestinal *E. coli*
FAO	Food and Agriculture Organisation of the United Nations
FDA	Food and Drug Administration
FOX	Cefoxitin
FPAs	Food Producing Animals
Fwd	Forward
g	gram(s)
GAP	Global Action Plan
gbk	GenBank
GC	Guanine Cytosine
gDNA	Whole Genome DNA
GENT	Gentamicin
GER	Germany
gff	General Feature Format
GFP	Green Fluorescent Protein
HCl	Hydrochloric Acid
HGT	Horizontal Gene Transfer
HP	Hypothetical Protein
HPLC	High Pressure Liquid Chromatography
HS	Heifer Shed
HS1	Heifer Shed 1 (older cows)
HUS	Haemolytic Uremic Syndrome

IMP	Imipenem
INT	Intermediate
IR	Inverted Repeat
IR_L	Inverted Repeat Left
IR_R	Inverted Repeat Right
IS	Insertion Sequence
K. ascorbata	*Kluyvera ascorbata*
K. cryocrescens	*Kluyvera cryocrescens*
K. georgiana	*Kluyvera georgiana*
K. oxytoca	*Klebsiella oxytoca*
K. pneumoniae	*Klebsiella pneumoniae*
KAN	Kanamycin
kb	kilobase
kg	kilogram
KPC	*Klebsiella pneumoniae* carbapenemase
L	Litre(s)
LB	Luria-Bertani
LEE	Locus of Enterocyte Effacement
Lft	Left
LMICs	Low Middle Income Countries
LOD	Limit of Detection
LT	Heat Labile Toxin
LUX	Luxembourg
M	molar
m	mole

Mac	MacConkey Agar
Mb	Megabase
MER	Meropenem
mg	milligram(s)
$MgCl_2$	Magnesium Chloride
MGE	Mobile Genetic Element
MH	Mueller Hinton
MHE	Muck Heap Effluent
MIC	Minimum Inhibitory Concentration
ml	millilitre(s)
MLST	Multi Locus Sequence Typing
mm	Millimetre
mM	Millimolar
MPI	Multi-Pin Inoculator
MRD	Maximum Recovery Diluent
MRSA	Methicillin resistance *Staphylococcus aureus*
n/a	Not Applicable
n/s	Non-Selective
NaCl	Sodium Chloride
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NAL	Nalidixic Acid
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
ND	Non-Defined
NDM	New-Delhi Metallo-Beta-lactamase

NDtc	Non-Detectable
NEB	New England Biolabs
NEO	Neomycin
NERC	Natural Environment Research Council
ng	nanogram
NIT	Nitrofurantoin
nl	nanolitre
nm	nanometre
nM	nanomolar
NMEC	Neonatal Meningitis-causing *E. coli*
No.	Number
NTL	Netherlands
NTS	Non-Type Specific
NZ	New Zealand
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
OIE	Office International des Epizooties
OM	Outer Membrane
ONT	Oxford Nanopore Technologies
ORF	Open Reading Frame
P	Potency
P. aeruginosa	*Pseudomonas aeruginosa*
PacBio	Pacific Biosciences
PBS	Phosphate-Buffered Saline

PCR	Polymerase Chain Reaction
PD	Pharmacodynamic
pg	picogram
pH	potential hydrogen
PK	Pharmacokinetic
pmol	picomolar
ppb	part per billion
psi	Pound per Square Inch
QAC	Quaternary Ammonium Compounds
QD	Quinupristin-Dalfopristin
qPCR	Quantitative Polymerase Chain Reaction
QRDR	Quinolone Resistance-Determining Region
Ref	Reference
RES	Resistant
RNA	Ribonucleic Acid
RO	Reverse Osmosis
RPM	Revolutions Per Minute
Rvs	Reverse
SA	South Africa
S. aureus	*Staphylococcus aureus*
S. dysenteriae	*Shigella dysenteriae*
S. enterica	*Salmonella enterica*
S. flexneri	*Shigella flexneri*
S. fradiae	*Streptomyces fradiae*
S. griseus	*Streptomyces griseus*

S. kanamyceticus	*Streptomyces kanamyceticus*
S. marcescens	*Serratia marcescens*
S. rimosus	*Streptomyces rimosus*
S. venezuelae	*Streptomyces venezuelae*
S/I/R	Susceptible/Intermediate/Resistant
S3	Sulphonamides
SEPEC	Human Sepsis-Associated *E. coli*
SGP	Singapore
SHV	Sulphydryl Variable
SI	Super Integron
SNP	Single Nucleotide Polymorphism
SPRI	Solid Phase Reversible Immobilization
SS	Slurry Solids
ST	Sequence Type
ST	Slurry Tank
STb	Heat-Stable Toxin
STEC	Shiga Toxin-Producing *E. coli*
STREP	Streptomycin
SUS	Susceptible
SXT	Trimethoprim-Sulfamethoxazole
T3SS	Type III Secretion System
T4SS	Type IV Secretion System
TA	Toxin-Antitoxin
TAE	Tris(hydroxymethyl)aminomethane Acetic Acid Ethylenediaminetetraacetic Acid

TBX	Tryptone Bile X-Glucuronide Agar
TCA	Tricarboxylic Acid Cycle
TE	Tris(hydroxymethyl)aminomethane Ethylenediaminetetraacetic Acid
TET	Tetracycline
TIG	Tigecycline
T_m	Melting Temperature
Tn	Transposon
TOB	Tobramycin
Tris	Tris(hydroxymethyl)aminomethane
TT	Transposition Transconjugant
U	Units
UDP	Uridine Diphosphate
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
UR	Underground Reservoir
US	United States
UV	Ultraviolet
v	Version
V	Volts
V. cholerae	*Vibrio cholerae*
VRE	Vancomycin-resistant enterococci
VTEC	Verotoxigenic *E. coli*
W	Weight
WGS	Whole Genome Sequencing

WHO	World Health Organisation
WOAH	World Organisation for Animal Health
WT	Wild Type
X	Times
x g	times gravity
µg	microgram
µl	microlitre(s)
µM	micromol

CHAPTER 1

INTRODUCTION

“One sometimes finds what one is not looking for.”

Alexander Fleming 1928 (Wennergren and Lagercrantz 2007)

“The thoughtless person playing with penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism.”

Alexander Fleming 1945 (Sillankorva et al. 2019)

1.1. ANTIBIOTIC DISCOVERY

During the "golden era" of antibiotic discovery from 1930s – 1960s, it was thought the fight against infectious diseases caused by bacteria was finally won. The landmark scientific discovery of penicillin was made in 1929 and by 1945 it was being mass produced and distributed, particularly as a treatment during World War 2 (Gaynes 2017). Penicillin's success was followed by the discovery of streptomycin in 1943 (Schatz et al. 1944) which was first administered to a patient to treat tuberculosis in 1944 (Murray et al. 2015), tetracycline in 1945 (Duggar 1948) which was first used in 1948, to treat an infection in a 5 year old patient caused by a ruptured appendix (Nelson and Levy 2011a; Ramachanderan and Schaefer 2021) and chloramphenicol in 1947 (Ehrlich et al. 1947) with its first use in medicine in 1949 (Aronoff 2019). The discoveries of antibiotics resulted in many previously untreatable infectious diseases becoming treatable, leading to a worldwide reduction in morbidity and mortality as a result of bacterial infections (Davies 2006). However, the increasing usage of antibiotics, both for therapeutic purposes and then subsequently their usage within animals for growth promotion, led to the appearance of antibiotic resistance and resistant pathogenic bacteria (Davies 2006; C Reygaert 2018). Concerns regarding resistance were reported even before the first antibiotics were brought into clinical practice; even so antibiotics were seen as "wonder drugs" and used extensively in both human and veterinary medicine (Abraham and Chain 1940b; Davies and Davies 2010; Moellering 2010; Nathan and Cars 2014; Martens and Demain 2017; Aslam et al. 2018). Indeed, Alexander Fleming and Howard Walter Florey both warned of the risks

of over usage of penicillin that could result in resistance at their 1945 Nobel Prize acceptance (Nathan and Cars 2014).

Another class of drugs that have lacked attention since their discovery, after they were overshadowed by penicillin's introduction, are the sulphonamides (Davenport 2012). The sulphonamides were developed in Germany in the early 1930s by IG Farben (Wood 1996). The original metabolite, sulphanilamide, was first tested in Britain in 1937 by Leonard Colebrook, who found it was successful against puerperal fever (Dunn 2008). Subsequent research in England by May and Baker, resulted in derivatives of the original metabolite sulphanilamide also being developed including sulfapyridine (MB-693) (Gaudillière 2009), which in 1943 was used to cure pneumonia in Winston Churchill (Davenport 2012).

Sulphonamides were widely and successfully used during WWII for common ailments associated with the battlefield such as infected wounds, gas gangrene and dysentery, that during WWI had no effective treatment options. Another important usage during WWII was against the infection gonorrhoea, but eventually sulphonamide application was phased out in favour of penicillin for gonorrhoea management. However, the widespread usage during WWII of sulphonamides resulted in the development of resistance and this was thought to have been hastened through their usage as both prophylactics but also through self-medication by both soldiers and civilians (Davenport 2012).

Since the initial boom of antibiotic discovery, the advent of new drugs has dwindled due to lack of interest from pharmaceutical companies who see antibiotic drugs as having reduced economic incentives, due to the likelihood of resistance appearing soon after the introduction of the drug (Gould and Bal 2013;

Wright 2014; Ventola 2015). In addition, any new antibiotic introduced that has a unique mode of action, is also likely to be reserved as a drug of last resort, creating a further disincentive for investment (Hutchings et al. 2019). The antibiotic resistance crisis is attributed to the over usage of antibiotics both in human and veterinary medicine, as well as their usage in farming for the purposes of growth promotion, and the lack of new antibiotics coming to market (Nathan 2004; Davies and Davies 2010; van Boeckel et al. 2015a; Aslam et al. 2018).

1.1.1. Antibiotic Discovery and The Discovery Void

In 1910 a breakthrough in the fight against the disease syphilis caused by the bacterium *Treponema pallidum* was made by Paul Ehrlich, through his theory that a "magic bullet" could be found that would target the invading bacteria. Ehrlich and his colleague Sahachiro Hata made the discovery by using the method of testing multiple compounds of arsenic, to eventually find the one that proved successful in a rabbit model and that was compound number 606 later named Salvarsan. Salvarsan was one of the first chemotherapeutic drugs to be discovered (Williams 2009; Mbaba et al. 2022) and continued to be used in the treatment of syphilis until the introduction of the less toxic penicillin (Gelpi et al. 2015; Vernon 2019). This method of empirical screening of compounds has continued since then, with many antibiotic discoveries made in this way (Silver 2011). Of all the antibiotic discoveries made from the mid 1940s until the late 1970s, 55% of these came from the bacteria of the genus *Streptomyces*. It was thought this wealth of antibiotic classes found within this species, was due to the complex soil environment these bacteria inhabited, where competition,

signalling and interaction with their Eukaryotic hosts required the use of these chemical type weapons. In conjunction with antibiotics, these bacteria also contain the protective measures to resist their own and others antibiotic weapons (Seipke et al. 2012; Klassen 2014; Traxler and Kolter 2015; Hutchings et al. 2019).

Within the wider environment of both the soil and also the marine microbiome, there is thought to be a wealth of undiscovered potential new bioactive agents, that may offer new novel agents that could prove beneficial in the fight against antibiotic resistance (Tortorella et al. 2018). An example of this is the marine actinomycete of the genus *Salinospora*, that has been shown to encode several novel bioactive compounds that have demonstrated promise as anti-cancer agents (Gulder and Moore 2010; Ziemert et al. 2014). Another example is *Candidatus* Entotheonella that produces bioactive polyketides and is an as yet uncultivated symbiont of the marine sponge *Theonella swinhoei* (Wilson et al. 2014).

There are thought to be many uncultured bacterial species currently in the environment, that could prove to be home to vital new life-saving antibiotics (Davies 2006; Bérdy 2012). New methods such as isolating soil bacteria through the use of a diffusion chamber and the use of the iChip, have been employed to isolate these hard-to-culture bacteria (Nichols et al. 2010). Through this method in 2012 the new antibacterial peptide Teixobactin was identified from *Eleftheria terrae* (Ling et al. 2015; Gunjal et al. 2020). This discovery showed that the soil microbiome still holds promise as an area for antibiotic discovery.

The World Health Organisation (WHO) (2021) reported there were 27 antibacterial agents in phase 1-3 clinical development, which target WHO

priority pathogens and a further 13 that target *Mycobacterium tuberculosis* and five against *Clostridium difficile*. It was reported by the Pew Trust (2021a), that as of March 2021, there were currently 43 potential new antibiotic candidates in clinical trial stages within the United States (US), including nine new beta-lactam/beta-lactamase inhibitor combinations, four new beta-lactams, three new tetracyclines, three new polymyxins, two new macrolides, two new fluoroquinolones, and a new aminoglycoside, carbapenem, quinolone, pleuromutilin and a distamycin to name a few of them. However, within this group of 43, there are no new classes of antibiotic. There are currently 38 companies involved in the production of the 43 new antibiotics in clinical development and only two of them rank as top pharmaceutical companies, with 70% of those 38 companies classed as pre-revenue with no previous products commercialised, marketed, or developed (PEW Trust 2021b).

Sadly, due to the appearance of antibiotic resistance, the input of Big Pharma to invest in new antibiotic discovery has dwindled since the golden age of discovery that peaked in the 1950s, leading to the discovery void of new antibiotic classes (Hutchings et al. 2019). Since the 1980s no new classes of antibiotics have been discovered (Plackett 2020).

Figure 1.1 details the timeline of discovery of each antibiotic class, the golden age period and also details when important resistance first appeared such as methicillin resistance in *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (adapted from (Hutchings et al. 2019)).

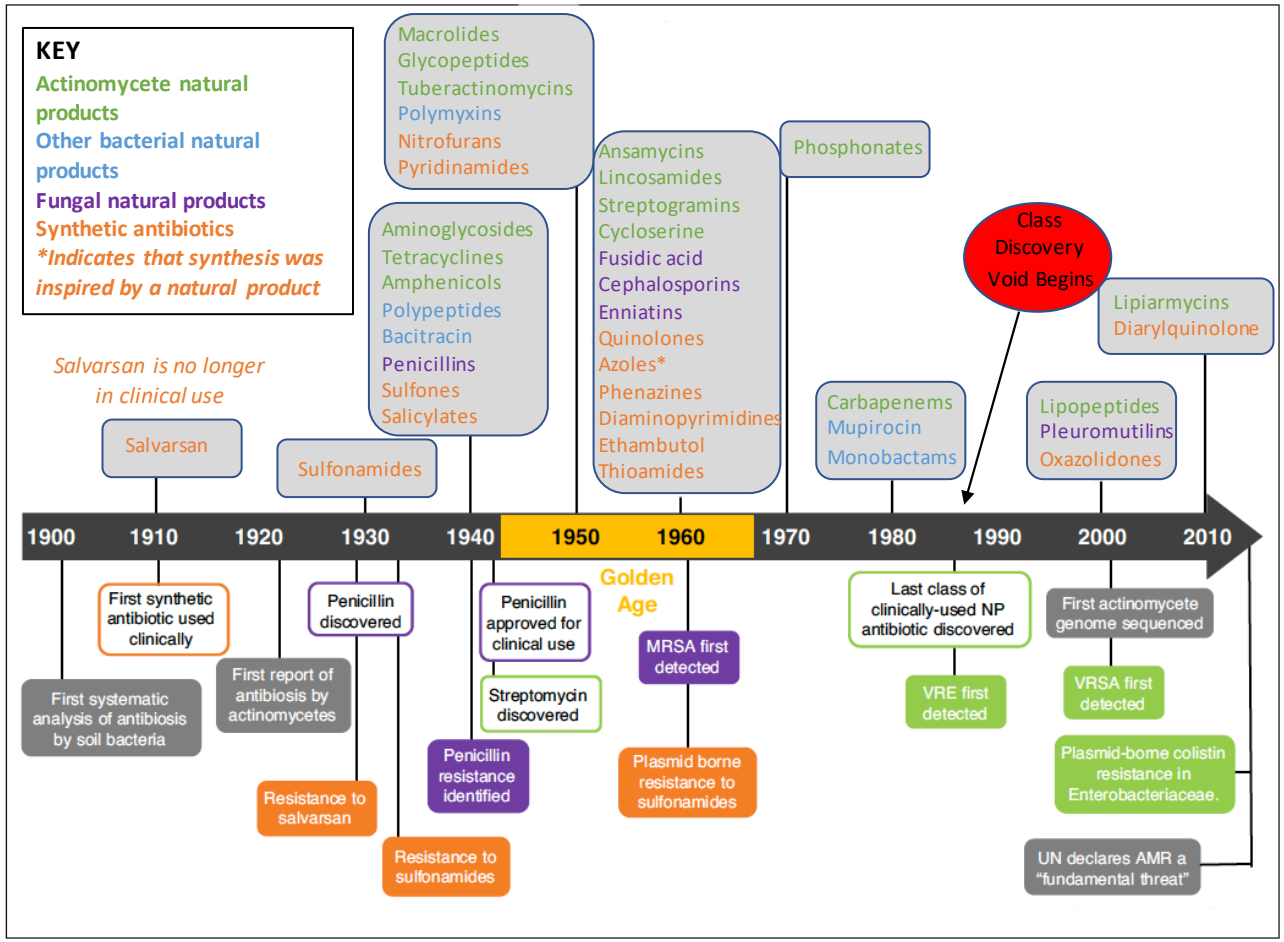


Figure 1.1: Antibiotic discovery timeline adapted from: (Hutchings et al. 2019)

1.1.2. Antibiotic Mechanisms of Action

Antibiotics have several mechanisms of action against bacteria that include different targets or pathways and that can be either bactericidal, in that they kill the bacteria, or bacteriostatic, in that they prevent growth. There are around six main mechanisms of action that include: targeting of cell wall synthesis, inhibition of protein synthesis, inhibition of DNA replication, inhibition of RNA synthesis, folic acid pathway metabolism inhibitors and disruption of the plasma membrane. **Table 1.1** lists the mechanisms of action and the associated antibiotics. **Figure 1.2** details a bacterial cell with the main target sites of antibiotics shown on the diagram, along with the antibiotics associated.

Table 1.1: Antibiotic Mechanisms of Action, Antibiotic Classes and Examples of Antibiotics

Mechanism of Action	**Antibiotic Class**	**Examples of Antibiotics**	**Bactericidal (BC) or Bacteriostatic (BS)**
Targeting Cell Wall Synthesis	Beta-lactams (including penicillins, cephalosporins, monobactams and carbapenems	Ampicillin, cefotaxime, aztreonam, imipenem	BC
	Glycopeptides	Vancomycin, teicoplanin	BC
Inhibit Protein Synthesis (30S Ribosomal Unit)	Aminoglycosides	Streptomycin, Gentamicin	BC
	Tetracyclines	Oxytetracycline	BS
Inhibit Protein Synthesis (50S Ribosomal Unit)	Phenicols	Chloramphenicol	BS
	Macrolides	Erythromycin, azithromycin	BS
	Oxazolidinones	Linezolid	BS

Inhibit DNA Replication	Quinolones and fluoroquinolones	Nalidixic Acid, Ciprofloxacin	BC
Inhibit RNA Synthesis	Rifamycins	Rifampicin	BC
Folic Acid Pathway Metabolism Inhibitors	Sulphonamides (Dihydropteroate Synthase Inhibitor)	Sulphonamide, Sulfamethoxazole	BS
	Dihydrofolate Reductase Inhibitor	Trimethoprim	BS
Disrupt Plasma Membrane	Polymyxins	Colistin	BC

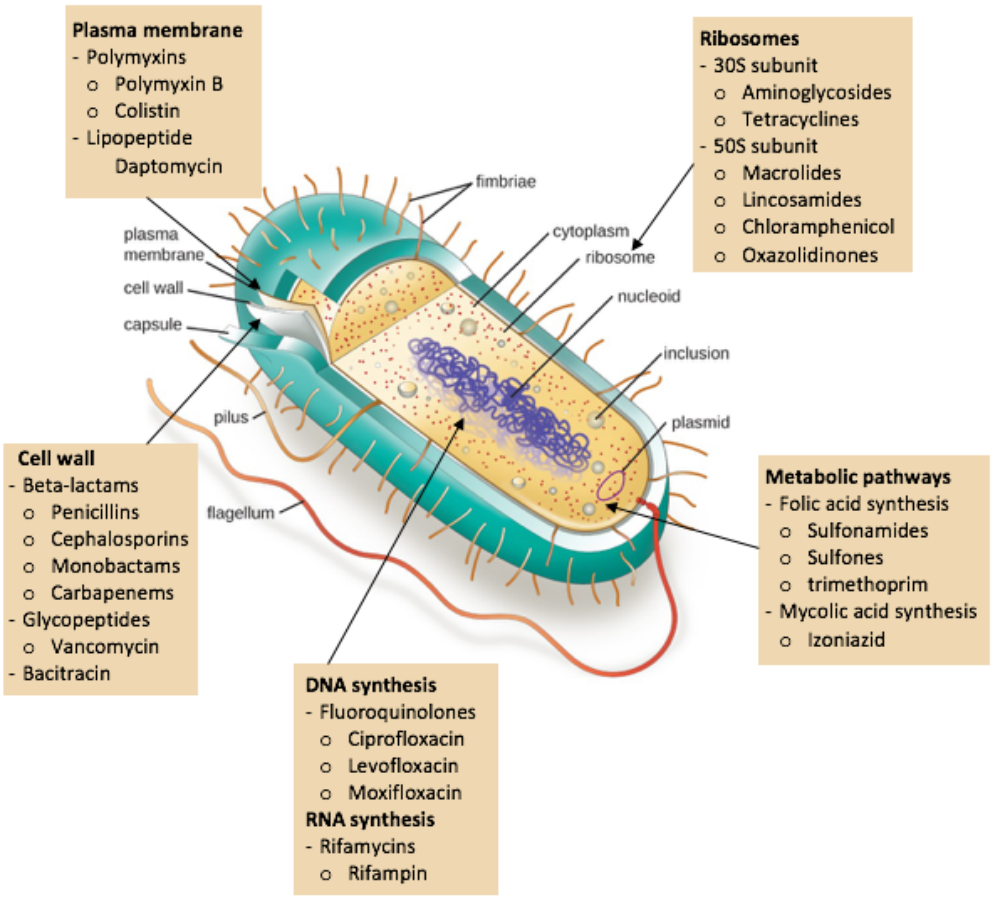


Figure 1.2: Target sites of antibiotics on the bacterial cell. Adapted from: (lumenlearning.com 2021a; 2021b)

1.2. ANTIBIOTIC USAGE IN FARMING

Soon after the introduction of antibiotics to human medicine, they began to also show promise in treating disease in livestock and companion animals, which all began with the introduction of the synthetic sulphonamides in the 1930s. From around 1938 onwards they were marketed for use as therapeutics within animals (Kirchhelle 2018). By the 1950s antibiotics were being used extensively to increase productivity of livestock. The surge of modern commercial farming brought with it a need for greater efficiency and the incorporation of confinement rearing. As a consequence of herds living in large

numbers and close proximity, infection rates and diseases able to spread easily increased (Gustafson and Bowen 1997; Sarmah et al. 2006a; Chattopadhyay 2014; Hedman et al. 2020). Antibiotics offered a solution to the consequences of commercial losses from disease but helped to fuel the global crisis of antibiotic resistance (Maron et al. 2013). It was reported early on in antibiotic history, that the use of antibiotics at sub-therapeutic doses was advantageous for the growth promotion benefits they provided in chickens (Moore et al. 1946) and pigs (Jukes et al. 1950). Today the exact mechanism behind antibiotics as growth promoters is still disputed, with several ideas proposed (Gadde et al. 2017). One of those ideas, is that subtherapeutic doses of antibiotics result in a reduction in the diversity and number of bacteria in the gut microflora, which in turn results in less competition for nutrients within the gut and also a reduction in growth-affecting microbial metabolites (Feighner and Dashkevicz 1987; Gaskins et al. 2002; Knarreborg et al. 2004).

The use of antibiotics as growth promoters however, has been banned throughout the European Union (EU) (Casewell et al. 2003a) and is heavily restricted in the US (FDA 2012b). However, antibiotic use within animals continues to rise in countries such as China, India, Russia, South Africa and Brazil, where meat production is now increasing due to rising incomes. Many of these developing countries where food production is increasing, are less regulated in their antibiotic use and are therefore believed to be fuelling the antibiotic resistance crisis (Maron et al. 2013). Van Boeckel et al. (2015a) produced a study of antibiotic consumption for livestock purposes in 228 countries and estimated that the total consumption in 2010 was around 63,151 tons. With the need for better infectious disease prevention and greater

productivity through the conversion of feed input to produce output, the global use of antimicrobials is estimated to increase by around 67% by 2030 (van Boeckel et al. 2015a; Wyrsch et al. 2016). Despite the growing concern over resistance, antibiotic use within farming is still an essential part of food production. **Figure 1.3** demonstrates the growing demand for food, which has increased since the 1960s and is predicted to increase yet further by 2050, due to the continuing surge in population numbers. In addition, **Figure 1.3** demonstrates meat demand is outpacing cereal demand (adapted from (McLaughlin and Kinzelbach 2015)).

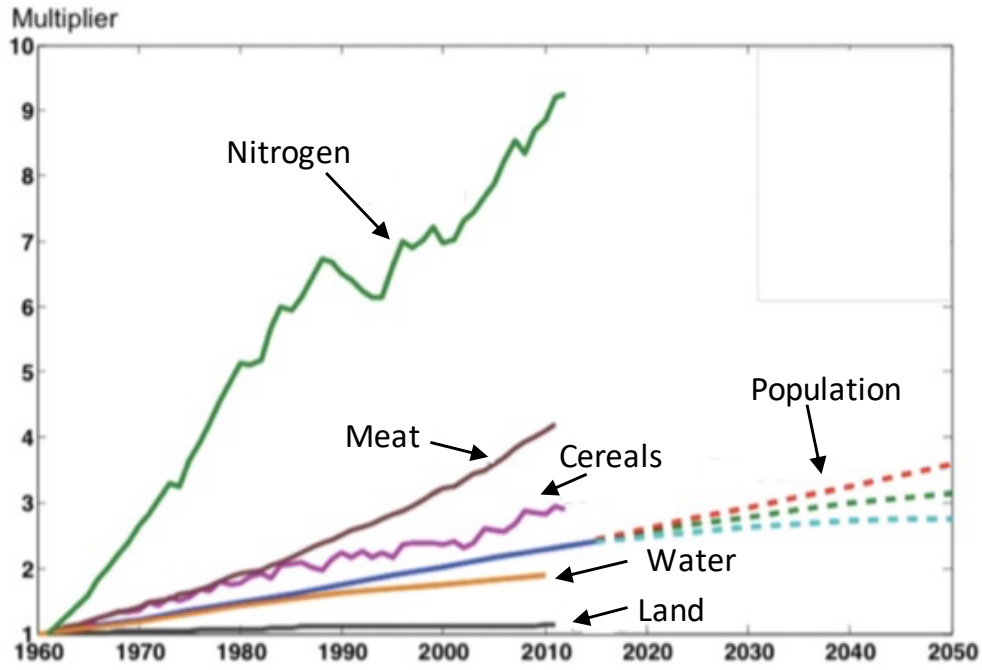


Figure 1.3: Global trends of food production and population growth (population plotted as high, median and low projections on red, green and blue dotted line respectively), along with nitrogen consumption from year 1960 to predictions by 2050. Adapted from (McLaughlin and Kinzelbach 2015)

It is predicted that population numbers will reach an estimated 9.7 billion people by 2050 and 11.2 billion by 2100 (Pandey and Upadhyay 2022a; UNPD 2022). This growth in population would require an increase in food production of 70% to meet the requirements of this greater number of people. Cereal production would need to reach 3 billion tonnes per year up from 2.1 billion tonnes and meat production would need to reach 470 million tonnes per year, an increase of over 200 million tonnes (FAO 2009a). Compared to 50 years ago, we now produce over 3 times the amount of meat, and in 2018 this production was around 340 million tonnes with 80 billion animals per year slaughtered for meat. Milk production has also increased and is now at a production level of around 800 million tonnes each year, which is more than double what was produced 50 years ago (Ritchie and Roser 2017). In 2000, the global cattle and goat & sheep population was 1.5 billion and 1.7 billion respectively. By 2050 the global population of cattle may reach 2.6 billion and sheep and goats may reach 2.7 billion (Thornton 2010; Pandey and Upadhyay 2022a). In the US alone milk production in July 2022 was a total of around 8.3 million tonnes, which was a 0.3% increase in production from July 2021 (USDA 2022b). There has been an increase of 13% over a 10 year period in milk production in the US (USDA 2022c). Global pork, chicken and beef production forecasts for 2022 were at 110.7, 101.0 and 58.7 million tonnes respectively. China in 2022 was the biggest importer of pork worldwide, making up 20% of shipments globally (USDA 2022a) and the EU's annual poultry meat production of 13.4 million tonnes, is one of the largest worldwide (European Commission 2022). With increasing food requirements from population growth, the need to

ensure food security through the prevention of losses from disease, becomes ever more important.

Even though the use of antibiotics as growth promoters has been banned within the EU since 2006 (Castanon 2007), the use of antibiotics as both prophylactics (treatment used to prevent disease occurrence) and metaphylactics (treatment used to treat a whole group of animals, after development of disease within a part of the group, with the aim of disease spread prevention) (Baptiste and Pokludová 2020) still continues (Woolhouse et al. 2015) and is typical for treatment of poultry flocks, where treatment is flock rather than individual based (Agyare et al. 2018; van Cuong et al. 2021; Gray et al. 2021). However, in 2018 new EU legislation was approved that will came into force in 2022, that banned the use of prophylactic antibiotic treatment in FPAs (EU 2018). Antibiotics still require a prescription from a veterinarian, although the decisions to treat individual animals within a herd and the administering of antibiotic medication is often carried out by farm staff, who have no veterinary training. Unfortunately, the cost of continual veterinary attendance often motivates the decision and attitude of farmers to follow their own judgement (Friedman et al. 2007; Landers et al. 2012a; Jones et al. 2015).

The global production of animal-based protein and products is ever increasing to meet the demand and in turn with it comes an increase in antimicrobial use in animals. The demand for poultry in South Asia has an expected increase by 2030 of 725%. Demand by 2030 is anticipated to be greatest in low middle income countries (LMICs) and this increase is seeing a move in methods of production from livestock, mixed crops and small holdings

to large industrialised intensive farming. With this push comes a greater risk for increased antibiotic usage, employed not only for therapeutic usage but sub and non-therapeutic usage for preventative disease control and growth promoter purposes. Even though many countries have banned the usage of growth promoters, it is often hard to enforce or monitor in LMICs (FAO 2009b; FAO 2012; van Boeckel et al. 2015b; Schar et al. 2018). Globally estimates for meat production are projected to grow with an increase of 17% for poultry production, 16% for sheep meat production, 13% for pork production and 6% for beef production by 2030. Income and population growth in developing countries is largely driving these increases in global meat consumption (FAO 2021).

1.2.1. Use of Human Critical Antibiotics in Animals

Both the WHO concerned with human health and the World Organisation for Animal Health which was formerly known as the Office International des Epizooties (OIE), produce lists of those antibiotics which are deemed important to either human or animal health respectively (WHO 2018; OIE 2021). Antibiotics included on these lists, are often referred to as "critically important antimicrobials" and therefore they will be abbreviated to CIA for the purposes of this thesis.

The OIE was founded in 1924 and formally adopted the name World Organisation for Animal Health (WOAH) in 2003 but is still often referred to and published as the OIE, however for the purposes of this thesis in subsequent text, it will be referred to as WOAH. Currently the WOAH includes 182 member states and is focussed on transparency of information relating to animal diseases and global animal health. In 2015, a global action plan (GAP) for antimicrobial resistance was adopted which was developed through the WHO world health

assembly, the World Assembly of WOAH Delegates and the Food and Agriculture Organisation (FAO) conference. WOAH began its data collection of antimicrobial usage in 2015, to detect trends and strengthen antibiotic stewardship and governance and monitoring of antimicrobial usage in animals and AMR (Pinto Ferreira et al. 2022). The WHO in comparison began publishing its CIA list in 2005 and currently has 194 member states (Scott et al. 2019a; Taylor 2021). The WHO's CIA list ranks medically important antimicrobials and monitors the risk of AMR due to non-human use of antimicrobials. The WHO CIA list is updated every 2 years and is reviewed and managed by the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), who monitor and develop programmes of integrated surveillance of AMR in line with the GAP adopted in 2015 (WHO 2017).

The classes of antibiotics listed as the highest priority CIA for human health include the quinolones, cephalosporins of 3rd or higher generation, macrolides and ketolides, glycopeptides and the polymyxins (WHO 2018). Currently there are many antibiotics on both the WHO's list of antibiotics critical to human health (WHO 2018) and the WOAH's list of antimicrobial agents of veterinary importance (OIE 2021b) which are used in both humans and animals and many antibiotics of the same class are used in both humans and animals (Scott et al. 2019a). **Table 1.2** shows the combined WHO and WOAH lists detailing the antibiotics (separated by class) which fall under one of three categories including critical (highlighted in red), highly important (highlighted in orange) and important (highlighted in yellow). As this list was extensive, only the 70 antibiotics that feature on both lists are shown in **Table 1.2** and antibiotic classes which are listed by WHO as highest priority critically important

antimicrobials are highlighted in purple with text in bold white. Antibiotic classes or individual antibiotics within a class, that are deemed not important to human health or that are solely used in animals are detailed in **Table 1.3**. In addition, there are antibiotics that are either banned or restricted for use in food producing animals. The FDA in the US prohibits "extra label" use (which is the application of the drug in a way which is not indicated on the drug information leaflet or label of the bottle) of chloramphenicol and the glycopeptides and lipoglycopeptides and restricts the extra label use of cephalosporins in food producing animals (FPA). Nitrofuran derivatives are banned from use in FPA in most countries and the use of nitroimidazoles is prohibited in FPA in the EU and US. As can be seen within **Table 1.2** almost every class of antibiotic has a drug within it that is used in FPAs, with the exception of the glycopeptides, nitrofurans and nitroimidazoles whose usage is heavily restricted in FPAs. It is not surprising therefore that the usage of antibiotics in animals has been demonstrated to result in the appearance of resistance within humans (Landers et al. 2012b; Tang et al. 2017; Pokharel et al. 2020).

Even though the WHO's list serves as a benchmark for categorising antimicrobials, international, regional and national guidelines and restrictions are created by individual organisations in different countries, which may not be in line with the WHO list. Therefore, there may be some discrepancies on restricted usage/categorisation of certain antimicrobials in different countries that either produce their own CIA list or have differing restrictions in place on certain antimicrobial usage (Scott et al. 2019a). There is also the difficulty of obtaining reliable surveillance of antimicrobial usage within certain countries especially LMICs, where several countries are under-represented, and resistance

and usage may both go either under reported or completely unreported. Southeast Asia for example houses greater than half the world's population and encompasses a very large geographical area with varying degrees of economic development (Hamers and van Doorn 2018; Browne et al. 2021). The antibiotic stewardship within LMICs is often extremely poor or even non-existent (Hamers and van Doorn 2018) and an example of this was shown in a study by Thu et al. (2012) in Vietnam hospitals, where it was found in 55% of indications, there was inappropriate empirical antibiotic therapy. Unfortunately, due to a lack of equipment, workers and the financial support, microbiological testing is often not performed and therefore the only option is empirical antibiotic therapy, which results in the misuse and overuse of the few antibiotics available to LMICs (Sartelli et al. 2020). Antibiotic consumption in LMICs is also beginning to catch up with higher income countries (Klein et al. 2018; Wilkinson et al. 2019).

Table 1.2: The 70 antibiotics that feature on both the WOAH'S list of antimicrobial agents of veterinary importance and WHO's list of antibiotics critical to human health. Those identified on the WHO list as being highest priority critically important antimicrobials are highlighted in purple with text in bold white

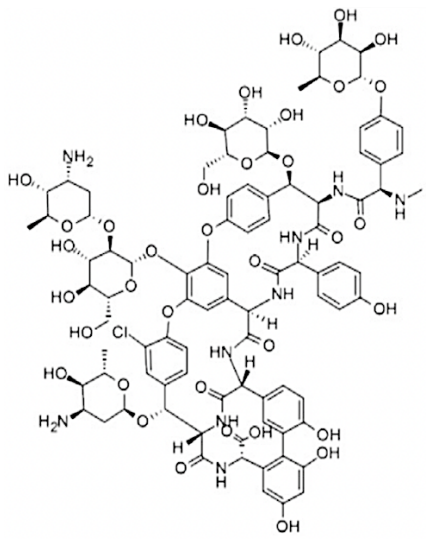
		WOAH List – Animals			WHO – Humans		
Antibiotic Class	**Antibiotic**	**Critical**	**Highly Important**	**Important**	**Critical**	**Highly Important**	**Important**
Aminoglycosides	Amikacin	✓			✓		
	Dihydrostreptomycin	✓			✓		
	Framycetin	✓			✓		
	Gentamicin	✓			✓		
	Kanamycin	✓			✓		
	Neomycin	✓			✓		
	Paromomycin	✓			✓		
	Spectinomycin	✓					✓
	Streptomycin	✓			✓		
	Tobramycin	✓			✓		
Amphenicols	Thiamphenicol	✓				✓	
Ansamycin- Rifamycins	Rifampicin		✓		✓		
	Rifaximin		✓		✓		
1st Generation Cephalosporins	Cefacetrile		✓			✓	
	Cefalexin		✓			✓	
	Cefalotin		✓			✓	
	Cefapirin		✓			✓	
	Cefazolin		✓			✓	
	Cefuroxime		✓			✓	
	Cefoperazone	✓			✓		
	Ceftriaxone	✓			✓		
Fusidic Acid	Fusidic Acid			✓		✓	
Macrolides and Ketolides	Erythromycin	✓			✓		
	Josamycin	✓			✓		

	Oleandomycin	✓			✓		
	Spiramycin	✓			✓		
Penicillins	Amoxicillin	✓			✓		
	Ampicillin	✓			✓		
	Benethamine Penicillin	✓				✓	
	Benzylpenicillin (Penicillin G)	✓				✓	
	Cloxacillin	✓				✓	
	Dicloxacillin	✓				✓	
	Hetacillin	✓			✓		
	Mecillinam	✓				✓	
	Nafcillin	✓				✓	
	Oxacillin	✓				✓	
	Phenethicillin	✓				✓	
	Phenoxymethylpenicillin (Penicillin V)	✓				✓	
	Procaine Benzylpenicillin	✓				✓	
	Ticarcillin	✓			✓		
Beta-Lactam/Beta-Lactamase Inhibitor Combinations	Amoxicillin and Clavulanic Acid	✓			✓		
	Ampicillin and Sulbactam	✓			✓		
Phosphonic Acid	Fosfomycin		✓		✓		
Polypeptides	Bacitracin		✓				✓
Polypeptides Cyclic	Colistin		✓		✓		
	Polymixin		✓		✓		
Quinolones and Fluoroquinolones	Ciprofloxacin	✓			✓		
	Difloxacin	✓			✓		
	Enrofloxacin	✓			✓		
	Flumequin		✓		✓		
	Marbofloxacin	✓			✓		
	Nalidixic Acid		✓		✓		
	Norfloxacin	✓			✓		
	Ofloxacin	✓			✓		

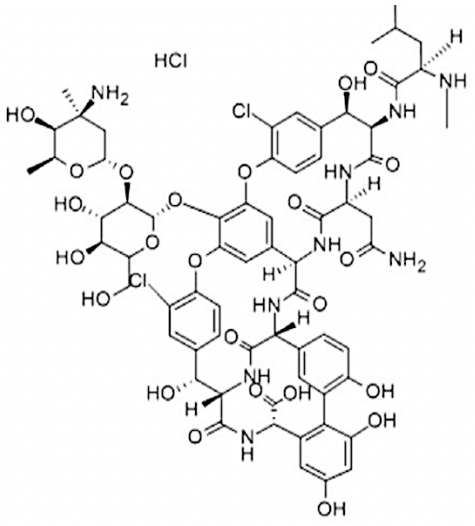
	Orbifloxacin	✓			✓		
	Oxolinic Acid		✓		✓		
Sulphonamides	Phthalylsulfathiazole	✓				✓	
	Sulfadiazine	✓				✓	
	Sulfadimethoxine	✓				✓	
	Sulfadimidine (Sulfamethazine, Sulfadimerazin)	✓				✓	
	Sulfafurazole	✓				✓	
	Sulfamerazine	✓				✓	
	Sulfamethoxypyridazine	✓				✓	
	Sulfanilamide	✓				✓	
	Sulfapyridine	✓				✓	
	Trimethoprim	✓				✓	
1st Generation Tetracycline	Chlortetracycline	✓				✓	
	Oxytetracycline	✓				✓	
	Tetracycline	✓				✓	
2nd Generation Tetracyclines	Doxycycline	✓				✓	

1.2.1.1. Risks Associated with Human Antibiotic Use in Animals and Cross Resistance

Cross resistance occurs when there is multiple resistance to antibiotics within or across antibiotic classes. This can happen due to the same intracellular target being altered, such is the case with ciprofloxacin and nalidixic acid which have cross resistance due to the similar mechanism of action these two drugs have (Lozano-Huntelman et al. 2020). Structural similarity between drugs also results in cross resistance. A classic example is apramycin which was used as a growth promoter in the 1980s, which led to apramycin resistant isolates of Enterobacteriaceae which had cross resistance to gentamicin through the enzymatic resistance gene *aac(3)-IV* (Chaslus-Dancla et al. 1991; Herrero-Fresno et al. 2016). Two other good examples of how the usage of antibiotics in animals may result in resistance to antibiotics in humans through cross resistance, are avoparcin and virginiamycin. Avoparcin was used in animals for the purposes of growth promotion but never used in humans, while vancomycin was reserved as a last-line-of-defence therapy for certain bacterial species in humans. The appearance of vancomycin resistant *Enterococcus* spp. in animals on farms where avoparcin was used in high levels as a growth promoter, led to the finding that resistance was due to the structural similarity of avoparcin with vancomycin (Acar et al. 2000). **Figure 1.4** shows the chemical structures of avoparcin and vancomycin and demonstrates just how similar the two structures of these antibiotics are. This resulted in avoparcin being banned as a growth promoter in 1997 to prevent the spread of vancomycin resistance (Pantosti et al. 1999; Casewell et al. 2003a; Kühn et al. 2005).



Avoparcin



Vancomycin

Figure 1.4: The chemical structures of avoparcin and vancomycin. Adapted from (Wielinga et al. 2014)

Virginiamycin was also banned as a growth promoter in 1997 due to the concerns of cross resistance to Quinupristin-Dalfopristin (QD) (trade name Synercid). Synercid is a combination of two streptogramin antibiotics, used for treating vancomycin-resistant enterococci (VRE) infections in humans and the use of virginiamycin as a growth promoter was believed to promote the emergence and dissemination of resistant *Enterococcus* spp. from FPAs, by selecting for virginiamycin resistant strains, which have cross resistance to Synercid (Hammerum et al. 1998; Jensen et al. 1998; Werner et al. 1998; Soltani et al. 2000; Donabedian et al. 2006).

The above examples of cross resistance, demonstrate why the use of antibiotics of the same class in animals and humans can be a significant cause of resistance development. This also demonstrates that the knowledge of crossover of resistance between antibiotics of the same structural class is not new. Following the 1997 ban of avoparcin and virginiamycin and then in 1999 the

ban of spiramycin, tylosin and bacitracin as growth promoters and in animal food products (Casewell et al. 2003c), all antibiotic usage for the purposes of growth promotion was banned in the UK and EU on 1 January 2006 and on 28 January 2022 this was extended to also include animals and animal products imported into the EU. A further restriction on the use of antibiotics for preventative treatment will be brought into effect in 2022 in the EU. In Australia in December 2017 a voluntary ban was introduced by the industry on the use of medically important antibiotics for the purposes of growth promotion (EPHA 2022). In 2017 in the US, the Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM) (2018) implemented "The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals" guidance document named GFI #209, which had begun in 2013. This process of implementing GFI #209 looked to eliminate the use of medically important antimicrobials in food producing animals (FPAs) for purposes such as growth promotion (FDA 2012b).

China is one of the largest contributors to antimicrobial usage in animals and back in 2013 almost half of the world's antibiotics equating to 162,000 tonnes was consumed by China, with 52% of this administered to animals (Tang et al. 2016). Between 2014 and 2018 this figure had decreased to less than 30,000 tonnes, a fall of 57% and the usage per tonne of animal of antibiotics was in line with figures reported in European countries. What China managed in 4 years was on par with what the Netherlands had also achieved in 5 years between 2007 and 2012. However, China remains a country where antibiotic resistance is severe, where the effects of AMR to public health are still under studied and antibiotic usage in animals remains high (Schoenmakers

2020). The transferable resistance gene *mcr-1* was discovered in China in 2015, which confers resistance to a last line antibiotic colistin and importantly was found within bacterial species causing infections in humans (Liu et al. 2016c; Wang et al. 2018; Schoenmakers 2020). As a result, the Chinese government formally banned colistin as a growth promoter on 30th April 2017 and further countries that included India, Japan and Brazil also enforced the ban. In the same year a national action plan in relation to antimicrobial resistance was published in China (Walsh and Wu 2016; Schoenmakers 2020; Wang et al. 2020). Within only 4 years of the discovery of *mcr-1*, bacteria positive for *mcr-1* were being reported in animals and meat products, humans and the environment across six continents in more than 50 countries (Wang et al. 2017; Sun et al. 2018; Wang et al. 2020a) and further derivatives of *mcr-1* including *mcr-2 – mcr-9* have also been identified (Kieffer et al. 2019a; Wang et al. 2020a).

In 2018, the amount of antibiotics used for growth promotion purposes accounted for 53% of the antibiotics used in Chinese farming (Schoenmakers 2020; FAIRR 2021). However, policing smaller farms in China is not always straightforward and a study by Xu et al. (2020), who conducted a survey of medium sized chicken farms in Ningxia, China, found that three-quarters still used banned antibiotics, without prescription and without records being kept as to their usage. Results such as these suggest there is still a long way to go to gain clear transparency on antibiotic usage, but also on antibiotic stewardship and the understanding of how antibiotics are used by the wider community of farmers outside of the bigger industrialised livestock farms.

Another example of the potential of agricultural usage of antibiotics to promote resistance in humans is the flavin-dependent monooxygenase *tetX*

(also described as tetracycline destructases) (Yang et al. 2004a; Gasparrini et al. 2020). *tetX* was first discovered in 1988 in *Bacteroides fragilis* (Park and Levy 1988; Speer and Salyers 1988), a commensal of the gut. Oddly owing to the fact that *tetX* activity requires oxygen (Yang et al. 2004b), the presence of *tetX* in *B. fragilis*, begs the question of why an oxygen dependent enzyme would be found in a strict anaerobe like *B. fragilis* (Baughn and Malamy 2004; Elsaghir and Reddivari 2022) and for this reason, when it was first discovered *tetX* was thought not to be of much clinical relevance (Chopra and Roberts 2001a). However, *tetX* is now found throughout many Gram negative species including *E. coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Cheng et al. 2022). Previously tetracycline resistance in clinical isolates had been mainly the result of either efflux or ribosomal protection rather than enzymatic activity (Thaker et al. 2010a). The appearance of *tetX* in clinical isolates resulted in resistance to newer tetracyclines including eravacycline and omadacycline but also the last line therapy drug tigecycline, vital in the treatment of multi-drug resistance in Gram negative bacteria such as carbapenem-resistant *Enterobacteriaceae* (CRE) (Gasparrini et al. 2020). The emergence of mobile *tetX* variants including *tet*(X3) to *tet*(X5) raised yet more concerns (He et al. 2019a; Li et al. 2021). Tetracycline accounts for 66% of the total livestock antibiotic usage worldwide (Ungemach et al. 2006) and the data collected for the WOAH'S 3rd, 4th and 5th Annual reports on antimicrobial agents intended for use in animals, has reported tetracycline as the most commonly reported antimicrobial class (OIE 2018; 2020; 2021a). Thus, *tet* genes which are readily transmissible would pose a serious threat to disease treatment.

1.2.2. Routes to the Environment

Antibiotics have existed within the natural environment far longer than man has been using them and along with these antibiotics are the natural mechanisms to resist them. The environment has a vast inventory of resistance genes and some of the resistant bacteria now of great clinical concern, are believed to have acquired their resistance genes from environmental sources. The beta-lactamase CTX-M believed to have originated from an environmental *Kluyvera* strain, is good example of environmental acquisition of resistance (Livermore and Brown 2001; Davies and Davies 2010; Forsberg et al. 2012). Antibiotic residues introduced to the natural environment will often favour selection for resistance mechanisms and one route for antibiotic environmental contamination is through the practice of spreading manure onto agricultural land (Wichmann et al. 2014; Bondarczuk et al. 2016). The spreading of animal manure onto agricultural land is a common practice throughout farming communities. This form of fertiliser is readily available on farms involved in animal rearing, recycles around 70% of the undigested nutrient minerals which would otherwise be lost and provides a good source of enrichment to soils in readiness for crop cultivation (Martinez et al. 2009a). Manure is often stored as liquid slurry and the storage stage is of vital importance to decrease the pathogen numbers within the liquid before spreading. Within the UK the Department for Environment, Food and Rural Affairs (DEFRA) has set out guidelines for farmers, which requires storage of slurry for at least 4 months prior to spreading (DEFRA 2015). Other EU countries such as Italy suggest storage times of around 3-6 months (Martinez et al. 2009a; Blaiotta et al. 2016). But, of course, other elements contained within the slurry such as antibiotic residues and trace

elements including heavy metals may still persist even after the extended storage time. These contaminants may then be free to interact with the microbial soil communities following the spreading of effluent onto farmland (Weber et al. 2007; Cortet et al. 2011; Youngquist et al. 2014; Bondarczuk et al. 2016). The EU sewage sludge directive of 1986 set out the allowed limits for heavy metal concentrations and banned the use of untreated sewage sludge on agricultural land. Thus, slurry is often subject to strict guidelines prior to spreading (European Commission 1986). However, tetracyclines and sulfadiazine have been found to be common contaminants of manure and treatment options such as vermiculture and storage processing are not always effective in eliminating the high levels often found in manure (Chen et al. 2012).

Many antibiotics are excreted from the animal either partially or unmetabolised in the faeces, due to poor absorption within the animal gut (Boxall et al. 2004). Enrofloxacin for example is only partially metabolised (<25%) in the liver and converted into ciprofloxacin, which is itself an active antimicrobial (Anderson et al. 2012; Berendsen et al. 2018).

Even though antibiotics show effectiveness at relatively low levels, the doses administered to FPAs are often much larger for adequate delivery of the drug, which can result in excretion of up to 90% of the drug (Sarmah et al. 2006a). Chee-Sanford et al. (2009) reported that around 75% of administered antibiotics are not absorbed by the animal gut and reported on studies that have suggested figures for active metabolite excretion in faeces within the region of 60% for tetracycline and around 67% for the macrolide tylosin. However, Sarmah et al. (2006b), Kemper (2008) and Zhou et al. (2012) all reported

anything from 30-90% of the original antibiotic compound can be excreted within the animal faeces following antibiotic therapy (Elmund et al. 1971; Sarmah et al. 2006a).

Huygens et al. (2021) investigated the presence of antibiotic residues and antibiotic resistant bacteria in manure from cattle intended for agricultural field fertilisation. A total of 69 antibiotic residues were examined in slurry and manure samples taken from a total of 34 farms, that included 9 mixed fattening calf and 25 mixed beef farms across Belgium. In the fattening calf slurry samples, sulfadiazine, doxycycline, oxytetracycline, ciprofloxacin, enrofloxacin, flumequine and lincomycin were all detected at mean concentrations of 10,895, 2776, 4078, 48, 31, 536 and 36 μg kg^{-1} manure, respectively. In 4 of the 25 manure samples from the beef cattle, oxytetracycline at concentrations of 471 and 28 μg kg^{-1} was detected in 2 samples. Ciprofloxacin and enrofloxacin and its metabolite at concentrations of 35 and 80 μg kg^{-1} respectively, were detected in the same sample and in another sample paromomycin at a concentration of 50 μg kg^{-1} was also detected. This study highlighted a possible route for antibiotic resistance from FPAs to the food chain, via the consumption of crop foods such as vegetables fertilised with cattle manure.

Berendsen et al. (2018) examined broilers and pig manure and liquid, semi-solid and solid manure from calves stored for 24 days, that had been fortified with 46 different antibiotics. The degradation time for 90% (DT90) of the antibiotics, was highest for quinolones at 100-5800 days, followed by lincosamides, pleuromutilins and macrolides with DT90s of 135-1400 days, 49-1100 days and 18-1,000 days respectively and tetracycline with a DT90 of up to

422 days, with rates dependent on manure type. Sulphonamides dissipated relatively quickly in all manure types, with a DT90 of 0.2-30 days. The study concluded that dissipation varied upon manure type and antibiotics that more readily dissipated during the manure storage period are less likely to reach the environment. However, it was found that doxycycline, oxytetracycline, flumequine, tilmicosin, tylosin and enrofloxacin were the most persistent after 4 months of manure storage, therefore these antibiotic residues would be the most likely to reach the environment.

It has been reported that cropland spread with dairy manure (Lupo et al. 2012; Economou and Gousia 2015a; Xie et al. 2018), nearby farmland and soils (Heuer et al. 2011a; Peng et al. 2017; Pollard and Morra 2018), groundwater near dairy operations (Li et al. 2015; Pan and Chu 2017b; Spielmeyer et al. 2017), irrigation water (Blaustein et al. 2016; Hafner et al. 2016; Palacios et al. 2017) and surface water (Pruden et al. 2012; Li et al. 2014; Kulesza et al. 2016), soil bacteria (Edrington et al. 2009; Yang et al. 2011; Fahrenfeld et al. 2014) and fertilized crops exposed to cattle manure (Tasho and Cho 2016; Pan and Chu 2017a), may all serve as a reservoir for both antibiotic resistant bacteria and antibiotic resistance genes (Oliver et al. 2020).

Carlson et al. (2004) sampled seven dairy lagoons and fresh manure stockpiles for the presence of tetracyclines, sulphonamides and macrolides. The reported figures from the lagoon samples found macrolide levels of 19 parts per billion (ppb) and both tetracyclines and sulphonamides ranging from non-detectable (NDtc) to 17 ppb. Within the manure sampled from the stockpiles, macrolides ranged from NDtc to 5 ppb, tetracycline ranged from NDtc to 5130 ppb and sulphonamides ranged from NDtc to 46 ppb. Sulphonamides and

tetracyclines were by far the antibiotics most commonly found throughout the different samples, with occurrence rates of 44% and 96% respectively.

Storteboom et al. (2007) showed that following treatment times of up to 6 months, levels of tetracycline in the stored slurry can be significantly reduced. This study emphasised the importance of manure management to decrease the possibility of leaching into the environment.

Watanabe et al. (2010) looked at the release of antibiotics from dairy concentrated animal feeding operations. In flush lane water and lagoon samples, tetracyclines, sulphonamides and trimethoprim and their isomers/epimers along with lincomycin were all detected at concentrations ranging from 0.012 to 267 $\mu g\ L^{-1}$. In addition, there were frequent detections of antibiotics from surface samples in the dairy hospital pens with sulfadimethoxine at 5.8-457 $\mu g\ kg^{-1}$ and tetracycline at 6.2-73 $\mu g\ kg^{-1}$.

Ince et al. (2013) looked at the concentration of oxytetracycline in cow faecal cecum samples following an intramuscular oxytetracycline dose of 8,800 mg (20 mg/kg). Over a 20 day period, samples were taken from the cecum and 20% of the original dose was detected in the pre-excreted faeces.

Not all antibiotics are administered orally however, and therefore may not be excreted within faeces. The third-generation cephalosporin ceftiofur (EFT) is an example of an antibiotic favoured for use within milk production dairy cows. EFT is often chosen for economical preference, as loss of milk production time is minimal. It is often administered in an injectable form directly into the udder and therefore the influences on bacterial gut flora in regard to EFT resistance may be limited. Excretion of EFT within urine however can still occur, furthering the possibility for the appearance of antibiotic resistant bacteria (Call

et al. 2013). Other cephalosporins favoured for injection include cefquinome (CFQ).

Ray et al. (2014) looked to analyse the excretion of cephapirin from dairy cattle following intramammary infusion. Only low traces (μg kg^{-1}) or around 1% of the initial dose was recovered from cattle faeces but very high levels of the excreted form desacetyl cephapirin, were detected in urine with concentrations ranging from 133 – 480 μg L^{-1}, which is around 50% of the initial dose. The metabolite desacetyl cephapirin has been shown to maintain up to 55% of the activity of the parent compound cephapirin (Jones and Packer 1984).

Findings on the selection of EFT resistant *Escherichia coli* within cattle following antibiotic treatment have revealed contrasting results. Lowrance et al. (2007) demonstrated that selection for resistance increased following the use of EFT therapy. However, Singer et al. (2008) and Mann et al. (2011) found no significant increase in resistance but did find a decrease in total *E. coli* levels. Elements including herd number, bedding material, dose and frequency of antimicrobial administration and manure disposal and/or the further application as fertiliser all need to be taken into consideration when assessing levels of EFT resistant *E. coli* (Call et al. 2013). Liu et al. (2016b) investigated antibiotic treatment in dairy calves and the subsequent shedding in calve faeces in a four-week trial and found that following treatment with both EFT and florfenicol, there was an increase in the number of resistant *E. coli* shed within the faeces. It was also discovered that the population of resistant *E. coli* remained stable within the soil of the pens for the duration of the four-week trial period. This study demonstrated that soil could possibly act as a long-term reservoir for the maintenance of resistant *E. coli*. Ohta et al. (2017) investigated the population

dynamics of antimicrobial resistance associated with *Salmonella* within beef cattle. It was observed that there was reduced *Salmonella* prevalence within cattle faecal samples, following treatment with both ceftiofur crystalline free acid (CCFA) and chlortetracycline (CTC) but within that same time frame, there was an increase of multi-drug resistant *Salmonella*, demonstrating that antimicrobial usage was able to shift the *Salmonella* population from pan susceptible, through the selection of the multi-drug resistant *Salmonella* serotypes. Ohta et al. (2019) quantified the number of non-type specific (NTS) *E. coli* and *Salmonella* within cattle faeces treated with CCFA and CTC. By using colony counting, the study demonstrated that with CCFA treatment, there was a significant decrease in both *Salmonella* and NTS *E. coli* quantities, but only *Salmonella* quantities were further decreased with CTC treatment, with NTS *E. coli* quantities unaffected. Following CTC and CCFA treatment, the population was dominated by resistant *Salmonella*. The NTS *E. coli* population behaved slightly differently, following CTC treatment, the tetracycline resistant *E. coli* population expanded in quantity, but EFT resistant *E. coli* did not.

Following antibiotic treatment, a withdrawal period is implemented that requires any produce from the animal to be discarded until the withdrawal period is over. The withdrawal period is a specific time, after the final dose of medicine is introduced, that must elapse before any produce from that animal can re-enter the food chain. The withdrawal period is determined by the establishment of what is known as the maximum residue limit for a particular medicine, which is the maximum allowed concentration of a drug that is thought to be non-hazardous, and which is permitted within food intended for animal or human consumption (Anika et al. 2019; Sachi et al. 2019). Milk from cows for

example treated with an antibiotic is discarded as waste milk (Brunton et al. 2012) and should this milk be disposed of within a slurry tank, this may add yet another source of antibiotic contamination.

1.2.3. Interaction of Resistance Genes with the Environmental Resistome

The survival of excreted bacteria and antibiotic residues within the environment is dependent upon a number of factors namely temperature, pH, weather (rainfall resulting in surface water and run-off, drops in temperature and sunlight), presence of metal ions and ionic strength present. Many resistant bacteria may well perish over time within the natural environment. However, many remain to integrate into the natural communities of microbes, contributing not only bacteria but also resistance genes along with them. Even if the bacteria are no longer viable over time, genetic elements relating to resistance may still persist within the environment and, with the selective pressure of antibiotics, resistance can persist and be advantageous to horizontal gene transferability (Kim et al. 2014; Bondarczuk et al. 2016). **Figure 1.5** demonstrates the path of antibiotic residues into the environment and the possible interaction within soil.

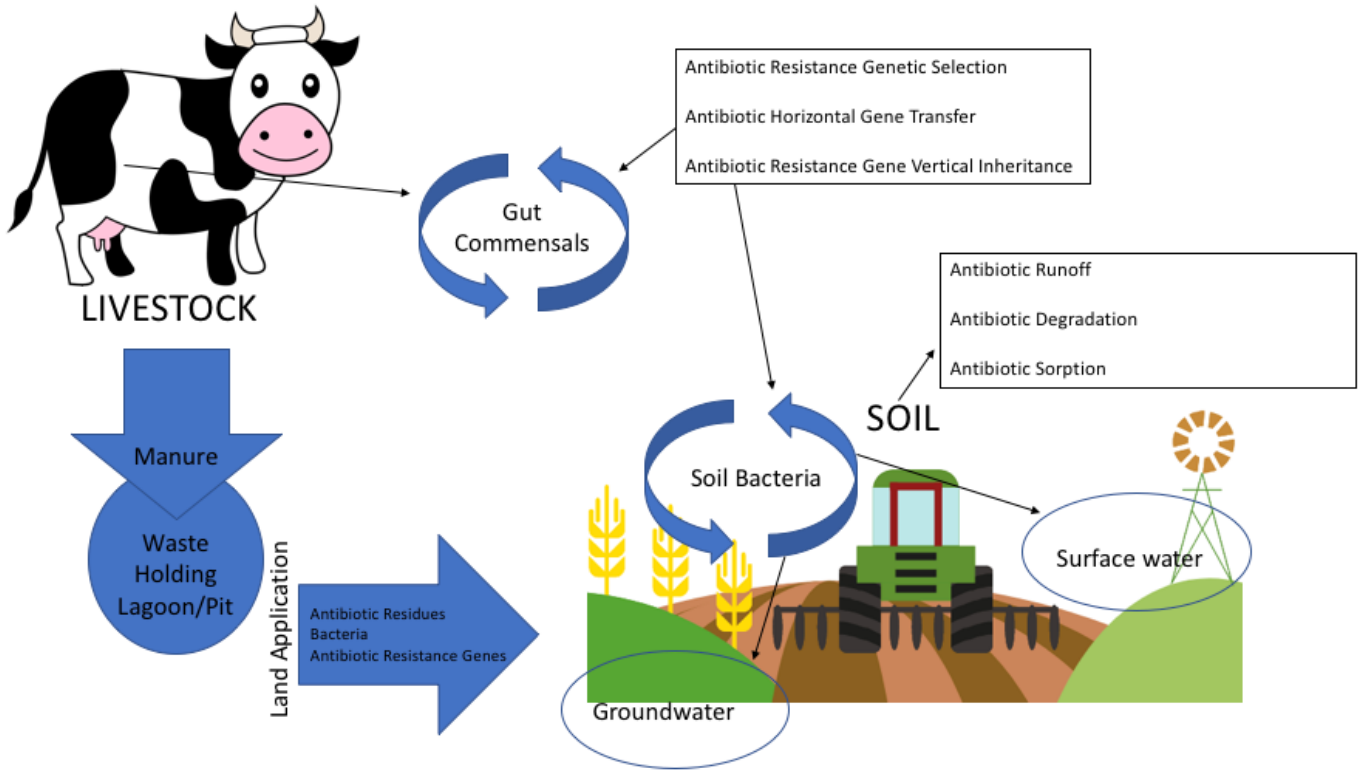


Figure 1.5: The path of antibiotic residues into the environment following the land application of manure and the interactions within soil and ground/surface water, including run-off, sorption and degradation along with resistance gene acquisition by resident bacteria. Based on: (Chee-Sanford et al. 2009)

A main source of antimicrobial resistance gene influx into the environment is through the spreading of slurry generated from animals reared in confinement. Demanding modern agriculture makes antibiotic use unavoidable in order to achieve high food production output requirements (Joy et al. 2013). Tetracycline is heavily favoured for use within food production animals, due to both its low cost and activity across a number of Gram negative and Gram positive bacteria (Chessa et al. 2016). However, as tetracycline is poorly absorbed by the animal gut, a large proportion is excreted within the animal faeces (O'Connor and Aga 2007). Tetracycline has been shown to strongly absorb to most types of commonly found soils such as clay, sediment and sand. Macrolides and sulphonamides in comparison exhibit poor absorption and in turn are likely to be better mobilised within the environment (Allaire et al. 2006; Chee-Sanford et al. 2009; Wang and Wang 2015).

Chessa et al. (2016) looked to discover whether tetracycline present in cow manure has an effect on the resident soil bacteria, following land application of manure as fertiliser. The results revealed that soil type and pollutant history of the land played significant parts in tetracycline sorption, which in turn impacted the availability of tetracycline to resident soil communities. Another point that was significant was that even in the absence of tetracycline, the repeated application of manure to soil resulted in an increase in the accumulation of both tetracycline and sulphonamide resistance genes. Within clay soils it was found that *tet* and *sul* could be co-selected for, which was likely due to both being present on the same mobile genetic element typically found to be an IncQ type plasmid. When tetracycline was present within manure the selection for IncP-1ε type plasmids was also increased but

only in sandy type soils. This research demonstrated that repeated application of manure to soils can result in increases in both resistance genes and the selection of mobile genetic elements (MGEs) such as plasmids when tetracycline was present.

1.3. ORIGINS OF RESISTANCE

Bacterial resistance is ancient and predates the selective pressures associated with human antibiotic usage, as has been discovered when looking at antibiotic resistant bacteria isolated within so called “pristine” environments with limited to no anthropogenic impact (D’costa et al. 2011; Bhullar et al. 2012; van Goethem et al. 2018; Scott et al. 2020); this includes sites such as the deep subsurface microbiome (Brown and Balkwill 2009), glacial ice cores from the Arctic and Antarctic (Segawa et al. 2013), deep oceans (Toth et al. 2010; Chen et al. 2013) and isolated caves (Bhullar et al. 2012). There is, however, still some debate over whether an area can be called truly “pristine” and without anthropogenic influence (Bhullar et al. 2012; Scott et al. 2020). However, under subzero conditions, the viability of microbial communities may be preserved for thousands to millions of years (Mindlin et al. 2008). It would therefore appear, that antibiotic resistance predates the human use of antibiotics (Wright 2007; 2010).

Several studies have demonstrated the discovery of resistance determinants in remote and isolated regions, such as Allen et al. (2009) who discovered within Alaskan soil, a reservoir of beta-lactamase type resistance genes through the use of functional metagenomic analysis. Mindlin et al. (2008)

tested resistance present in bacteria isolated from Eastern Siberian permafrost sediments aged from 3,000 to 3 million years old and found both Gram negative and Gram positive bacteria with resistance to kanamycin, streptomycin, gentamicin, chloramphenicol and tetracycline. Bhullar et al. (2012) investigated antibiotic resistance occurring within the culturable microbiomes within the Lechuguilla cave, New Mexico, which had been isolated for more than 4 million years. A total of 93 strains of bacteria, isolated from areas of the Lechuguilla cave with low anthropogenic antibiotic exposure, were screened against 26 antibiotics which included naturally occurring, semisynthetic and completely synthetic antibiotics. The results revealed that an average of 70% of the Gram positive strains were resistant to around 3-4 antibiotic classes, with three strains of *Streptomyces* spp. showing resistance to 14 antibiotics. Within the Gram negative samples, 65% were resistant to 3-4 antibiotic classes that included the antibiotics trimethoprim, sulfamethoxazole and fosfomycin. When looking at the actions of the beta-lactams ampicillin (AMP), piperacillin and the cephalosporin cephalexin against the samples, 22-62% of the Gram positive strains were able to inactivate these antibiotics. Each of these cited studies was able to reveal that resistance has deep evolutionary origins and that bacteria living within secluded environments potentially already possess a multitude of resistance genes.

Resistance as a mechanism in soil bacteria, for example, is often an advantageous requirement due to the competitive environment they live in with other neighbouring bacteria that produce antibiotics, where so called “chemical warfare” can occur between competing bacteria (Nesme and Simonet 2015; Granato et al. 2019; Westhoff et al. 2020). Within an ecosystem, bacteria may

employ resistance either as a form of self-protection if they are producers of antibiotics themselves or for protection against other bacteria producing antibiotics as a means to co-exist (Séveno et al. 2002; D'Costa et al. 2007; Laskaris et al. 2010; Martínez 2012). Many resistance genes conferring resistance to currently used antibiotics have been identified within environmental biomes (D'Costa et al. 2006). Stubbendieck and Straight (2015) demonstrated competitive interaction between *Streptomyces* spp. and *Bacillus subtilis*, whereby lytic linearmycins produced by the *Streptomyces* spp. resulted in spontaneous resistant mutants of *B. subtilis* to begin appearing, suggesting the activation of a defensive response by *B. subtilis* to counter the lytic stress from the competitor. In fact, two thirds of the clinically relevant antibiotics used in both human and veterinary medicine are synthesised by soil bacteria belonging to the genus *Streptomyces*, including neomycin isolated from *S. fradiae*, kanamycin isolated from *S. kanamyceticus*, tetracycline isolated from *S. rimosus*, chloramphenicol isolated from *S. venezuelae* and the antibiotic named for the genus, streptomycin, isolated from *S. griseus* (Izard and Ellis 2000; Yagüe et al. 2012). But if bacteria have the ability to both produce antibiotics and counter them with resistance, it is only a matter of time before resistance can occur and indeed this has been demonstrated with the speed at which resistance has appeared, soon after an antibiotic has been introduced for clinical usage (Davies and Davies 2010).

1.3.1. Types of Resistance

Bacterial resistance incorporates both natural resistance (intrinsic and induced) and acquired resistance where genes may be acquired from horizontal

gene transfer (HGT) via MGEs. Bacteria employ a host of different mechanisms for resistance including: membrane permeability changes thereby restricting access of antimicrobials into the cell, removal from the cell via efflux pumps, antibiotic modification through enzymatic action, antibiotic degradation, antibiotic target modification and overexpression of enzymes (van Hoek et al. 2011).

Both intrinsic and induced resistance are types of resistance that are naturally occurring within a particular bacterial species and are not attributed to the horizontal acquisition of resistance genes. The differences between these two types of natural resistance are that intrinsic resistance is always expressed and is independent of antibiotic selective pressure. Induced resistance, however, requires the presence of an antibiotic for gene expression (Fajardo et al. 2008; Cox and Wright 2013). A good example of intrinsic resistance is within *Mycoplasma* spp. that lack a cell wall and are therefore resistant to antimicrobials that target the cell wall such as beta-lactams (Bébéar et al. 2011). Another example of intrinsic resistance is within Gram negative bacteria, which are intrinsically resistant to many antibiotics including glycopeptides such as vancomycin and teicoplanin, the oxazolidinone linezolid and the lipopeptide daptomycin (C Reygaert 2018). This intrinsic resistance in Gram negative bacteria such as *E. coli* is attributed to the impermeable outer membrane (OM) and indeed most intrinsic mechanisms involve OM permeability reduction. The cytoplasmic membrane (CM) of bacteria forms a barrier between the cytoplasm and the outside environment. Gram positive bacteria possess only the CM and a thick outer layer of peptidoglycan, which allows small molecules to permeate through, with the result that Gram positive bacteria are susceptible to many

antibiotics. Gram negative bacteria however possess an additional impermeable OM of lipopolysaccharide, that acts as a formidable barrier (Wiener and Horanyi 2011), resulting in the high levels of insusceptibility to many antibiotics (Cox and Wright 2013). In addition to the OM in Gram negatives, there are water-filled channels called porins, that act as pathways for essential nutrients to gain access to the cell. The presence of porins within the protective OM, provide a pathway for the flow of nutrients crucial to sustaining the life of the bacterium (Galdiero et al. 2013). However, porins also restrict the influx of antibiotics, through charge repulsion (Bajaj et al. 2017), hydrophobicity (Wiener and Horanyi 2011) and pore size limitation (Nikaido 2003; Chowdhury et al. 2018), which contributes to the intrinsic resistome of Gram negative bacteria. Under expression of porins has also been indicated as a complementary resistance mechanism towards hydrophilic antibiotics that include beta-lactams, fluoroquinolones, tetracyclines and chloramphenicol (Pagès et al. 2008; Delcour 2009; Fernández and Hancock 2012; Kong et al. 2018). Porin loss is also a contributor to the now growing and worrying problem of carbapenemase-producing *Enterobacteriaceae* (CPE) (Cantón et al. 2012a; Codjoe and Donkor 2017; van der Zwaluw et al. 2020).

Both Gram positive and Gram negative bacteria also possess efflux pumps, and these are highly conserved within all members of the same species with tightly regulated expression (Martinez et al. 2009b). Antibiotic resistance association with efflux pumps was first described in 1980 (McMurry et al. 1980) but efflux pumps are believed to be important for a number of intracellular processes besides just providing an antibiotic resistance mechanism, including: the removal of toxic substances including antiseptics (Chuanchuen et al. 2001;

Sanchez et al. 2005; Pumbwe et al. 2007), solvents (Ramos et al. 2002), detergents (Zgurskaya and Nikaido 2000) and heavy metals (Silver and Phung 1996; 2005) along with waste products from the cell, cell signalling, cell homeostasis and virulence (Martinez et al. 2009b). Efflux pumps that have a broad-spectrum substrate specificity are often associated with multi-drug resistance, due to their ability to remove various antibiotics from the cell and it appears bacteria have repurposed this mechanism that served a different purpose in their natural ecosystem, to provide resistance. Many of the genes associated with efflux are encoded on the chromosome, are conserved in arrangement and structure, and found within environmental bacteria that are not antibiotic producers and in areas where there is a low antibiotic selective pressure. This provides more evidence that efflux likely was not originally designed as an antibiotic resistance mechanism (Webber 2003; Martinez et al. 2009b).

Efflux pumps when expressed at natural activity levels would be classed as intrinsic but can be classed as induced if the activity is increased following exposure to an antibiotic (Cox and Wright 2013).

Some bacteria display intrinsic resistance throughout the species or in individual strains to certain antibiotics and there are examples of these detailed in **Table 1.3**.

Table 1.3: Intrinsic resistance within certain types of bacteria and examples of intrinsic resistance

Organism(s)	**Intrinsic Resistance**
All Gram positives	Aztreonam, colistin
All Gram negatives	Glycopeptides, oxazolidinones, lipopeptides
P. aeruginosa	Tetracyclines, chloramphenicol, trimethoprim-sulfamethoxazole (SXT), several beta-lactams including ampicillin and 1st and 2nd generation cephalosporins,
Enterococci	Cephalosporins, ertapenem, macrolides, clindamycin, SXT, fluoroquinolones, lincosamides, aminoglycosides
Acinetobacter spp.	Amoxicillin, ampicillin, ertapenem, glycopeptides
Staphylococcus spp.	Macrolides, fluoroquinolones
Serratia spp.	Ampicillin, amoxicillin/clavulanic acid, macrolides
Klebsiella spp., *Proteus* spp.	Ampicillin
E. coli	Macrolides
Listeria monocytogenes	Cephalosporins
Bacteroides	Quinolones, several beta-lactams, aminoglycosides
Stenotrophomonas maltophilia	Carbapenems, quinolones, beta-lactams, aminoglycosides

***Footnote for Table 1.3**: Table adapted from:* (C Reygaert 2018) *and https://www.uspharmacist.com/article/understanding-antimicrobial-resistance.*

Bacteria can also display transient resistance produced by phenotypic variability and gene expression, which is independent of genetic change (el Meouche et al. 2016) and is generally only a temporary resistance in response to

external stimuli such as antibiotic exposure or exposure to noxious substances such as bile (Rosenberg et al. 2003; Viveiros et al. 2007). A good example of transient resistance in *E. coli* is the overexpression of efflux pumps such as AcrAB and under expression of porins such as OmpF and OmpC in response to bile salts in the natural environment of the gut (Thanassi et al. 1997).

1.3.1.1. Co-Selection

Biocide/metal resistance genes (BMRGs) and antimicrobial resistance genes (ARGs) often occur together, an example of which includes the genes *qacE* and *qacEΔ1* which encode resistance to quaternary ammonium compounds (QACs) through an efflux pump.

QACs have been used extensively in healthcare to try and combat the problem of antibiotic resistance. However, as with antibiotics, the intensive use of disinfectants by clinicians has only resulted in disinfectants that no longer work, due to bacterial resistance with decreased or no susceptibility to these compounds (Kücken et al. 2000; Romao et al. 2011). The genes *qacE* and *qacEΔ1* are frequently found associated with class 1 integrons and are widely disseminated throughout Gram negative bacteria (Jechalke et al. 2013; Bragg et al. 2014).

Pal et al. (2015) investigated the co-occurrence of BMRGs and ARGs from fully sequenced genomes and plasmids isolated from a variety of different environments. Five percent of the 4,582 plasmids and 17% of the 2,522 genomes analysed were found to be carrying a minimum of one BMRG and one ARG with increased frequency of ARG carriage in the presence of BMRG. A large proportion (47%) of the plasmids were isolated from Proteobacteria such as *E.*

coli and *Klebsiella* spp. Resistance towards metals including arsenic, cadmium, copper and mercury and antibiotics including sulphonamides, beta-lactams, aminoglycosides and tetracyclines was found at high frequency within plasmids along with resistance towards biocides such as QACs, acridines, biguanides, diamidines, phenanthridines and xanthenes. Clusters between ARGs and BMRGs were found which included strong correlations between mercury resistance, *qacE*Δ*1* and multiple ARGs. The integrase *intI1* was also shown to be strongly correlated with aminoglycoside resistance and the *qacE*Δ*1*/mercury resistance genes. These findings reveal that QACs and metals may provide strong selective pressure towards class 1 integron promotion and co-selection of ARGs and BMRGs. If the genes for each resistance are carried on the same MGE, only one of the compounds needs to be present for multi-resistance to be maintained.

Copper has also been found to be significant in co-selection with BMRGs and ARGs. Fang et al. (2016) investigated 25 IncHI2 plasmids from *E. coli* isolates from FPAs. From 25 plasmids analysed, genes conferring resistance to third generation cephalosporins, fosfomycin, amphenicols, quinolones, aminoglycosides and olaquindox, as well as *pco* and *sil* conferring resistance to copper and silver, were found. Plasmids of the IncHI2 type have also been shown to be associated with metal resistance, an example of which is R478, which carries efflux systems allowing for the detoxification of silver, copper and arsenic and resistance to mercury via a Tn*1696*-like mercury operon along with resistance towards tellurite (Cusumano et al. 2010). Genes such as *tcrB*, conferring transferable resistance to copper, have been shown within several studies to be linked to co-transfer of antibiotic resistance genes including AMP,

erythromycin, gentamycin, tetracycline and vancomycin within enterococci isolated from pigs, poultry and cattle (Hasman and Aarestrup 2002; Amachawadi et al. 2013; Silveira et al. 2014; You and Silbergeld 2014). Huysman et al. (1994) looked at bacteria isolated from agricultural land, where pig manure had been spread that was contaminated with copper. When a comparison was made between copper-sensitive and copper-resistant bacteria, an increased frequency of resistance to other antimicrobials within copper-resistant bacteria was found. This comprised resistance towards metals including cadmium, cobalt, nickel and zinc and antibiotic resistances including AMP, olaquindox, spiramycin and streptomycin. Studies such as these show the importance of considering multiple factors within the multi-drug resistance problem.

Metals even at sub-lethal levels are known to persist within manure, which is then spread onto agricultural land, thereby gaining access to the environment (Wales and Davies 2015).

1.3.2. Acquired Resistance and Mobility

Acquired resistance is another mechanism for resistance, whereby a previously susceptible bacterium gains resistance either through the acquisition of resistance genes or through mutations. A good example of acquired resistance are the acquisition of the progenitors to the CTX-M beta-lactamase genes from *Kluyvera* spp. by human pathogenic bacteria (Humeniuk et al. 2002b; Poirel et al. 2002; Bonnet 2004a; Rodríguez et al. 2004; Lartigue et al. 2006a; Rossolini et al. 2008a; Literacka et al. 2009; Cantón et al. 2012b; Bevan et al. 2017a). Another good example of acquired resistance is, the acquisition of the quinolone

resistance gene *qnrA* from *Shewanella algae* (Poirel et al. 2005e). Original acquisition of the *qnr* genes was believed to have been from the chromosomes of aquatic bacteria (Strahilevitz et al. 2009; Jacoby et al. 2014). The first report of transferable plasmid-mediated quinolone resistance (QnrA) was in 1994 from a *Klebsiella pneumoniae*, identified within a urine sample, that provided low-level resistance to quinolones including ciprofloxacin and nalidixic acid (Martínez-Martínez et al. 1998; Jacoby et al. 2014). QnrS is another transferable quinolone resistance determinant that is often reported flanked by Tn*3* (Monárrez et al. 2018). Qnr determinants which include *qnrA*, *qnrB* and *qnrS* have now been detected and reported worldwide and are frequently found associated with extended spectrum beta-lactamase (ESBL) type genes (Nordmann and Poirel 2005). The *qnr* genes act to protect the primary target of quinolones, DNA gyrase, from the inhibitory effects of the antibiotic. This is thought to be through at least two different mechanisms, including lowering the availability of chromosomal target enzymes by decreasing binding of topoisomerase IV and gyrase to DNA or by inhibiting quinolone entry to enzyme cleavage complexes by binding to gyrase and topoisomerase IV (Aldred et al. 2014).

Resistance to quinolones however, mostly arises through the actions of combined acquired mutations, rather than through the *qnr* genes alone, as *qnr* genes generally only provide low level resistance (Strahilevitz et al. 2009; Salah et al. 2019). For high level resistance, the actions of multiple different acquired mutations alone or in tandem with *qnr* are required. An example of acquired quinolone resistance through mutation, would be mutation in DNA gyrase (topoisomerase II) which is the target of the fluoroquinolone antibiotics.

Mutations at particular regions of *gyrA* are associated with low level resistance to fluoroquinolones. However, when mutations in *gyrA* are coupled to mutations in the DNA topoisomerase IV subunit A *parC*, at specific regions known as the quinolone resistance-determining region (QRDR) (present within codons 67–106 in *gyrA* and 56–108 in *parC* according to *E. coli* numbering), the result is increased resistance to fluoroquinolones (Hopkins et al. 2005; Woodford and Ellington 2007; Johnning et al. 2015).

The tetracycline resistance genes are another important and large group of acquired resistance genes. Tetracycline resistance genes have three main mechanisms of action, which include active efflux, ribosomal protection and enzymatic inactivation of the target antibiotic. Tetracycline resistance genes include at least 63 genes and 11 mosaic genes (according to: http://faculty.washington.edu/marilynr/). This group of 63 genes and 11 mosaic genes, consists of 36 efflux genes (examples include *tetAB*), 13 ribosomal protection genes (an example of which is *tetM*), 13 antibiotic enzymatic inactivation genes (an example of which is *tetX*), 11 mosaic protection genes (an example of which is *tetO/32/O*) and 1 gene classed as "unknown" (*tetU*) due to it being unrelated to either ribosomal protection or efflux. A full list of the 63 genes and 11 mosaic genes is available (http://faculty.washington.edu/marilynr/tetweb1.pdf) (Roberts 2005; Jones et al. 2008a; Thaker et al. 2010b).

Tetracycline genes are also commonly found associated with MGEs including the 11,139 bp Tn*1721* that encodes *tetAR* (Allmeier et al. 1992) (**Figure 1.6**) (accession number: X61367.1). It is well reported that tetracyclines persist within the environment, adding selective pressure to antibiotic resistance

gene maintenance and potential for MGE encoding tetracycline resistance dissemination (Scaria et al. 2021).

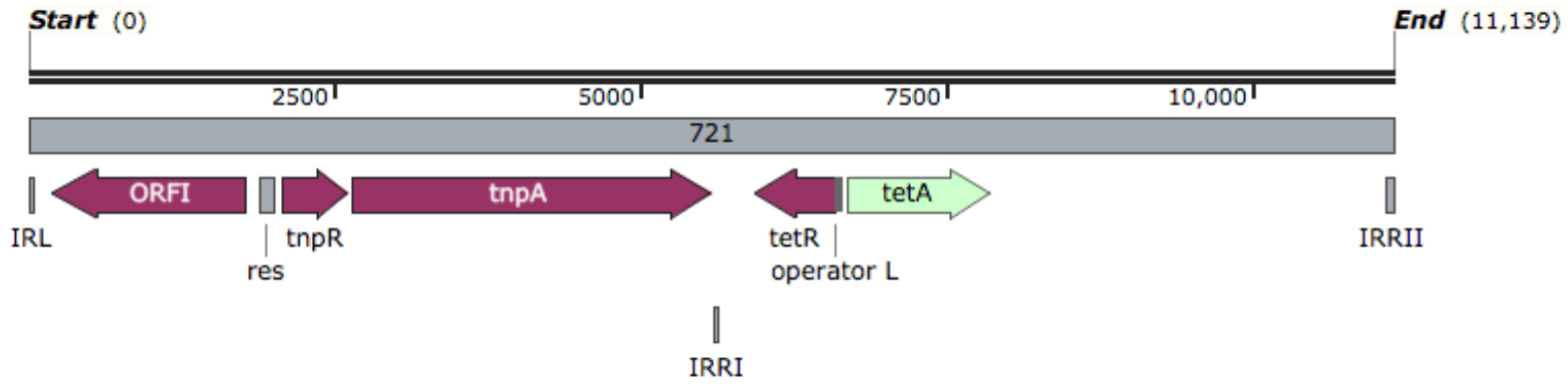


Figure 1.6: The 11,139 bp Tn*1721* that encodes the tetracycline resistance genes *tetAR*. Adapted from: (Allmeier et al. 1992)

There are several routes to acquired resistance in association with MGEs, namely through plasmids, transposable elements, bacteriophage transduction and transformation and all come under the term HGT. Bacterial genomes are dynamic and exhibit plasticity through genomic rearrangement via the activity of HGT (Darmon and Leach 2014). Bacteria gain their resistance to antibiotics predominantly by HGT, thus expanding their gene repertoire and allowing increased survival under antibiotic pressure and the exploitation of niches. Three main mechanisms exist for HGT; namely transformation, transduction and conjugation. Natural transformation involves competent bacteria taking up DNA from the environment and incorporating it into their own genome, with a good example being the naturally competent *Acinetobacter* spp. (C Reygaert 2018). Transduction mediated by bacteriophage is another possible route but the major contributor is conjugation, which requires the actions of a conjugative plasmid to deliver MGE such as transposons or plasmids and even entire chromosomes to a target cell (Norman et al. 2009; Guglielmini et al. 2013; Huddleston 2014).

1.3.2.1. Transduction Mediated by Bacteriophage

Transduction mediated by bacteriophage occurs when a bacteriophage can replicate and package any part of the host bacterial genome (that can be either chromosomal or plasmid) and transfer this to another bacterium. If the cell is one that confers resistance to an antibiotic, upon infecting another cell the bacteriophage can potentially transfer the resistance. This process of genetic transfer is completely accidental and three main types of transduction are currently described in the literature. When the genes packaged

into the bacteriophage head during phage assembly are host-only DNA and completely random through aberrant host DNA packaging, this is known as generalised transduction which is mediated by virulent phage and was first described in *Salmonella* phage P22 (Zinder and Lederberg 1952; Thierauf et al. 2009; Chiang et al. 2019). Specialised transduction mediated by temperate phage, was first identified in Coliphage λ and involves phage lysogeny through integration into the host genome at specific prophage attachment sites and the subsequent transfer of specific bacterial gene sets that are close to this prophage site. The phage genome integrates into the host genome and a DNA hybrid transducing molecule is formed from both host and viral DNA combined known as a prophage. When the phage becomes lytic, phage excision from the host genome then occurs and in this process, through imprecise excision, adjacent bacterial genes are also packaged into the phage head, to be delivered to a new bacterium (Morse et al. 1956; Schneider 2017). A more recently described method of phage transduction, known as lateral transduction, is mediated by temperate phage and was discovered in *Staphylococcus aureus* (Chen et al. 2018). During lateral transduction, following induction into the bacterial chromosome, the phage do not excise, but instead generate capsids with the capability to package downstream insertion site bacterial DNA. The process of lateral transduction has the ability to transfer host chromosomal DNA at up to 1000-fold greater frequencies than has previously been described. These three methods of transduction by phage show the possibilities for antibiotic resistance spread and the capabilities of phage as a potential driver towards transfer of resistance genes, owing to the fact they are one of the most abundant biological entities.

An experiment by Schmieger and Schicklmaier (1999) showed that transduction of resistance to tetracycline, chloramphenicol and AMP was possible using bacteriophage within *Salmonella enterica* serovar Typhimurium DT104. Fard et al. (2011) looked to demonstrate the ability of bacteriophage to transfer resistance genes via transduction within the same and different *Enterococcus* spp. Gentamicin was transduced between strains of *Enterococcus faecalis* and also to other species *Enterococcus hirae/durans* and *Enterococcus casseliflavus* and resistance to tetracycline was transduced from *Enterococcus gallinarum* to *E. faecalis*. The results show interspecies transduction by bacteriophage is a possibility. Both of these studies could offer better understanding about the mechanisms by which resistance arises and how it can be mediated by bacteriophage. Transduction does not require that the donor and recipient be present together and due to the nature of the capsid of the bacteriophage protecting the transduced DNA and the long-lived nature of bacteriophage, persistence within an environment can occur (Muniesa et al. 2013a; 2013b). Bacteriophage-mediated transduction of antibiotic resistance between different taxa is considered to be a rare event, however the idea that bacteriophage could be a link between environmental reservoirs of resistance and human or animal biomes is considered to be a possibility (Muniesa et al. 2013a; 2013b).

Several studies have demonstrated the mobilization of resistance genes by phage including the P1 phage of *E. coli* mobilising the beta-lactamase gene bla_{SHV} (Billard-Pomares et al. 2014) and fosfomycin resistance propagated by the Wβ phage in *Bacillus anthracis* (Schuch and Fischetti 2006; Brown-Jaque et al. 2015)

1.3.2.2. Plasmid Conjugation

Conjugation, whereby a plasmid is transferred via a conjugative pilus through the actions of the Type IV Secretion System, is not a rare event and occurs quite readily among bacterial populations (Huddleston 2014).

Plasmids are extrachromosomal circular DNA, that autonomously replicate and can readily transfer between species of bacteria. Plasmids can be both broad host and narrow host range and can act as vehicles for other mobile elements such as insertion sequences and transposons (Carattoli 2013). Broad host range plasmids are one of the chief vectors key to promoting antibiotic resistance as they can conjugate into a large variety of different host bacteria (Klümper et al. 2015).

Plasmids often contain an array of genes that contribute to the evolution of bacteria, aiding their survival and allowing them to exploit particular niches or survive within hostile environments (Norman et al. 2009; Guglielmini et al. 2013). Plasmids are also instrumental in the spread of antibiotic resistance in both human and veterinary medicine (Darphorn et al. 2021).

Plasmids transfer readily into a variety of host bacteria and a good example are the IncP plasmids, which are subdivided into the two classes, IncP alpha: examples of which include R18, R68, RK2, RP1 and RP4 and IncP-1beta examples of which include pB10, pKJK10 (Yakobson and Guiney 1983; Pansegrau et al. 1994). IncP-1beta plasmids can replicate and transfer into almost any species of proteobacteria including *alpha*, *beta* and *gamma* classes due to being highly promiscuous (Jain and Srivastava 2013).

Genes encoded by plasmids often include not just antibiotic resistance genes but also genes conferring resistance to toxic metals such as silver, mercury or cadmium (Bennett 2008). The incorporation of resistance genes onto plasmids, which can also include integrons, insertion sequences (IS) and transposons (Kim et al. 2014; Ho et al. 2015) and the acquisition of advantageous traits such as virulence determinants is also an essential part of bacterial evolution. This acquisition and mobility potential gives rapid adaption capability thus allowing bacteria to thrive, exploit novel niches and persist in almost any environment (Beceiro et al. 2013). Environments are often continually changing and are exposed to antibiotic residues, metals and other biocides which in turn can result in potential increases of antibiotic resistance and the maintenance of resistance genes (Levy 2002).

Plasmids are ancient and play a key role in bacterial adaption to environmental change and to the dissemination of resistance genes. Genes encoded on plasmids often complement the function of genes encoded on the chromosome, providing bacteria with a successful strategy at colonizing a variety of environments (Wein et al. 2019) including soil (Heuer and Smalla 2012) and even areas rich in heavy metals (Dziewit et al. 2015).

Plasmids are found in abundance within manure and are therefore the perfect vehicle to transport resistance genes to the environment when manure is spread as fertiliser (Heuer et al. 2011b; Wolters et al. 2014). Kim et al. (2014) looked to discover if even low levels of antibiotics, in the parts per billion (ppb) concentration, could contribute to the persistence of antibiotic resistance within the environment through processes such as HGT. Previous studies had revealed promotion of plasmid transfer at 100 ppb concentrations (Al-Masaudi et al.

1991; Ohlsen et al. 2003). The findings of the study by Kim et al. (2014) however, revealed that tetracycline and sulfamethoxazole levels as low as 10 ppb were enough to promote the transfer of the plasmid pB10 via conjugation to both commensal and enteric bacteria. This study demonstrates that in the presence of antibiotics even at sub lethal levels, HGT can be promoted leading to antibiotic resistance dissemination within the environment.

Tetracycline is a commonly used antibiotic within the EU in FPAs and high levels of resistance towards it has been described in both the EU and the US (Szmolka et al. 2015). Szmolka et al. (2015) described two plasmids of IncF and IncI1 type responsible for multidrug resistance via co-transfer with *tetA*, demonstrating transferability of tetracycline and multi-drug resistance. With continued use of tetracycline within farm animals and the risk of contamination to the environment following manure spread, selective pressures towards persistence will likely continue to maintain resistance plasmids (Michalova et al. 2004).

Plasmids are now playing a worrying role in the dissemination of both ESBLs and CPE. ESBLs such as *bla*$_{\text{CTX-M}}$ are now of worldwide concern (Cantón and Coque 2006a; Livermore et al. 2007a; Cantón et al. 2012b), with plasmids playing an important role in their dissemination (Carattoli 2009a) with *bla*$_{\text{CTX-M}}$ frequently reported in association with plasmids of IncFII type, (Novais et al. 2007; Coque et al. 2008a; Villa et al. 2010; Partridge et al. 2011; Zhang et al. 2013a; Agyekum et al. 2016a) which are narrow host-range and limited to the Enterobacteriaceae genera (Carattoli 2009b; Bonnin et al. 2012; Toukdarian 2014).

In addition to ESBLs, CPE emergence and dissemination in humans is of worldwide concern (Nordmann et al. 2011; Rolain and Cornaglia 2014; van Duin and Doi 2017; Bonomo et al. 2018; Hansen 2021), and in addition it has also been reported within FPAs (Köck et al. 2018; Taggar et al. 2020). Carbapenemase genes such as *bla*$_{\text{NDM-1}}$ have been reported in association with a variety of plasmid replicon types including IncF, IncL/M, IncA/C and IncHI1, with IncHI1 and the broad host range IncA/C the most frequently reported types (Carattoli 2013). In addition to *bla*$_{\text{NDM-1}}$, IncA/C plasmids can carry a multitude of additional resistance genes including those conferring resistance to sulphonamides, aminoglycosides, trimethoprim, and chloramphenicol along with genes for persistence and maintenance promotion such as partitioning systems and antirestriction DNA methylases (Colinon et al. 2007; Poole et al. 2009).

1.3.2.3. Transposition

Transposition refers to a genetic event whereby, a piece of DNA sequence is translocated from one site to another. Transposition generally involves genetic structures that contain defined ends, and which are able to mediate self-transposition. Generally, when an element only encodes functions required for transposition and is around 1-2 kb in size, it will be classed as an insertion sequence (IS). Transposons in comparison are generally at least 3-4 kb in size and encode functions, not essential to transposition, which may include antibiotic resistance for example (Bennett 2004). Transposition can occur by several mechanisms and some of these will be explored in further detail in the subsequent sections with examples of the associated MGE.

1.3.2.4. Insertion Sequences

IS are some of the most abundant and smallest autonomous mobile genetic elements, defined to be simply containing only genes required for transposition (Campbell et al. 1979; Chandler and Siguier 2013). The discovery of IS occurred in the 1960s from observing the generation of mutations associated with them (Jordan et al. 1968; Hirsch et al. 1972). IS are an important factor when considering antibiotic resistance gene carriage and mobility, due to their impact on genome evolution (Siguier et al. 2014a; Vandecraen et al. 2017; Razavi et al. 2020). IS are part of what is known as the mobilome, where variability can be found, which contains accessory genes that form the pan genome of a bacteria. Unlike plasmids and bacteriophage, which are self-transmissible from bacteria to bacteria, IS require integration into a plasmid or uptake by a bacteriophage for transferability (Siguier et al. 2014a; Carr et al. 2021). IS can create truncation/interruption/loss of genes such as porins (Wolter et al. 2004), the introduction of a new stronger promoter sequence or the creation of hybrid promoters for increased gene expression and the activation of neighboring genes (Glansdorff et al. 1981; Prentki et al. 1986) and also increases to efflux activity; an example of the latter is within the AcrAB efflux system, where the insertion of IS*186* was shown to result in inactivation of the repressor AcrR (Jellen-Ritter and Kern 2001). Hawkey et al. (2020) examined the genome sequences of around 120 *Shigella sonnei* and *S. dysenteriae* and 343 *S. flexneri* and demonstrated that IS provide a substantial impact on *Shigella* spp. evolutionary history and diversification. Large scale genome reduction and convergent evolution of *Shigella* spp. was also suggested to have occurred though IS expansion. Within *Shigella* spp., loss mediated by IS of the flagella

operons curli *csg* and *flhDC* has also been reported (Pupo et al. 2000; Prosseda et al. 2012). Hernández-Allés et al. (1999) detailed the loss of porin function through the insertion of IS into the *ompK36* gene of *K. pneumoniae*, resulting in increased resistance to cefoxitin (FOX).

Several IS are known to be associated with antibiotic resistance and are frequently reported, such as IS*26* (He et al. 2015; García et al. 2016; Harmer and Hall 2016; Wong et al. 2017; Harmer and Hall 2019), which has also been reported in association with $bla_{\text{NDM-1}}$ (Weber et al. 2019).

1.3.2.5. Transposons

Transposons play a key role in the uptake of resistance genes and the carriage and transport of integrons (see **Section 1.3.2.6**) (Thenmozhi et al. 2014). Integrons are often found associated with transposons, allowing their mobility from the environment and integration into bacterial DNA. Transposons are also commonly associated with other antimicrobial resistance genes such as resistances to metals and biocides.

Transposons may be classed as either composite or complex both of which will be explored in further detail in the following sections, with a few examples of each outlined.

1.3.2.5.1. Composite Transposons

Composite transposons are modular structures containing a copy of the same IS element at either end forming either terminal inverted repeats (IR) or direct repeats (DR). The transposase required for transposition is provided by either one or both of the IS elements, with the short IR sequences flanking the

IS elements utilised for end recognition. The whole structure between the two terminal IRs then moves as one unit (Bennett 2004; Clark et al. 2019). A few examples include Tn*10* that is flanked by two copies of IS*10* and Tn*5* that is flanked by two copies of IS*50*. The terminal elements of both Tn*5* and Tn*10* form IR. Both Tn*5* and Tn*10,* in association with their respective IS elements IS*10* and IS*50*, mobilise by a cut and paste mechanism of transposition (Bennett 2004; Haniford and Ellis 2015). Tn*9* in comparison is flanked by two copies of IS*1* and these terminal elements form DR (these DR face each other in the same direction, unlike the IR that are in an opposite orientation) (Clark et al. 2019). IS elements that have become part of a composite transposon do not necessarily lose their own ability to function independently. However, through a process called *coherence*, fusion of the components of the transposon may occur, leading to loss of independent function of the flanking IS elements (Bennett 2004).

Many composite transposons encode genes associated with antibiotic resistance. For example, the composite transposon IS*10* Tn*10* encodes inducible tetracycline resistance (Chalmers et al. 2000), the composite transposon IS*50* Tn*5* includes an operon which encodes kanamycin/neomycin, streptomycin and bleomycin resistance (Reznikoff 1993) and the composite IS*1* Tn*9* encodes chloramphenicol resistance (Clark et al. 2019).

1.3.2.5.2. IS*Ecp1*

Another composite transposon formation known as IS*Ecp1* has gained much attention, through its frequent association with *bla*$_{\text{CTX-M}}$ type beta-lactamases worldwide (Bou et al. 2002; Chanawong et al. 2002; Poirel et al.

2005a; Lartigue et al. 2006a; Zong et al. 2010a; Dhanji et al. 2011b; Bevan et al. 2017a; Hu et al. 2018; Irrgang et al. 2018; Singh et al. 2018a).

IS*Ecp1* is weakly related to IS*1380* and, as a composite transposon, it is unusual as only a single copy of the terminal sequence, IS*Ecp1,* is present, providing the transposition function and the IR sequence. The additional IR sequence originates not from an additional copy of IS*Ecp1*, but rather through the recognition of an imperfect IR, that the IS*Ecp1* transposase mistakes for an IS*Ecp1* IR (Bennett 2004). This means IS*Ecp1* is able to mobilise downstream genes in a one-ended transposition mechanism, following recognition of the imperfect IR right (IR_R) in conjunction with its IR left (IR_L) (Poirel et al. 2005a). This results in a *bla*$_{CTX-M}$ flanked by IS*Ecp1* and an IS*Ecp1* IR homolog. The result of this recognition of a new IR_R is that IS*Ecp1* can collect downstream genes, forming transposition units that can potentially mobilise into plasmids for resistance dissemination. IS*Ecp1* is also known to bring promoter sequences in the form of a new hybrid -35 promoter box, that results in high level expression of downstream genes.

IS*Ecp*1 has also been reported in association with a number of other resistance determinants including *bla*$_{OXA}$ (Potron et al. 2011; Liu et al. 2015; Sonnevend et al. 2017; Izdebski et al. 2018), *bla*$_{CMY-2}$ (Verdet et al. 2009; Fang et al. 2018; Chiu et al. 2020) and *bla*$_{KPC}$ (Martínez et al. 2014), as well as broad-spectrum cephalosporin resistance and increased expression of fosfomycin resistance (Kieffer et al. 2020) and association with the *E. coli* pandemic clone ST131 (Hirai et al. 2013a; Stoesser et al. 2016; Ludden et al. 2020).

It has also been demonstrated that enhanced transposition of IS*Ecp1* can occur, in response to sub-lethal levels of antibiotics including ceftazidime

(CAZ), cefotaxime (CTX) and piperacillin (Lartigue et al. 2006a; Nordmann et al. 2008a). One potential reason for this enhanced transposition could be, that the stress from sub-lethal levels of certain antibiotics may result in the induction of the SOS response, which can increase genetic variability and result in increased transposition (Capy et al. 2000a; Foster 2007)**.** Antibiotics including beta-lactams, trimethoprim and quinolones and environmental contaminants such as metals, are known to promote the SOS response (Beceiro et al. 2013; Kim et al. 2014).

1.3.2.5.3. Complex Transposons

Complex transposons differ from composite transposons in that they do not have a modular structure or the long terminal repeats and are more complex and often contain resistance genes that form part of the body of the transposon. An example of a complex transposon includes the widely distributed Tn*3*, which encodes *bla*$_{\text{TEM-1}}$ conferring resistance to certain beta-lactam antibiotics including AMP and the early cephalosporins (Bennett 2004). Tn*3* encodes the transposase of approx. 1,000 amino acids TnpA and also a resolvase TnpR, involved in the "copy-in" mechanisms of transposition, known as replicative transposition. A cointegrate is formed during transposition, which connects the donor and the target DNA molecule, producing repeat copies of the transposon. Both the transposase and the host machinery are involved in this replicative process and the resolvase TnpR completes the process by site-specific recombination, at a resolution site between the two copies of the duplicated transposon (Nicolas et al. 2015).

1.3.2.5.4. Tn*21* and Tn*1696*

The transposons Tn*21* and Tn*1696*, both carry the mercury resistance operon and mercury was once commonly used as an antimicrobial agent both in human medicine and agriculture. The use of mercury however has now dwindled due to the high levels of toxicity it confers, but resistance to it within bacteria is prevalent and so is the influence mercury resistance plays in the co-selection with antibiotics and biocides.

The transposons Tn*21* and Tn*1696* are large in size and confer resistance to mercury and multiple antibiotics. Despite their similarities, their evolutionary origins are different as are the integrons they carry. Tn*21*, which was first isolated in Japan in the 1950s within the plasmid NR1 (R100) isolated from a strain of *S. flexneri*, is around 19.7 kb in size and carries the class 1 integron In*2* conferring resistance to streptomycin/spectinomycin, quaternary ammonia compounds, sulphonamides and chloramphenicol (de la Cruz and Grinsted 1982; Liebert et al. 1999; Hobman and Crossman 2015). In comparison, Tn*1969*, which was discovered in the 1970s within the plasmid R1033 isolated from a strain of *P. aeruginosa*, is approximately 16kb in size and carries the class 1 integron In*4* conferring resistance to gentamicin, streptomycin, spectinomycin and chloramphenicol (Rubens et al. 1979; Gómez-Lus 1998). Both carry the mercury resistance operon and the integrons within them contain site-specific recombination systems that allow for the capture of resistance gene cassettes (Martinez and de la Cruz 1988; Gómez-Lus 1998). The insertion of the different class 1 integrons is an example of the independent evolution of these two transposons (Partridge et al. 2001).

The mercury transposon has been shown to play a pivotal role in the uptake and integration of resistance genes located on integrons. Integrons associated with Tn*21*-like mercury transposons have been found to confer resistance to multiple antibiotics including beta-lactams. Pathogenic strains are often found to be harboring mercury resistance and those carrying ESBL types such as *bla*$_{CTX}$, *bla*$_{OXA}$ and *bla*$_{TEM}$ have also been found to be associated with mercury transposons such as Tn*21* and Tn*1696* (Novais et al. 2006; Cantón et al. 2008; Novais et al. 2010; Evans and Amyes 2014a).

1.3.2.5.5. Tn*4401*

Another transposon that has gained much attention within the literature due to its association with CPE, namely *bla*$_{KPC,}$ is Tn*4401*. Tn*4401* is a Tn*3* type composite transposon, that is approximately 10 kb in size and the major transposable element associated with the carbapenemase *bla*$_{KPC}$ (Cuzon et al. 2011). Association of *bla*$_{KPC}$ with Tn*4401* often results in high level expression of the carbapenemase gene (Cheruvanky et al. 2017; Decraene et al. 2018). Tn*4401* is flanked by 39 bp imperfect IRs, creates 5 bp target site duplications and appears to have no target site specificity (Cuzon et al. 2011; Stoesser et al. 2020). Tn*4401* was associated with an extensive outbreak of *bla*$_{KPC}$ in Manchester Hospitals within the North West of England and a study by Stoesser et al. (2020), which whole genome sequenced 604 *bla*$_{KPC}$ positive isolates, found Tn*4401* within 97% of the isolates. Cuzon et al. (2011) experimentally showed transposition rates of Tn*4401* to be at a frequency of 4.4 x 10^{-6}, demonstrating Tn*4401* as an active transposon with high rates of mobility found in association with *bla*$_{KPC}$, which is now rapidly spreading worldwide.

1.3.2.6. Integrons

Integrons are site-specific recombination units, containing a recombinase site capable of capturing gene cassettes to express an array of functions and traits within bacteria. Integrons can be divided into five classes, which relates to the integrase gene they are carrying. Unlike transposons, integrons are transposition defective and therefore are required to be in association with either plasmids or transposons for mobility (Cambray et al. 2010; Moura et al. 2012b; 2012a; 2014). Integrons have an extensive variety of at least 130 gene cassettes and when integrated into plasmids and transposons, they can provide bacteria with resistance to almost every antibiotic, many metals, biocides and detergents (Partridge et al. 2009; Gaze et al. 2011). Classes 1 – 3 have been shown to be associated with multi-drug resistant bacteria and are known to be commonly associated with particular transposons. Kargar et al. (2014) looked for the presence of integrons of classes 1 – 3 within multi-drug resistant diarrhoeagenic *E. coli* faecal samples. Of the 69 samples identified as being multi-drug resistant, class 1 was found within 78.26%, class 2 within 76.81% and class 3 within 26.09%. de la Torre et al. (2015) discovered multi-resistant commensal bacteria isolated from piglets, which were carrying both class 1 and class 2 integrons. The examples of HGT within this study indicated the potential routes integron-mediated resistance could take, from commensal bacteria to zoonotic pathogen, possibly allowing resistance to enter both the environment and the food chain. Gaze et al. (2011) reported on the anthropogenic activity affecting the dissemination of class 1 integrons into the environment following land application of slurry or sludge. Reported figures based on data from their study predicted that within each ton of slurry, the

bacterial load incorporating class 1 integrons is potentially >1 x 10^{13} bacteria. The resulting figure being applied to UK agricultural land each year therefore equates to >1.5 x 10^{16} bacteria, which are likely to be capable of carrying resistant MGE. Should resistant bacteria find their way into waterways through agricultural practices, their dissemination to human populations becomes an ever more likely occurrence.

Another type of integron that is chromosomally encoded and known as the super integron (SI) confers even greater plasticity to the bacterial genome, with the possibility to carry up to 200 cassettes. One such SI associated with the pathogenic bacterium *Vibrio cholerae* carries 175 cassettes making up 3% of the *V. cholerae* genome (Mazel et al. 1998). This example of extensive and varied gene-carrying capability represents the importance integrons play in bacterial adaption and survival. Rowe-Magnus et al. (2002) demonstrated that through the application of an antibiotic selective pressure, mobile resistance integrons were able to recruit directly from the SI gene cassette of *V. cholerae*. This recruitment resulted in the acquisition of a chloramphenicol acetyltransferase gene. Following this they also demonstrated that the acquired resistance traits could be successfully conjugated to other relevant bacteria. This study demonstrated that environmental conditions such as antibiotic selective pressure, can play a key role in the acquisition of resistance traits from chromosomally encoded SIs.

Integron recombination has also been shown to trigger what is termed the bacterial SOS response, which can promote HGT. This can further increase the likelihood of gene cassette integration into bacterial cells and result in co-selection of resistance genes (Aminov 2011a). Guerin et al. (2009) found the

excision and gene cassette integration rate rose by 340 following induction of the SOS response in relation to antibiotic exposure.

1.4. VIRULENCE AND PATHOTYPES

Virulence factors of *E. coli* are involved in many processes of the pathogenic pathway including colonisation, invasion, mobility, adhesion, cell entry, secretion of effectors, immune evasion, immunosuppression and nutrient acquisition. Pathotypes are mostly designated according to the site of isolation of the bacteria, for example from within the intestinal tract or from a blood or urine sample and then further differentiated according to the preferred host colonisation site, virulence factors carried and resultant clinical symptoms and outcomes. Those responsible for causing disease in the intestinal tract are designated diarrhoeagenic *E. coli* (DEC) and include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), the EHEC type known as Shiga toxin-producing *E. coli* (STEC) (also referred to as Verocytotoxin-producing *E. coli* (VTEC)), enteroaggregative *E. coli* (EAEC), diffusely-adherent *E. coli* (DAEC) and adherent-invasive *E. coli* (AIEC) (Nataro and Kaper 1998; Kaper et al. 2004; Gomes et al. 2016). Those responsible for causing disease outside the intestinal tract are known as extraintestinal pathogenic *E. coli* (ExPEC) types. Unlike DEC pathotypes, ExPEC are generally described as opportunistic and are placed into the ExPEC category due to isolation site and grouped depending on the host and disease caused, which is followed by subdividing into pathotypes depending on the virulence-associated traits. However, a urinary tract infection (UTI) causing

strain may also cause an infection in the human or animal body at a different location, therefore the ExPEC classification is often more appropriate. ExPEC pathotypes include uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), sepsis-associated *E. coli* (SEPEC) and avian pathogenic *E. coli* (APEC) (Sarowska et al. 2019a; Santos et al. 2020).

Pathotypes associated mostly with humans rather than animals include NMEC, SEPEC, EAEC, EIEC and UPEC. UPEC is a frequently reported cause of UTIs in humans, but it has been found to be a problem within companion animals such as cats and dogs too (Kuhnert et al. 2000; LeCuyer et al. 2018; Zogg et al. 2018; Salgado-Caxito et al. 2021). ETEC, the common cause of travellers' diarrhoea, is also known however to be one of the principle causes of porcine postweaning diarrhoea (Turner et al. 2006) and calf scouring diarrhoea (Nagy and Fekete 2005; Cho and Yoon 2014).

E. coli pathotypes that are often associated with animals include APEC, EHEC and ETEC. ETEC is the most common cause of *E. coli* associated neonatal diarrhoea in sheep and calves and post weaning diarrhoea in piglets (Dubreuil et al. 2016). Healthy cattle are a major reservoir of EHEC types such as the STEC pathotype O157:H7 and are therefore a potential source for food contamination with resulting disease outbreaks (Lim et al. 2010a; Rahal et al. 2012; Beauvais et al. 2018; Kolodziejek et al. 2022). In addition, sheep and goats are asymptomatic shedders of non- O157 STEC and are therefore sub-clinical carriers (la Ragione et al. 2009; Shahzad et al. 2021). APEC are a frequently reported cause of colibacillosis in poultry resulting in significant losses and mortality rates of up to 20%. Mortality in adult swine, as a result of UTIs caused by ExPEC infections have also been reported (Bélanger et al. 2011).

Abri et al. (2019) conducted surveillance of ETEC and EPEC strains among 120 raw meat samples in the form of fresh beef, ground beef and hamburger meat and 102 dairy product samples including yoghurt, cheese and raw milk and *E. coli* was found in 49% of the meat products and 42.2% of the dairy products, of which two were ETEC and four were EPEC. Further studies have also shown that raw meat and milk can be a source of pathogenic *E. coli* such as EPEC and ETEC (Paneto et al. 2007; Mohammed 2012; Canizalez-Roman et al. 2013; Bonyadian et al. 2014; Abri et al. 2019). Paneto et al (2007) looked at 50 samples of raw cheese and found 6% and 2% respectively, as being VTEC and ETEC contaminated. Mohammed looked at 32 *E. coli* isolated from meat products found 15.63% were ETEC, 9.38% were EHEC and 6.26% were EPEC. Canizalez-Roman et al. (2013) reported on the presence of DEC strains in 5,162 food items consumed in Sinaloa between 2008 and 2009 and found, of the 409 *E. coli* detected from food samples, 13.6% were of DEC type, with EPEC the most commonly isolated pathogenic type at 78.5%, followed by EAEC at 10.7%, STEC at 8.9% and ETEC at 1.7%. Bonyadian et al. (2014) found 21.6% of the *E. coli* isolated from 24 unpasteurised cheese samples and 96 raw milk samples contained *E. coli* encoding the enterotoxins enteroaggregative heat-stable toxin 1 (EAST1), heat-stable toxin (STb) and heat labile toxin (LT).

DEC strains have been shown to cause outbreaks and disease cases which may extended to neighbouring countries. An EHEC outbreak in May 2011 in Germany due to the serotype O104:H4 which originated from contaminated sprouts and extended to 15 countries across Europe, caused 3,842 disease cases and resulted in 855 (20% of total cases) cases of severe haemolytic uremic syndrome (HUS) with 35 deaths amongst the HUS patients (4.1%) and 53 deaths

in total, making this outbreak one of the largest caused by EHEC (Buchholz et al. 2011; Frank et al. 2011; Grad et al. 2012). An outbreak in October 2016, in Kanagawa, Japan as a result of EHEC O157:H7 affecting 61 patients with 24 hospitalisations and 4 cases of HUS, was found to be the result of supermarket bought uncooked meat cutlets made from a mixture of minced pork and beef, eggs and onions (Furukawa et al. 2018). An earlier outbreak of EHEC O157:H7 in Sakai City, Osaka, Japan in 1996 which most effected school children, resulted in 9,451 cases between May and December 1996 and 12 deaths, with the source found to be white radish sprouts shipped from one specific farm in July 1996 (Michino et al. 1999). In 2006, a multistate investigation by the Utah and New Mexico health departments involving 26 states, was conducted following 205 patient cases of O157:H7 with 29% developing HUS. This Utah and New Mexico outbreak was found to be attributed to bagged spinach and subsequent field investigations by the FDA was able to link the contamination of the spinach to samples taken from wild pig faeces, cattle manure and a stream on ranches in the Salinas Valley, California (Grant et al. 2008). Annually it is estimated there are around 2.8 million cases worldwide of acute illness as a result of O157:H7 (Majowicz et al. 2014; Dejene et al. 2022). In the US it is estimated there are around 73,480 cases each year of O157:H7 resulting in the hospitalisation of 2,168 people and 61 deaths (Rangel et al. 2005; Scallan et al. 2011; Dejene et al. 2022) with an economic burden of $607 million (Scharff 2012; Dejene et al. 2022). O157:H7 has firmly established itself as a significant global zoonotic food-borne pathogen (Chekabab et al. 2013; Munns et al. 2015; Zhang et al. 2018; Bolukaoto et al. 2019).

Most pathotypes may be identified due to the carriage of specific virulence genes, resulting in the development of disease and symptoms seen in the host. This is not the case in AIEC and DAEC however, as virulence genes found in these strains may be common to other strains isolated from extraintestinal infections or even commensal strains. An example is afimbrial adhesins in DAEC, which might be found in both extraintestinal and intestinal pathogens but also commensal *E. coli*. In addition, genes traditionally associated with a particular pathotype may be found among different pathotypes, therefore the presence of a particular virulence gene does not necessarily designate an *E. coli* to one pathotype (Santos et al. 2020).

E. coli is associated with a variety of plasmids, some essential to the virulence of individual strains. Some pathotypes owe part or all of their virulence to HGT by plasmids including ETEC, EPEC, EIEC, ExPEC, EHEC (Johnson and Nolan 2009a)) and UPEC (Cusumano et al. 2010).

Amongst the DEC pathotypes, certain genes are known to be plasmid associated and others are known to be encoded chromosomally. ETEC STb and LT enterotoxins and fimbrial adhesion (K antigens), are plasmid encoded and solely responsible for ETEC pathogenicity (So et al. 1976; So et al. 1978; Zamboni et al. 2004; Qadri et al. 2005; Tobias et al. 2016; Sahl et al. 2017). EAEC carries the pAA plasmid which encodes the Pet (cytotoxin), EAST-1 toxin gene *astA* and *aggR* which regulates aggregative adherence fimbrial expression (Zamboni et al. 2004; Zhang et al. 2013b; Boisen et al. 2014b; Berger et al. 2016a; Jønsson et al. 2017a; Prieto et al. 2021a). EAEC can also have the ShET1 enterotoxin encoded on the chromosome (Harrington et al. 2006; Meza-Segura et al. 2020). However, *astA* can also appear in a range of pathotypes such

as ETEC, APEC, EPEC and ExPEC on both pAA and other plasmids (Paiva De Sousa and Dubreuil 2001a; Paiva De Sousa and Dubreuil 2001b; Yatsuyanagi et al. 2003; Maluta et al. 2017a). EIEC carries the invasion plasmid pINV which is solely responsible for EIEC pathogenicity (Lan et al. 2004; Fung et al. 2015; Pasqua et al. 2017a; Dhakal et al. 2019; Dranenko et al. 2022). Both EPEC and EHEC have the initial adherence factor plasmid EAF which carries the gene for the bundle forming pili *bfpA* (Okeke et al. 2001; Brinkley et al. 2006; Bugarel et al. 2011; Teixeira et al. 2015). The major pathogenicity genes of EPEC and EHEC however are on the chromosome (Pakbin et al. 2021), including the *eae* gene (intimin) and *tir* (intimin receptor), which are on the LEE pathogenicity island in the chromosome. The LEE pathogenicity island also has genes for the type 3 secretion system and signaling pathways which are all needed for pedestal formation and the attaching and effacing mechanism (Mohammadzadeh et al. 2013; Franzin and Sircili 2015). The *stxAB* genes of EHEC are found on a lysogenic phage which is in the chromosome (Nakao and Takeda 2000; Iversen et al. 2015; Berger et al. 2019a; Sy et al. 2020). All these examples demonstrate, within the classically described pathotypes, virulence can be both chromosomally and plasmid encoded.

1.5. BETA-LACTAMASES

Beta-lactamases have been in existence for what has been estimated to be 2 billion years (Hall and Barlow 2004). These ancient enzymes existed long before the selective pressure of mass-produced antibiotics, but soon after the introduction of penicillin, Abraham and Chain reported on 28th December

1940, the discovery of a penicillin destroying enzyme (Abraham and Chain 1940a). Hamilton-Miller was quoted as saying in 1979 that the "penicillinase was born on December 28, 1940" (Hamilton-Miller 1979). Today there is a huge diversity of beta-lactamases which are widespread globally. In particular, ESBLs have become a global health concern due to the spectrum of resistance they confer (Gharavi et al. 2021). It was reported by Day et al. (2019), that within the UK *E. coli* carrying ESBLs are responsible for more than 5,000 cases of bacteraemias annually. Beta-lactamases are enzymes produced by a variety of different Gram negative bacteria, that possess hydrolytic activity towards the amide bond of beta-lactam antibiotics (Bush 2018a; Tooke et al. 2019).

Beta-lactamase enzymes with the increased spectrum to hydrolyse oxyimino-cephalosporins were initially called 'extended broad-spectrum beta-lactamases' which would eventually lead to the term now used, ESBL. Initially ESBLs consisted of beta-lactamases from functional group 2be, which were all mutant derivatives of TEM and SHV types (Livermore 2008). The term ESBL now encompasses many other types such as OXA-11 derived from OXA-10, which was the first OXA type to be designated an ESBL (Evans and Amyes 2014b) and the huge number of CTX-M variants (Livermore 2008).

The TEM type beta-lactamase $bla_{\text{TEM-1,}}$ which was initially described as a plasmid-mediated narrow spectrum beta-lactamase, was first identified in the 1960s (Datta and Kontomichalou 1965a) within a patient in Greece known as Temoniera, resulting in the designation TEM-1 (Medeiros 1984). This was followed by the plasmid mediated sulphydryl variable (SHV) type beta-lactamase $bla_{\text{SHV-1}}$ in the 1980s (Kliebe et al. 1985). Through point mutation at specific loci in the original TEM and SHV types, this gave rise to several

variations of TEM and SHV, resulting in the ESBL phenotype (Bradford 2001; Cantón et al. 2012b). In the 1980s ESBLs established themselves as a major cause of hospital-acquired infection, mainly as a result of *E. coli* and *Klebsiella* spp. producing TEM and SHV type ESBLs (Rawat and Nair 2010). In 1989, the CTX-M type ESBLs were discovered (Bauernfeind et al. 1990; 1992) and by the 1990s, were becoming more prevalent. The name CTX-M originated from both the extended activity towards CTX compared to CAZ and from the location of the original isolation, which was Munich, Germany (Birbrair and Frenette 2016; Ur Rahman et al. 2018).

By the 2000s, the prominence of CTX-M type ESBLs had become a globally reported problem, with evolutionary acceleration of different CTX-M types observed (Cantón et al. 2012b; Cantón 2014). It was believed unlike TEM and SHV types, CTX types did not evolve through point mutation but rather through the acquisition from chromosomally encoded *bla* genes from *Kluyvera* spp. via mobile genetic elements such as IS*Ecp1* (Humeniuk et al. 2002; Poirel et al. 2002; Bonnet 2004; Rodríguez et al. 2004; Lartigue et al. 2006; Rossolini et al. 2008a; Literacka et al. 2009; Bevan et al. 2017).

Table 1.4 which was adapted from Bush (2018b) shows a chronological list of just some of the major beta-lactamases, with their original name and the now currently recognised name, the year they were first identified, the bacterial species they were first identified in, the location and the first description in the literature.

The chemical structures of the 1st – 4th generation penicillins: penicillin, cloxacillin, ampicillin and piperacillin respectively, 1st – 5th generation cephalosporins: cefalexin, cefaclor, cefotaxime, cefquinome and

ceftaroline respectively, the cephamycin cefoxitin and the monobactam aztreonam are shown in **Figure 1.7**. This highlights the beta-lactam ring (encircled in red on each structure) present within all of the chemical structures, which is targeted by beta-lactamases.

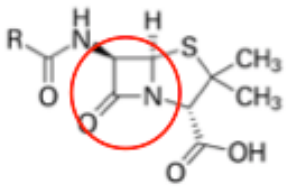
Table 1.4: A chronological list of some of the major beta-lactamases, detailing their original name and now currently recognised name, year of isolation and location, year first described in the literature and reference. Adapted from Bush (2018b)

Beta-Lactamase - original name and (currently recognised name)	Verified Year of First Isolation	Bacterial Species	Location	Year First Described in the Literature	Reference
Penicillinase (chromosomal AmpC)	1940	*Bacillus coli* (*E. coli*)	England	1940	(Abraham and Chain 1940a)
Penicillinase	1942	*S. aureus*	England	1942	(Rammelkamp and Maxon 1942)
OXA	1962	*Salmonella enterica* serovar Typhimurium, *E. coli*[a]	England	1965 1967	(Anderson 1965; Egawa and Sawai 1967)

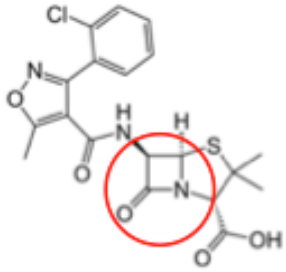
TEM-1	1963	*E. coli*	Greece	1965	(Datta and Kontomichalou 1965b)
SHV-1	1972	*K. pneumoniae*	Unknown	1972	(Pitton 1972)
Transferable ESBL (SHV-2)	Pre-1983	*K. pneumoniae*	Germany	1983	(Knothe et al. 1983)
Serine (class A, group 2f) carbapenemase (SME-1)	1982 1985	*Serratia marcescens*	England (London) USA (Minnesota)	1990 1986	(Medeiros and Hare 1986; Yang et al. 1990)
Plasmid-encoded AmpC (MIR-1)	1988	*K. pneumoniae*	USA (Massachusetts)	1990	(Papanicolaou et al. 1990)
Plasmid-encoded MBL (IMP-1)	1988	*P. aeruginosa*	Japan	1991	(Watanabe et al. 1991)
CTX-M	1989	*E. coli*	France	1990	(Bauernfeind et al. 1990b)

Inhibitor-resistant TEM (TEM-30)	1991	*E. coli*	France (Paris)	1994	(Xiang Yang Zhou et al. 1994)
KPC-type (KPC-2)	1996	*K. pneumoniae*	USA (North Carolina)	2000	(Yigit et al. 2001)
NDM-1	2006	*K. pneumoniae*	India (New Delhi)	2009	(Yong et al. 2009; Castanheira et al. 2011)

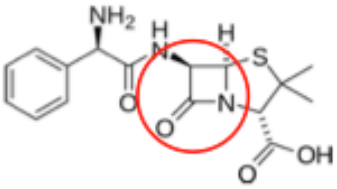
***Footnote for Table 1.4**: [a]Anderson and Datta described a Salmonella Typhimurium isolate from 1962 that later was confirmed to produce the bla$_{OXA-2}$ enzyme ((*Anderson 1965)*). Egawa et al. described an E. coli isolate in 1967 that produced the bla$_{OXA-1}$ enzyme ((*Egawa and Sawai 1967)*).*



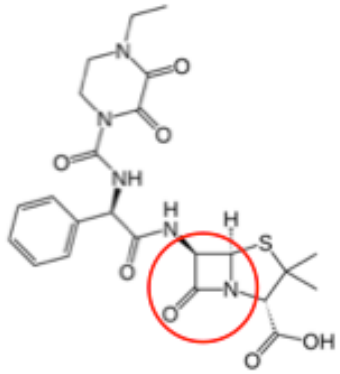
Penicillin
(1st generation penicillin)



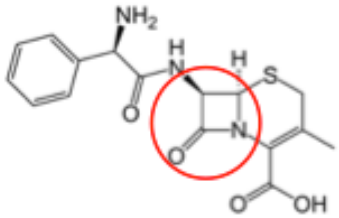
Cloxacillin
(2nd generation penicillin)



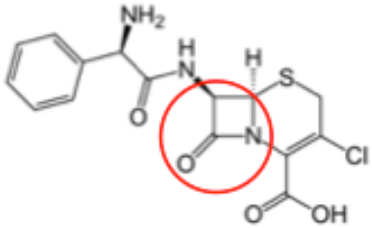
Ampicillin
(3rd generation penicillin)



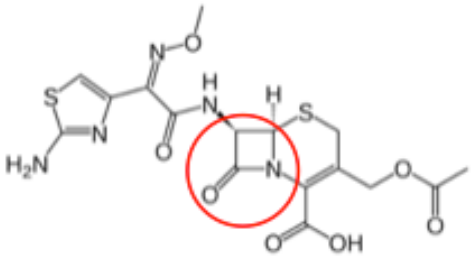
Piperacillin
(4th generation penicillin)



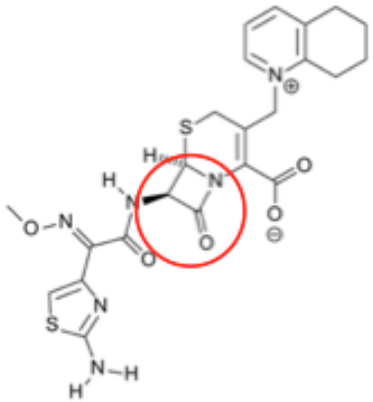
Cefalexin
(1st generation cephalosporin)



Cefaclor
(2nd generation cephalosporin)



Cefotaxime
(3rd generation cephalosporin)



Cefquinome
(4th generation cephalosporin)

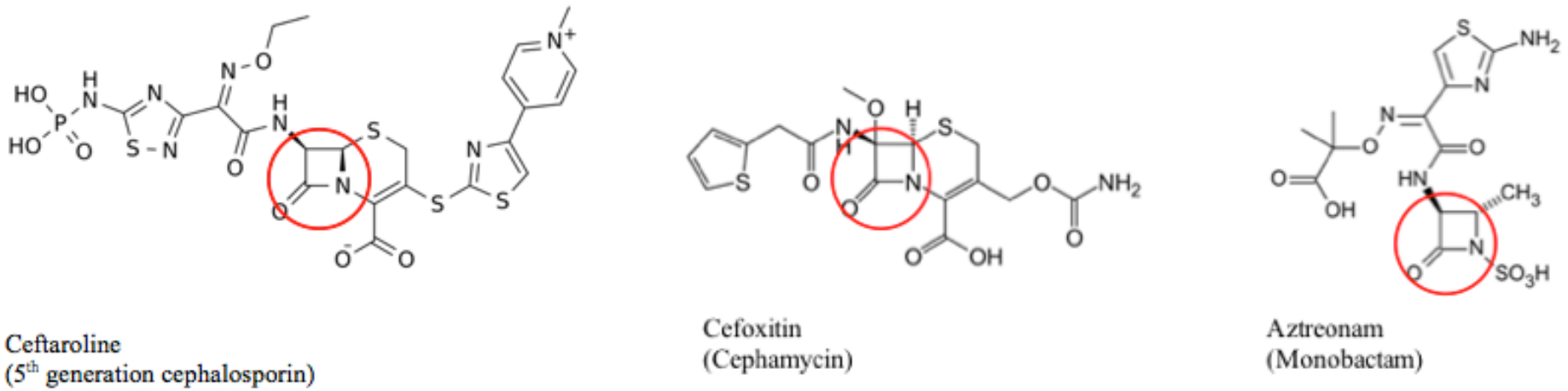


Figure 1.7: Chemical structures of the 1st – 4th generation penicillins, 1st – 5th generation cephalosporins, the cephamycin cefoxitin and the monobactam aztreonam with the beta-lactam ring encircled in red on each structure. Adapted from (Turner et al. 2022)

1.5.1. Beta-Lactamase Classification Schemes

Currently there are two schemes used to classify the enormous variety of beta-lactamases which include the Bush-Jacoby system and the Ambler system. The Bush-Jacoby system is based on a functional classification and includes Groups 1-3, which incorporates substrate and inhibitor profiling, allowing grouping of the enzymes correlated with phenotype. Group 1 includes cephalosporinases, which are often found chromosomally encoded in many *Enterobacteriaceae*, with an example being AmpC. Group 1 also includes plasmid encoded enzymes including *bla*$_{CMY}$, *bla*$_{FOX}$ and *bla*$_{MIR}$. Group 2 is the largest group of beta-lactamases and includes the serine beta-lactamases and a number of subgroups which divide the enzymes based on function classification. Finally Group 3 incorporates the metallo-beta-lactamases with examples including *bla*$_{IMP}$ and *bla*$_{VIM}$.

The Ambler system is based on a molecular classification that incorporates amino acid sequence and has the groups A, C and D, which all require serine for beta-lactamase hydrolytic activity and group B, which are all metalloenzymes and utilise zinc as the hydrolytic substrate (Bush and Jacoby 2010; Bush 2018a). The Bush-Jacoby system was updated in 2010, as detailed in Bush and Jacoby (2010) from the original Bush et al. (1995) classification scheme, to include major subgroups that divided the molecular classes based on enzyme specifics, including inhibitor and substrate profiles. (Bush 2013; Bush 2018a). Group 2 is further subdivided and incorporates derivatives of TEM, SHV and CTX. Group 2b includes the early TEM and SHV enzymes, *bla*$_{TEM-1}$, *bla*$_{TEM-2}$ and *bla*$_{SHV-1}$ that hydrolyse the penicillins and the earlier cephalosporins. Group 2be includes the ESBLs, which are broad spectrum

enzymes that hydrolyse the penicillins, cephalosporins and may have activity against the oxyimino beta-lactams such as ceftazidime, cefotaxime or aztreonam. Examples of group 2be ESBLs include CTX-M and the TEM and SHV derivatives that have a broadened substrate specificity for the group 2b enzymes including the examples, *bla*$_{\text{TEM-3}}$, *bla*$_{\text{TEM-10}}$ and *bla*$_{\text{TEM-26}}$ and *bla*$_{\text{SHV-2}}$, *bla*$_{\text{SHV-3}}$ and *bla*$_{\text{SHV-115}}$ respectively. Group 2d includes the OXA type beta-lactamases which are so called due to their ability to hydrolyse oxacillin (Bush and Jacoby 2010).

There are further functional groups that incorporate other beta-lactamases and derivatives and examples of these are shown in **Figure 1.8**, which details a tree of the molecular and functional relationships of the beta-lactamase with representative example of enzymes and enzyme families.

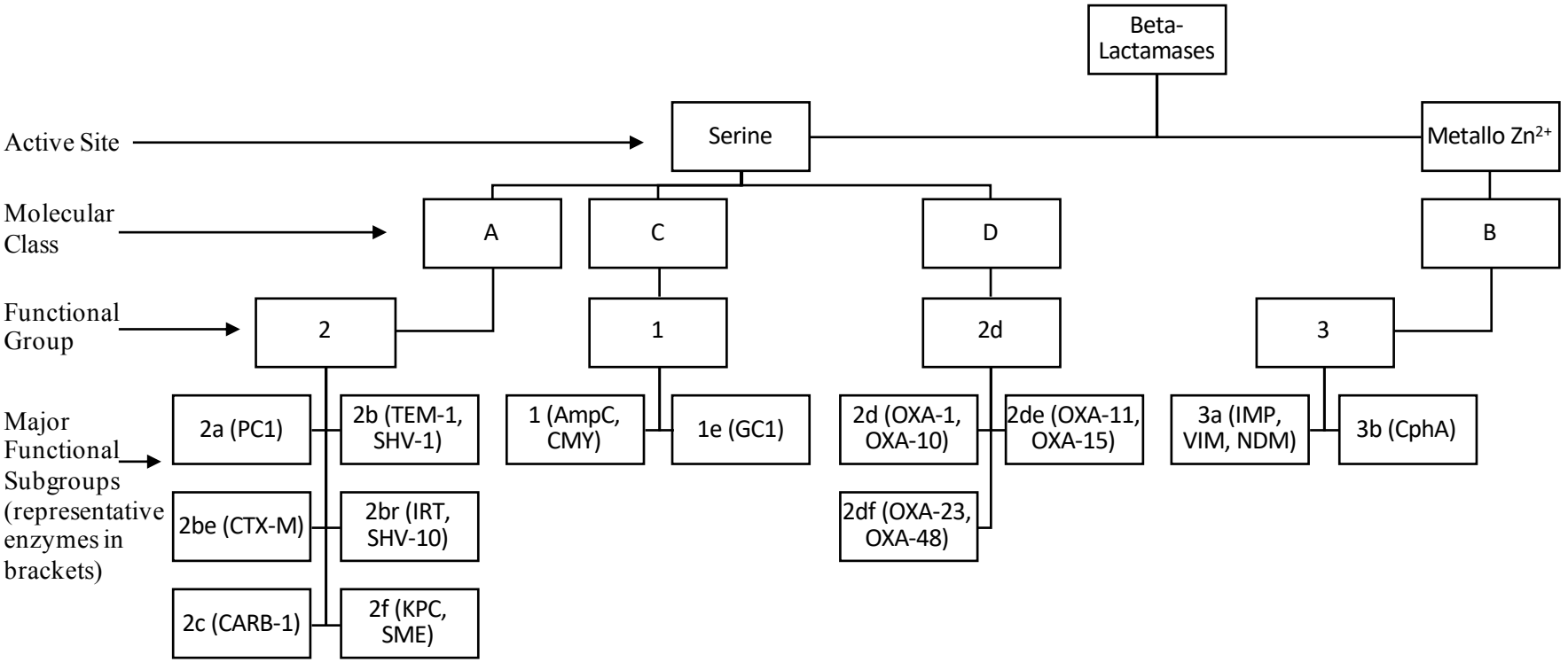


Figure 1.8: The beta-lactamase functional and molecular relationships, detailing the molecular classes, functional groups and major functional subgroups and representative examples of enzymes or enzyme families. Based on: (Bush 2018)

1.5.2. CTX-M type ESBLs

Amongst the CTX-M type ESBLs there are now at least 170 distinct allelic variants, clustered on sequenced-based homology into the five main groups including CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (D'Andrea et al. 2013). **Figure 1.9** shows the similarities between the groups of CTX-M enzymes based on amino acid sequence alignments, with some examples of CTX-M types within each group, along with the *Kluyvera* spp. putative progenitors within each group (which are highlighted in pink on the figure) (adapted from (D'Andrea et al. 2013)). CTX-M variant type ESBLs have almost surpassed TEM and SHV variant types to become the most predominant and globally distributed ESBL (Coque et al. 2008b; Ángel Díaz et al. 2009; Hawkey and Jones 2009; Bush 2010; Rodriguez-Villalobos et al. 2011; Cantón 2014). A possible reason for the displacement of TEM and SHV types by CTX-M types, could be due to the dissemination of the *bla*$_{\text{CTX-M}}$ genes on mobile genetic elements and within highly successful clones and clonal groups such as O25b-ST131 and epidemic plasmids and transposons (Cantón and Coque 2006a; Clermont et al. 2009; Rogers et al. 2011; Woodford et al. 2011; Can et al. 2015; Bevan et al. 2017a; Giedraitienė et al. 2017; Li et al. 2017; Hu et al. 2018; Begu et al. 2019; Demirci et al. 2019). The clonal group ST131 was first identified on three continents in 2008. Previously mostly unknown, ST131 soon became globally widespread and a predominant lineage of ExPEC *E. coli* commonly associated with *bla*$_{\text{CTX-M-15}}$ and fluoroquinolone resistance and responsible for multi-drug resistant infections within healthcare facilities and the community (Nicolas-Chanoine et al. 2014) but has also been seen within animals (Platell et al. 2011) and the environment (Amos et al. 2014; Zurfluh et al. 2014a). Sequence

data analysis of ST131 isolates puts the likely origin data of ST131 at around 1991 in North America (Stoesser et al. 2016). It was believed that the capture of *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$ and also fluoroquinolone resistance by ST131 was likely through the actions of existing plasmids carried by ST131 via IS*26*-mediated transposition; clonal dissemination followed soon after (Bevan et al. 2017a). Co-resistance with fluoroquinolones, aminoglycosides, tetracyclines and sulphonamides is also believed to play a significant role in the now widespread scourge of CTX-M type resistance within Enterobacteriaceae, creating increased therapeutic challenges (Nachimuthu et al. 2020; Yasir et al. 2020).

Increased reports of widespread *bla*$_{\text{CTX-M}}$ type ESBLs within Enterobacteriaceae are being made worldwide within animals (Shiraki et al. 2004; Kojima et al. 2005; Pitout et al. 2005; Cantón and Coque 2006a; Girlich et al. 2007; Li et al. 2007; Livermore et al. 2007a; Schmid et al. 2013). Beta-lactam antibiotics are a commonly used therapy in cattle particularly for the treatment of mastitis (Blum et al. 2014; Kempf et al. 2016). With increasing usage of beta-lactam antibiotics however, the emergence of multi-drug resistance determinants such as broad spectrum *bla*$_{\text{CTX-M,}}$ which hydrolyses many beta-lactam antibiotics, may occur and is now of worldwide concern (Rossolini et al. 2008a; Lynch et al. 2013; McDanel et al. 2017; Afema et al. 2018). The association of *bla*$_{\text{CTX-M}}$ with mobile genetic elements such as plasmids and transposable elements, allows for transmission and sometimes expression of *bla*$_{\text{CTX-M}}$ within environments such as dairy farms (Eckert et al. 2004; Liebana et al. 2013; Irrgang et al. 2017a). One mobile element that has been increasingly reported in association with *bla*$_{\text{CTX-M}}$ is IS*Ecp1* (Poirel et al.

2005a; Rossolini et al. 2008a; Zong et al. 2010a; Agyekum et al. 2016b; Karami et al. 2017; Singh et al. 2018b; Widyatama et al. 2021a; Sultan et al. 2022).

It remains clear that with the continued overuse of antibiotics within both human and animal medicine, the success of resistance genes such as *bla*$_{\text{CTX-M-15}}$ will continue to thrive. Increasing population numbers, global migration and the potential contamination of the food chain and the environment, all play a part in promoting antibiotic resistance maintenance and mobility. Concerns regarding carbapenemase resistance worldwide are now adding to the increasing worry, that antibiotics may in the future be lost as a treatment option in the fight against infectious disease. Carbapenem antibiotics are often reserved for usage against ESBL type infections and the loss of this treatment option would be devastating (Meletis 2016; Codjoe and Donkor 2017; Elshamy and Aboshanab 2020). It is essential that antibiotic stewardship in both humans and animals in a one health approach, is utilised as a strategy to combat antibiotic resistance (McEwen and Collignon 2018a; Hernando-Amado et al. 2019a; Thakur and Gray 2019).

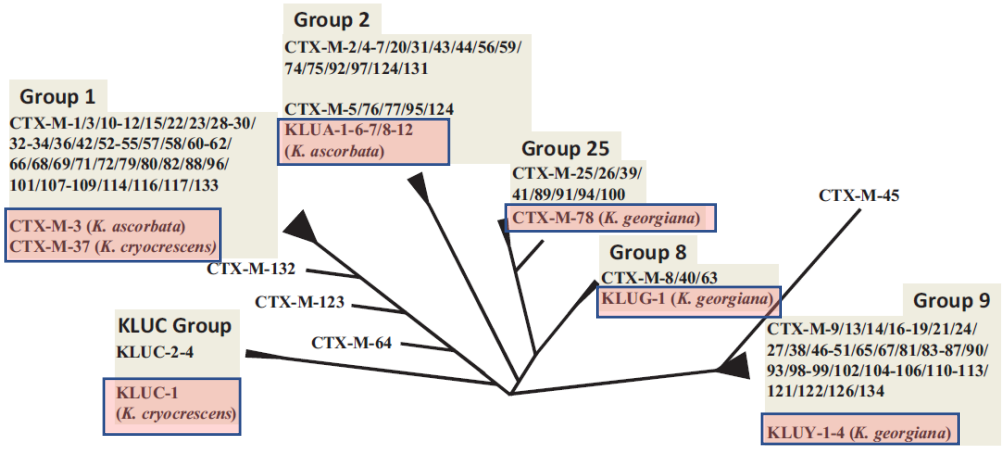


Figure 1.9: The different groups of CTX-M enzymes showing similarities based on amino acid alignment. The *Kluyvera* spp. progenitors are shaded in pink within each group. Adapted from (D'Andrea et al. 2013)

1.5.3. Extended Spectrum Cephalosporinases (ESCs)

Many Gram negative organisms including *E. coli* encode a chromosomal *ampC*, with some examples from the Enterobacteriaceae family being *Shigella* spp., *Citrobacter freundii*, *Enterobacter cloacae*, *Providencia stuartii* and *Klebsiella aerogenes*. In contrast some species from the Enterobacteriaceae family completely lack chromosomal *ampC.* This group includes *Salmonella* spp., *Proteus mirabilis*, *Citrobacter sedlakii*, *Edwardsiella ictaluri*, *Kluyvera ascorbata*, *Klebsiella oxytoca* and *K. pneumoniae* as some examples. Resistance mediated by *ampC* can still occur however, in organisms lacking chromosomal *ampC* such as *Salmonella* spp. and *K. pneumoniae* via plasmid mediated *ampC* such as bla_{CMY} and bla_{FOX}. Chromosomal *ampC* is also found in other species of bacteria not in the Enterobacteriaceae family including *A. baumannii*, *P. aeruginosa*, *S. marcescens*, *Hafnia alvei* and *Morganella morganii* as some examples (Jacoby 2009; Tamma et al. 2019).

AmpC may have a similar hydrolytic profile to ESBLs when expression is increased from the normal low level of expression (Bajaj et al. 2016). The expression level change may be due either to inducible or non-inducible mechanisms and these will be explored in further detail in the subsequent sections. When AmpC production is increased due to the overexpression of *ampC*, the result is resistance to the aminopenicillins such as ampicillin and amoxicillin, earlier generation cephalosporins such as cephalexin and cefapirin, the cephamycins such as cefoxitin and cefotetan and reduced susceptibility to extended spectrum cephalosporins such as the 3rd generation cephalosporins ceftazidime, ceftriaxone and cefotaxime (Mammeri et al. 2008a). When overexpression of *ampC* is present, isolates are often termed extended

spectrum cephalosporinases (ESCs). In comparison ESBLs such as *bla*$_{CTX-M}$ are susceptible to cephamycins and amoxicillin/clavulanic acid but unlike ESCs, are resistant to cefquinome (Caroff et al. 1999a; Drawz and Bonomo 2010a; Peter-Getzlaff et al. 2011a; Haenni et al. 2014a).

1.5.3.1. Inducible Chromosomal *ampC*

Inducible chromosomal *ampC* resistance resulting in hyperexpression of *ampC*, is seen in a variety of Gram negative bacteria with some examples being *P. aeruginosa*, *C. freundii*, *Enterobacter* spp., *Morganella* spp., *Providencia* spp. and *S. marcescens* (Jones et al. 1997) . In bacteria with inducible *ampC*, the expression of *ampC* under normal conditions is regulated by the *amp* operon which consists of *ampC*, *ampD*, *ampR*, *ampE*, and *ampG* (Yu et al. 2009a). The repressor *ampR*, under normal conditions with no beta-lactam exposure, regulates translation of *ampC* and keeps expression at low levels (Schmidtke and Hanson 2006; Tamma et al. 2019). However, in the presence of some beta-lactams, *ampR* can act as a transcriptional activator which in an *in vitro* model of *C. freundii* was shown to increase the expression of *ampC* by more than 11-fold (Lindberg et al. 1985; Tamma et al. 2019). Examples of antibiotics that are potent inducers of the *ampC* hyperexpression pathway include cephamycins such as cefoxitin, 1st generation cephalosporins, aminopenicillins such as ampicillin and the beta-lactam/beta-lactamase inhibitor combination amoxicillin-clavulanic acid. Following exposure to the antibiotic inducers, cell wall degradation products accumulate and competitively bind to the transcriptional regulator AmpR resulting in reduced uridine diphosphate (UDP) -N- acetylmuramic acid peptides bound to AmpR and disabling its

function to regulate *ampC* expression. AmpD, an *N*-acetylmuramyl-L-alanine amidase works alongside AmpR, to cleave the residues of cell wall degradation 1,6-anhydromuropeptides into 1,6-anhydromuramic acid and peptides and recycle them back into the cell-synthesis pathway, thus preventing them from binding to AmpR. However, with increases of cell wall degradation products, as a result of antibiotic exposure, AmpD can no longer cleave the peptides sufficiently to prevent binding to AmpR. Mutations in the *amp* operon most commonly occur in *ampD* followed by *ampR* which may result in a derepression of *ampC* and subsequent overtranscription of *ampC*, which does not require a beta-lactam to trigger it. Deletion mutations in *ampR* can generate a non-inducible *ampC* with expression two to three times higher than normal (Sanders et al. 1997; Schmidtke and Hanson 2006; Tamma et al. 2019).

1.5.3.2. Non-Inducible Chromosomal *ampC*

There is a marked difference in the regulation of expression of *ampC* in some species of bacteria, as it is non-inducible. The regulation of *ampC* in non- inducible *ampC* bacteria such as *Shigella* spp., *A. baumannii* and *E. coli*, is not via the regulator *ampR*, as bacteria in this category lack *ampR*. Rather *ampC* is constitutively expressed but at a low level, due to the regulation of expression through the combination of weak promoters and a strong attenuator. *E. coli ampC* promoters consist of two hexamers of sigma 70-type, with a spacer sequence between them of 15-21 bases, followed by an attenuator of 21 bp (Forward et al. 2001; Peter-Getzlaff et al. 2011a). Mutations within specific areas of either the promoter or attenuator may result in overexpression of *ampC* (Schmidtke and Hanson 2006; Peter-Getzlaff et al. 2011a) . Promoter strength is

generally correlated to the level of homology to the consensus sequence for both the -35 (TTGACA) box and the -10 (TATAAT) box (also known as the Pribnow box), which are crucial for RNA Polymerase σ subunit fixation (Caroff et al. 2000; Mandal et al. 2016). There is also an optimal distance between the two promoter boxes of 17 bp, which plays an important role in promoter strength. The strong promoter pair and ideal spacer length consisting of -35 <spacer> -10 has sequence homology to the consensus sequence of TTGACA-17 bp spacer-TATAAT. The two promoters in wild type *E. coli* differ enough from the *E. coli* consensus sequence, that *ampC* expression is kept to a low level (Caroff et al. 2000; Mulvey et al. 2005a; Haenni et al. 2014b).

1.5.3.3. Plasmid Mediated AmpC

Plasmid mediated AmpC beta lactamases are believed to have originated from chromosomally encoded AmpC beta-lactamases and are therefore often found to be very closely related. Plasmid mediated AmpC, are found globally and have been arranged into several families, however, those from the same families can sometimes have different origins, for example, *bla*$_{\text{CMY-1}}$, -8, -9, -10, -11, and -19 are closely related to the chromosomal AmpC of *Aeromonas* spp., whereas *bla*$_{\text{CMY-2}}$ has a different origin, being related to AmpC beta lactamases of *Citrobacter freundii* (Jacoby 2009). The main families of plasmid mediated AmpC, include *bla*$_{\text{CMY-1}}$ (origin *Aeromonas hydrophilia*) and *bla*$_{\text{CMY-2}}$ (origin *C. freundii*), *bla*$_{\text{LAT}}$ (also origin *C. freundii*), *bla*$_{\text{ACC}}$ (origin *Hafnia alvei*), *bla*$_{\text{DHA}}$ (origin *M. morganii*), *bla*$_{\text{MOX}}$ and *bla*$_{\text{FOX}}$ (origin *Aeromonas* spp.) and *bla*$_{\text{MIR}}$ and *bla*$_{\text{ACT}}$ (origin *Enterobacter* spp) (Bush and Bradford 2020). The *bla*$_{\text{CMY}}$ family is the most populated and has a great

diversity of alleles, with *bla*$_{\text{CMY-164}}$ the most recently released to the GenBank database. The latest releases to the GenBank database for the other plasmid encoded AmpC families include *bla*$_{\text{LAT-3}}$, *bla*$_{\text{ACC-8}}$, *bla*$_{\text{DHA-28}}$, *bla*$_{\text{MOX-25}}$, *bla*$_{\text{FOX-21}}$, *bla*$_{\text{MIR-25}}$ and *bla*$_{\text{ACT-111}}$.

A number of mobile genetic elements have been implicated to have possibly mobilised AmpC genes away from the chromosome and into a plasmid, with one being IS*Ecp1*, which has been associated with a variety of different *bla*$_{\text{CMY}}$ variants including *bla*$_{\text{CMY-2}}$ (Giles et al. 2004; Kang et al. 2006; Haldorsen et al. 2008), *bla*$_{\text{CMY-4}}$ (Nakano et al. 2007), *bla*$_{\text{CMY-5}}$ (Wu et al. 1999), *bla*$_{\text{CMY-7}}$ (Hossain et al. 2004), *bla*$_{\text{CMY-12}}$, *bla*$_{\text{CMY-14}}$ and *bla*$_{\text{CMY-15}}$ (Literacka et al. 2004), *bla*$_{\text{CMY-16}}$ (D'Andrea et al. 2006) and *bla*$_{\text{CMY-21}}$ (Hopkins et al. 2006). A variety of plasmid types have been found associated with *bla*$_{\text{CMY-2}}$, however those of Inc A/C and IncI are most common (Accogli et al. 2013; Ingti et al. 2018; Carattoli et al. 2021). The *bla*$_{\text{CMY-2}}$ confers resistance to broad-spectrum beta lactams including the penicillins, cephamycins, third generation cephalosporins including ceftiofur and ceftriaxone and also to the beta lactamase inhibitors clavulanic acid, tazobactam and sulbactam. However, *bla*$_{\text{CMY-2}}$ has little to no effect to the susceptibility of cefepime or the carbapenems (Heider et al. 2009; Jacoby 2009; Deng et al. 2015; Bush and Bradford 2020).

Of all the plasmid encoded AmpC types, *bla*$_{\text{CMY-2}}$ is the one most commonly detected and has been found globally in both humans and FPAs (Pérez-Pérez and Hanson 2002a; Liu et al. 2007; Mataseje et al. 2010; Accogli et al. 2013; Bortolaia et al. 2014; Ingti et al. 2018; Bush and Bradford 2020; Carattoli et al. 2021; Kim et al. 2021).

In the US in the early 2000s public health concerns were raised, when the stool culture of a patient suffering with acute abdominal pain with fever and diarrhoea, yielded *Salmonella enterica* serotype Typhimurium which was positive for *bla*$_{\text{CMY-2}}$. The *S. enterica* was found to have an animal origin, having come from cattle on the family ranch (Fey et al. 2000; Carattoli et al. 2021). In 2010, *bla*$_{\text{CMY-2}}$ positive *E. coli* was found in imported chickens in Sweden, which generally had a low prevalence of Enterobacteriaceae positive for transferable ESC resistance, as antimicrobial use in broiler production was rare and cephalosporins were never used. It was found that imported chickens for breeding purposes, had introduced the *bla*$_{\text{CMY-2}}$ when a single clone was found to be present within all levels of the production pyramid (Nilsson et al. 2014; 2020).

1.5.4. Extended Spectrum AmpC Beta-Lactamases (ESACs)

The spectrum of hydrolysis can be further increased in *ampC*, when mutations are present in the coding region which results in ESACs. The increased catalytic efficiency has been found to be associated with insertions, deletions, or substitutions to amino acids in the vicinity of the active site (Nordmann and Mammeri 2007). Structural modifications included changes at residues 189 to 225 within the R1 Ω-loop, residues 280 to 292 of the H10 helix of R2 and residues 286 to 310 of the R2 loop. ESACs though structurally related to wild type cephalosporinases, have an increased catalytic profile to ESCs, which is evident with cefepime, a 4$^{\text{th}}$ generation cephalosporin and to a lesser extent imipenem (Mammeri et al. 2008b; Philippon et al. 2022). The first ESAC to be identified was in Japan in 1992 in a strain of *Enterobacter cloacae* known

as GC1, that had three amino acid duplications within the Ω-loop at positions 208 to 210 and was constitutively expressed (Nukaga et al. 1995; Philippon et al. 2022). Since then, ESACs have been reported in a wide variety of *Enterobacteriaceae* including *Enterobacter aerogenes* (Barnaud et al. 2004), *C. freundii* (Ahmed and Shimamoto 2008), *S. marcescens* (Hidri et al. 2005) and *E. coli* (Mammeri et al. 2008b; Bogaerts et al. 2010) including *E. coli* from animals (Haenni et al. 2014a; Santiago et al. 2018) but also *P. aeruginosa* and *A. baumannii* (Rodríguez-Martínez et al. 2009; Rodríguez-Martínez et al. 2010). ESACs are a significant concern considering their contribution to reduced susceptibility to carbapenems (Nordmann and Mammeri 2007; Mammeri et al. 2008b; Sawa et al. 2020).

1.6. ONE HEALTH

As has been outlined in many of the sections of this introduction, we are fast moving towards a decline in treatment options when it comes to multi-drug resistant infections. In addition, food requirements are increasing year on year as population numbers continue to climb and as a result antibiotic use in animals has also continued to climb. The One Health approach was set up to be multisectoral, transdisciplinary and collaborative, to tackle not only global health security in humans but also animals and the environment, to address issues such as AMR, zoonotic disease, food safety and disease emergence and dissemination. The original concept considered not only human and animal health but also the environmental ecosystem in which both exist. One health collaboration works at not only the local and regional level but also the national

and global level (Ryu et al. 2017; McEwen and Collignon 2018b; Walsh 2018; Hernando-Amado et al. 2019b; Sinclair 2019; Aslam et al. 2021; Ratnadass and Deguine 2021; CDC 2022). It is important to consider when looking at zoonotic epidemics, how much can be attributed to negative anthropogenic activities, such as agriculture, the extraction of raw materials such as coal, iron and oil, building of infrastructure such as road and railways and the degradation of natural habitats from activities such as logging and the construction of dams (Patz et al. 2004; Li et al. 2022). All these activities affect the ecosystem by decreasing biodiversity, introducing environmental pollution and potentiating climate change. With increasing land use and habitat degradation, animal populations are pushed closer to human populations, thus increasing contact and the potential for exposure to pathogens for which no natural immunity is present and zoonotic disease transmission (Cunningham et al. 2017). Globally, approximately 60% of emerging infectious disease and 75% of zoonotic disease is believed to have originated from animals (Taylor et al. 2001; Jones et al. 2008b; Wu et al. 2016; Li et al. 2022).

The One Health approach to AMR, looks to preserve our existing antimicrobial therapies, by taking steps in both human and animal health sectors to reducing their inappropriate use, encourage antimicrobial stewardship, disease surveillance, epidemiology, animal management practices and alternatives to antimicrobials. In human health this involves improving infection control, hygiene and sanitation and preventing over prescribing. In animal health it involves reducing the mass medication of food producing animals, preventing use of antibiotics for prophylactic and metaphylactic treatment in healthy animals, preventing the use of antimicrobials that are critical to human medicine

such as 3rd generation cephalosporins and fluoroquinolones, and preventing the use of antimicrobials as growth promoters. In the environment it involves adequate farm, industrial and hospital waste management, reducing the potential for pollution and leaching into the environment, efficient sewage systems, clean drinking water and careful management of manure fertilisation of farmland.

The 'One Health triad' aims to emphasise how important it is to understand that each of the three members of the triad: animals, humans and the environment, all interact with each other and must therefore be considered when tackling AMR related issues. **Figure 1.10** shows the path of AMR through the food chain via food producing animals, towards the environment via the spreading of animal manure onto land and crops, potential introduction to the food chain and towards humans and finally back towards the environment and animals. The One Health collaboration needs to involve many sectors not just farming, veterinary and human medicine but also the consumer, companion animal owners and others involved in the food industry and food production. With better surveillance, communication, education and understanding, all can work together towards the One Health goal (McEwen and Collignon 2018b; Walsh 2018; Aslam et al. 2021).

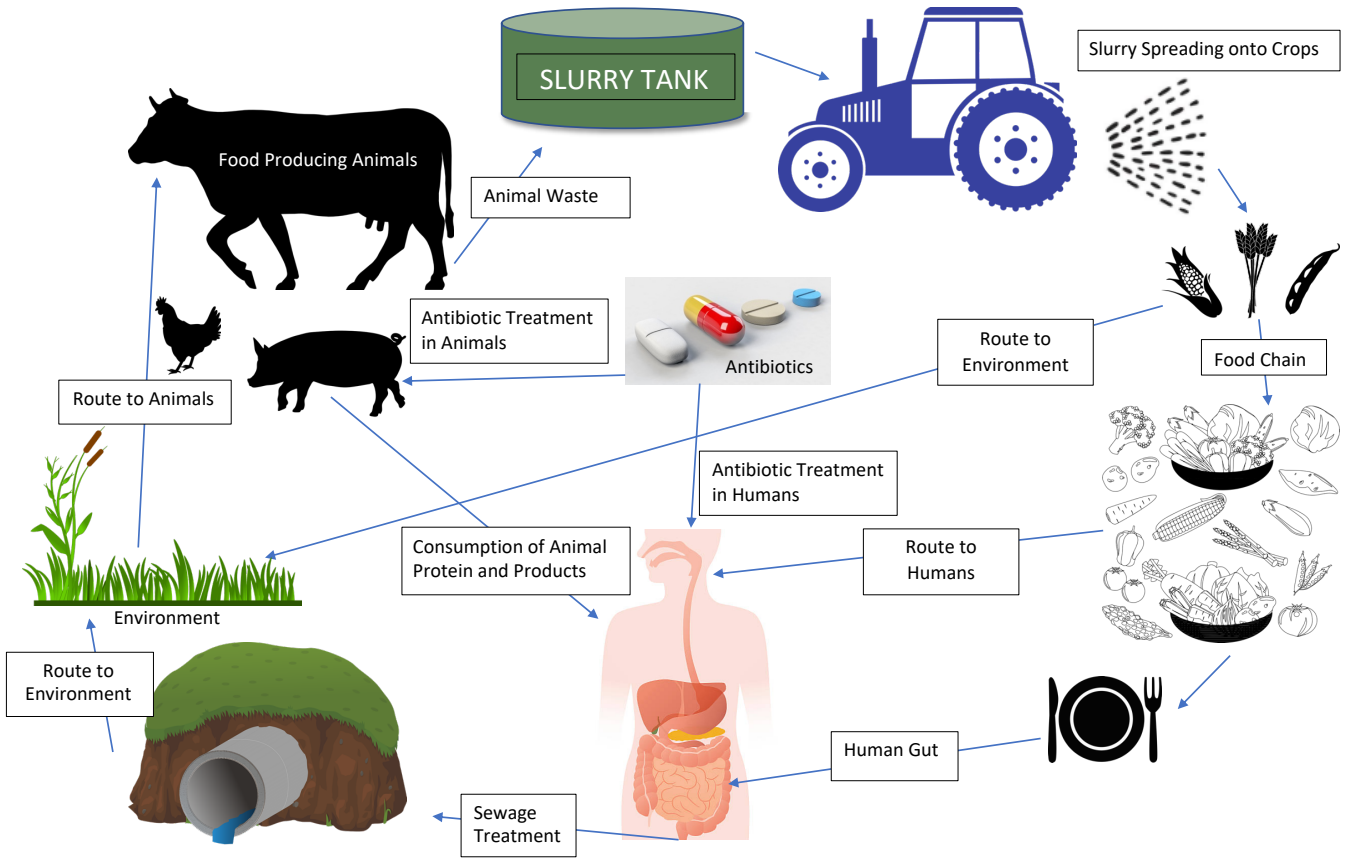


Figure 1.10: A schematic showing the potential transmission paths between animals, humans and the environment and how AMR may travel through the food chain. Adapted from (Walsh 2018)

1.7. AIMS AND OBJECTIVES

The introduction has highlighted the discovery of antibiotics that was quickly followed by the emergence of antibiotic resistance. Also highlighted was the role that MGEs play in resistance dissemination within the environment and why the One Health approach is so important when understanding how to manage AMR. Of particular concern are the ESBLs such as *bla*$_{\text{CTX-M}}$ with *bla*$_{\text{CTX-M-15}}$ now found worldwide in both humans and animals. This study looked to investigate the presence of beta-lactamase type resistance from *E. coli* isolated from a UK dairy farm, with particular focus on the mechanisms involved in the mobility of the associated resistance genes. A previous study by Ibrahim et al. (2016) had discovered the presence of chromosomally encoded *bla*$_{\text{CTX-14}}$ (in association with IS*Ecp1*) and *bla*$_{\text{CTX-M-32}}$ within this UK dairy farm environment. Further investigation using whole genome sequencing had shown a multi-drug resistant plasmid to be present that was carrying a variety of both antibiotic and metal resistance genes. It was therefore thought that plasmids along with IS*Ecp1*, may be playing a key role, in the resistance dissemination of *E. coli* on the dairy farm.

The main objectives included:

i. Further phenotypic characterisation of a number of dairy farm *E. coli* isolates through MIC assays, that had previously been analysed for antibiotic resistance phenotypically through the disc diffusion method as part of the EVAL farms project.

ii. Genotypic characterisation of both individual resistance genes through PCR and Sanger sequencing and also through investigation of whole genome sequences through Illumina short

read and MinION long read whole genome sequencing with hybrid assembly

iii. Characterising the MGEs within the *E. coli* isolates

iv. Understanding the clonality of the *E. coli* isolates through whole genome sequencing, MLST and phylogenetics along with SNP analysis for smaller groups of isolates that appeared to be related

v. Assessing the mobility of *bla*$_{\text{CTX-M}}$ within the *E. coli* isolates, whether resident plasmids were playing a key role in this mobility and whether sub-lethal levels of antibiotics might enhance mobility of MGEs

vi. Looking at potential alternative mechanisms of beta-lactamase type resistance not associated with *bla*$_{\text{CTX-M}}$

The first undertaking which is detailed in **Chapter 3**, was to understand the phenotypic resistance picture, through antimicrobial susceptibility testing via MIC assays. This phenotypic analysis hoped to give an initial indication of the types of resistance mechanisms that might be present. Following phenotypic analysis, the genotypic mechanisms could then be determined, through initial PCR characterisation and Sanger sequencing for individual genes, followed by whole genome sequencing. Whole genome sequencing was undertaken on all isolates, for gene typing and to understand the types of MGEs present and where resistance genes were encoded within the *E. coli* isolates. It was also important to understand whether isolates were related through their association with MGEs and resistance genes or through clonal expansion and this was achieved through both whole genome phylogeny analysis and SNP distance comparison analysis.

The next important question to answer was whether *bla*$_{\text{CTX-M}}$ within the *E. coli* isolates was mobile and able to readily mobilise under laboratory conditions and what type of MGEs were being utilised by the *E. coli* isolates to mobilise *bla*$_{\text{CTX-M}}$. An additional question was also investigated, as to whether sub-lethal levels of antibiotics, similar to those that might be found within the dairy farm environment, might enhance mobility of MGE. Both of these questions were investigated in **Chapter 4**.

Another important question to answer was whether multiple mechanisms, creating beta-lactam resistance phenotypes, were present such as *bla*$_{\text{CTX-M}}$ and overexpression of *ampC*, which both create reduced susceptibility to penicillins, 3rd generation cephalosporins and the monobactam aztreonam and this was investigated in **Chapter 5**.

CHAPTER 2

MATERIALS AND METHODS

2.1. BACTERIAL GROWTH MEDIA

Unless specified otherwise, all growth media, reagents, chemicals, and antibiotic powders were obtained from Sigma-Aldrich, UK.

Growth media for the purposes of bacterial cell culture were prepared in Schott Duran® glassware with reverse osmosis (RO) water, according to manufacturer's instructions. Media were sterilised via autoclaving by heating to 121 °C at 15 psi for 15 minutes (Astell, Kent, UK).

2.1.1. Luria-Bertani (LB) Broth and Agar

LB broth was prepared to a final concentration of 10 g L^{-1} NaCl, 10 g L^{-1} tryptone and 5 g L^{-1} yeast extract, with the addition of 15 g L^{-1} bacteriological agar for LB agar. RO water was added to bring to required volumes prior to autoclaving.

2.1.2. Mueller Hinton (MH) II Agar

MH Agar was prepared to a final concentration of 2 g L^{-1} beef heart infusion, 17.5 g L^{-1} casein acid hydrolysate, 1.5 g L^{-1} starch, soluble and 17 g L^{-1} agar. RO water was added to bring to required volumes prior to autoclaving.

2.1.3. Maximum Recovery Diluent (MRD)

MRD (Oxoid, UK) was prepared from a composition of 1 g L^{-1} of peptone and 8.5 g L^{-1} of NaCl, to a final concentration of 9.5 g L^{-1}. RO water was added to bring to required volumes prior to autoclaving.

2.2. *Escherichia coli* ISOLATES

2.2.1. Dairy Farm Isolates

Dairy farm *E. coli* isolates utilised for this study were sampled and supplied courtesy of EVAL Farms: Evaluating the Threat of Antimicrobial Resistance in Agricultural Manures and Slurries, NERC, Project Reference NE/N019881/1 (EVAL farms) (full details are available at: https://gtr.ukri.org/projects?ref=NE%2FN019881%2F1) and from a previous study by Ibrahim et al. (2016), from various locations around a UK dairy farm and selected on TBX, MacConkey and CHROMagar™ ESBL agars supplemented with and without various concentrations of CTX and AMP. Isolates utilised within this study were selected from a collection of over 1,000 dairy farm *E. coli* samples, that had been characterised phenotypically via the disc diffusion method according to Clinical & Laboratory Standards Institute (CLSI, 2012; CLSI, 2015) by EVAL farms. In addition, all isolates were confirmed as *E. coli* as part of the EVAL farms study by utilising biochemical tests that included oxidase, indole, and catalase tests. **Table 2.1** details the sampling locations around the dairy farm and the abbreviations of these locations used within isolate codes. Isolates selected from the EVAL farms collection, were initially divided into two groups based on their presenting phenotype from the disc diffusion assay data provided by EVAL farms. The two groups were defined as those with resistance to AMP, CTX and ATM but with susceptibility to AMC and FOX, that were likely the result of an ESBL such as bla_{CTX} and those with resistance to AMP, AMC, FOX and CTX, that were likely the result of an alternative mechanism such as an extended spectrum cephalosporinase

(ESC) like overexpression of *ampC*. These two groups are listed in **Tables 2.2** which details those suspected to be encoding an ESBL type genotype (most likely $bla_{\text{CTX-M}}$) and **Table 2.3** which details those likely to be encoding an alternative beta-lactamase type resistance mechanism such as overexpression of *ampC*. The isolates selected were all sequenced via MinION Oxford Nanopore Technologies (ONT) long read and Illumina short read platforms and subject to hybrid assembly and are listed in **Tables 2.2** and **2.3**. Details of sequencing platforms are outlined in **Section 2.7.3**.

Antibiotic usage data was kindly provided for the years 2016-mid 2018 by the dairy farm unit. Graphs produced from the usage data were compiled using Microsoft Excel software.

Table 2.1: EVAL farms sampling locations and abbreviations

Sample Locations	Abbreviations
Slurry Tank	SL or ST
Dairy shed scraper channel	DSSC
Underground reservoir	UR
Heifer shed	HS
Heifer shed 1 (older cows)	HS1
Muck heap effluent	MHE
Slurry solids	SS
Dairy Lane Outside	DLO
Bulling Heifer Shed Scraper Channel	BHSSC

Table 2.2 The 39 *E. coli* EVAL farms dairy farm isolates sampled between 2017-18 and used in the *bla*$_{CTX-M-15}$ and IS*Ecp1* studies explored in Chapters 3 and 4 respectively

Isolate Name	Isolation Date	Sampling Location	Selective Media
EcoSL1010-687	10/10/2017	Slurry Tank	CA*→TBX*
EcoSL1710-726	17/10/2017	Slurry Tank	Mac*+CTX*→TBX+CTX
EcoSL3110-774	31/10/2017	Slurry Tank	Mac+CTX→TBX+CTX
EcoHS11212-873	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-874	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-875	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-876	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-877	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-878	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-879	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-880	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX
EcoHS11212-881	12/12/2017	Heifer shed 1 (older cows)	CA→TBX+CTX

EcoMHE1212-939	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-940	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-941	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-942	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-944	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-945	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-946	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-947	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-948	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1212-949	12/12/2017	Muck heap effluent	CA→TBX+CTX
EcoMHE1801-950	18/01/2018	Muck heap effluent	CA→TBX+CTX
EcoMHE1801-951	18/01/2018	Muck heap effluent	CA→TBX+CTX
EcoMHE1801-952	18/01/2018	Muck heap effluent	CA→TBX+CTX

EcoMHE1801-953	18/01/2018	Muck heap effluent	CA→TBX+CTX
EcoMHE1801-955	18/01/2018	Muck heap effluent	CA→TBX+CTX
EcoMHE1801-956	18/01/2018	Muck heap effluent	CA→TBX+CTX
EcoMHE1801-957	18/01/2018	Muck heap effluent	CA→TBX+CTX
EcoSS2501-958	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-959	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-960	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-961	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-962	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-963	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-964	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-965	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-966	25/01/2018	Slurry solids	CA→TBX+CTX
EcoSS2501-967	25/01/2018	Slurry solids	CA→TBX+CTX

***Footnote for Table 2.2**: Mac – MacConkey Agar, CTX – cefotaxime (at a working concentration of 2 mg L^{-1}), TBX - Tryptone Bile X-Glucuronide Agar, CA – CHROMagar ESBL. The → signifies the isolate was initially isolated on one medium and was then restreaked onto a second medium for E. coli confirmation.*

Table 2.3: The 47 *E. coli* EVAL farms dairy farm isolates sampled between 2017-18 placed into the *ampC* group which is explored in Chapter 5

Isolate Name	Isolation Date	Sample Location	Selective Media
EcoSL2906-99	29/06/2017	Slurry Tank	TBX+Amp100
EcoSL1107-125	11/07/2017	Slurry Tank	TBX+Amp16
EcoSL1107-127	11/07/2017	Slurry Tank	TBX+Amp16
EcoSL1107-152	11/07/2017	Slurry Tank	TBX+Amp16
EcoSL1107-157	11/07/2017	Slurry Tank	TBX+Amp16
EcoSL1807-183	18/07/2017	Slurry Tank	TBX+Amp16
EcoSL0407-209	04/07/2017	Slurry Tank	TBX
EcoSL2906-253	04/07/2017	Slurry Tank	TBX+Amp16
EcoSL2906-295	25/07/2017	Slurry Tank	TBX+Amp16
EcoSL0108-308	01/08/2017	Slurry Tank	TBX
EcoSL0108-309	01/08/2017	Slurry Tank	TBX
EcoSL0108-320	01/08/2017	Slurry Tank	TBX+Amp16
EcoSL0108-326	01/08/2017	Slurry Tank	TBX+Amp16
EcoSL1608-408	16/08/2017	Slurry Tank	TBX+Amp16
EcoSL1608-410	16/08/2017	Slurry Tank	TBX+Amp16
EcoSL2208-431	22/08/2017	Slurry Tank	TBX+Amp16
EcoSL0509-486	05/09/2017	Slurry Tank	TBX+Amp16
EcoSL0509-495	05/09/2017	Slurry Tank	TBX+Amp16
EcoSL0509-508	05/09/2017	Slurry Tank	TBX+Amp16
EcoSL0509-514	05/09/2017	Slurry Tank	TBX+Amp16
EcoSL0509-518	05/09/2017	Slurry Tank	TBX+CTX
EcoSL2209-536	22/09/2017	Slurry Tank	TBX+Amp16
EcoSL2906-582	27/09/2017	Slurry Tank	CA→TBX

EcoDLO2111-825	21/11/2017	Dairy Lane Outside	TBX+Amp16
EcoHS2111-826	21/11/2017	Heifer Shed	TBX+Amp16
EcoST2111-828	21/11/2017	Slurry Tank	TBX+Amp16
EcoMHE2111-833	21/11/2017	Muck Heap Effluent	TBX+Amp16
EcoDSSC2111-854	21/11/2017	Dairy shed scraper channel	TBX+CTX
EcoDSSC2111-855	21/11/2017	Dairy shed scraper channel	TBX+CTX
EcoDSSC2111-856	21/11/2017	Dairy shed scraper channel	TBX+CTX
EcoST2111-859	21/11/2017	Slurry Tank	TBX+CTX
EcoST2111-860	21/11/2017	Slurry Tank	TBX+CTX
EcoST2111-862	21/11/2017	Heifer Shed	TBX+CTX
EcoST2111-863	21/11/2017	Heifer Shed	TBX+CTX
EcoBHSSC2111-864	21/11/2017	Bulling Heifer Shed Scraper Channel	TBX+CTX
EcoBHSSC2111-865	21/11/2017	Bulling Heifer Shed Scraper Channel	TBX+CTX
EcoBHSSC2111-866	21/11/2017	Bulling Heifer Shed Scraper Channel	TBX+CTX
EcoBHSSC2111-867	21/11/2017	Bulling Heifer Shed Scraper Channel	TBX+CTX
EcoUR2111-868	21/11/2017	Underground Reservoir	TBX+CTX
EcoUR2111-869	21/11/2017	Underground Reservoir	TBX+CTX
EcoUR2111-870	21/11/2017	Underground reservoir	TBX+CTX

EcoDSSC2111-871	21/11/2017	Dairy shed scraper channel	TBX+CTX
EcoHS2111-872	21/11/2017	Heifer shed	TBX+CTX
EcoHS1212-887	12/12/2017	Heifer shed 1 (older cows)	TBX+Amp16
EcoST2501-968	25/01/2018	Slurry tank	TBX+CTX
EcoST2501-969	25/01/2018	Slurry tank	TBX+CTX

***Footnote for Table 2.3**: Amp100 – 100 mg L^{-1}Ampicillin and Amp16 – 16 mg L^{-1} Ampicillin as supplements for TBX agar*

2.2.1.1. Control Strains

The control strains utilised for Minimum Inhibitory Concentration (MIC) assays and PCR applications are listed in Table 2.4.

Table 2.4: Strains utilised as controls for MIC assays and PCR

Strain Name	Description	Obtained From	Applications
ATCC25922	*E. coli* FDA strain Seattle 1946 [DSM 1103, NCIB 12210]. Pan susceptible negative control	American Type Culture Collection (ATCC)	MICs and PCR
BCC2	Environmentally isolated *E. coli* encoding $bla_{\text{CTX-M-14}}$, sequenced via PacBio by EVAL Farms	The study by Ibrahim et al. (2016)	MICs and PCR
EcoSL3110-774	Environmentally isolated *E. coli* encoding $bla_{\text{CTX-M-15}}$, sequenced via PacBio by EVAL farms	EVAL farms study	MICs and PCR
EcoUR2111-869	Environmentally isolated *E. coli* encoding an overexpression of *ampC*, sequenced via PacBio by EVAL farms	EVAL farms study	MICs and PCR

2.2.1.2. Recipient Strains

Two recipient strains were used for conjugation assays. These were: *E. coli* CV601 and TG2. CV601, a K-12 derivative, is resistant to both kanamycin and rifampicin and encodes an additional marker of a green fluorescent protein (GFP) encoding gene for correct identification of true transconjugants and was obtained from Prof. Kornelia Smalla (Smalla et al. 2000). TG2 (Gibson 1984), a K-12 derivative resistant to tetracycline, was obtained courtesy of Dr. Jon Hobman.

2.3. BUFFERS AND SOLUTIONS

All buffers were prepared in Schott Duran® glassware and brought to required volumes using RO water prior to sterilisation by autoclaving.

2.3.1. DNA Preparation, PCR and Agarose Gel Electrophoresis

Tris-HCl

Tris-HCl was prepared as a 1 M solution by dissolving 121.1 g of Tris base in 800 ml of RO water, adjusted to pH 8.0 with concentrated HCl, brought to a final volume of 1 L with RO water and sterilised by autoclaving.

EDTA

EDTA was prepared as a 0.5 M solution by dissolving 186.1 g of EDTA in 800 ml of RO water, adjusted to pH 8.0 with 1M NaOH, brought to a final volume of 1 L with RO water and sterilised by autoclaving.

TE Buffer

TE buffer was prepared as a 50 X working stock to a final concentration of 500 mM Tris-HCl and 50 mM EDTA, adjusted to pH 7.5 with either concentrated HCl or 1 M NaOH, brought to a final volume of 1 L with RO water and sterilised by autoclaving. Working stocks of TE buffer were prepared by dilution with HPLC water to 1 X from the 50 X stock.

TAE Buffer

TAE buffer was prepared to a final concentration of 40 mM Tris-HCl, 1 mM EDTA and 20 mM acetic acid, adjusted to pH 8.0 with either concentrated HCl or 1 M NaOH, and brought to a final volume of 1 L with RO water.

2.3.2. Antibiotic Diluent and PBS for MIC Assays

0.1M Acetic Acid

An acetic acid solution was prepared to a final concentration of 0.1M at pH 5.0, by adding 5.72 ml of glacial acetic acid to 500 ml of HPLC water in a 1 L volumetric flask. The solution was swirled gently to mix and then brought to a final volume of 1 L with HPLC water.

Dulbecco's Phosphate Buffered Saline (PBS)

Dulbecco's PBS was supplied as a sterile-filtered complete formulation from Sigma-Aldrich, of 8 g L^{-1} sodium chloride, 0.2 g L^{-1} potassium chloride, 1.15 g L^{-1} sodium phosphate (dibasic) and 0.2 g L^{-1} potassium phosphate (monobasic).

2.3.3. Primers

Primers detailed within this thesis were either utilised from existing publications or designed as novel primers for use in this study. Novel primers were designed by cutting and pasting the nucleotide sequence of the target gene into SnapGene Viewer and then selecting forward and reverse primers covering a minimum amplicon size of 750 bp, with primers of 18-30 nucleotides in length, with an equal mix of GC and AT regions but avoiding dinucleotide repeats, a GC content of between 40 and 60 %, a melting temperature (T_m) of between 65 °C and 75 °C and, to promote primer binding, both primers G or C clamped at the 3' end. Melting temperatures were calculated using the NEB Tm Calculator (available at: https://tmcalculator.neb.com/#!/main) with the following input parameters: Product Group: Taq DNA Polymerase, Polymerase/Kit: Taq 2X Master Mix, Primer Concentration (nM): 200.

Primers were supplied by Eurofins Genomics, Germany in a lyophilized form, requiring rehydration with a volume of HPLC water specified by Eurofins, to give a final stock concentration of 100 pmol μl^{-1}. Rehydrated primer stocks were diluted 1:10 to give a final working concentration of 10 pmol μl^{-1} with both stock and working concentrations stored at -20 °C until required. All primers used within this study are listed within **Table 2.5**.

Table 2.5: Primer sets utilised within PCR analyses

Primer Name	Direction	Sequence (5'-3')	Product Size	Ref (if applicable)
CTX-Fwd	Forward	ATGTGCAGYACCAGTAARGTKATGGC	593 bp	(Dierikx et al. 2012)
CTX-Rvs	Reverse	TGGGTRAARTARGTSACCAGAAYSAGCGG		
ISEcp1-Fwd	Forward	CTCTGCGGTCACTTCATTGG	846 bp	Designed for study
ISEcp1-Rvs	Reverse	CACCGCCATGTCGTATTTGG		
GFP-F	Forward	ATATAGCATGCGTAAAGGAGAAGAACTTTTCA	714 bp	(Andersen et al. 1998)
GFP-R	Reverse	CTCTCAAGCTTATTTGTATAGTTCATCCATGC		
T3SS Lft-ISEcp1-Fwd	Forward	GGACCATTGAATGGATGCGA	807 bp	Designed for study
T3SS Lft-ISEcp1-Rvs	Reverse	CGCACCTTCTTGATGACCT		
prom-AmpC-Fwd	Forward	GATCGTTCTGCCGCTGTG	271 bp	(Peter-Getzlaff et al. 2011b)
prom-AmpC-Rvs	Reverse	GGGCAGCAAATGTGGAGCAA		

2.3.4. Antibiotic Stocks

All antibiotic stocks were prepared in sterile plastic Universal containers (25 mL Bibby Sterilin, UK) as a 1000 X stock using antibiotic powders weighed using a fine balance. Antibiotics requiring preparation with HPLC water were filter sterilised using a 0.22 µM Sartorius Minisart® syringe filter (Scientific Laboratory Supplies Ltd, UK). Antibiotics prepared with ethanol (ETOH), dimethyl sulfoxide (DMSO) or 0.1M acetic acid were not filter sterilised. Antibiotic stocks were stored at -20 °C. Antibiotic stocks are listed in **Tables 2.6** and **2.7**.

Table 2.6: Antibiotics stock solutions along with working concentration and application

Antibiotic	Abbreviation	Stock Concentration and Solvent	Working Concentration	Application
Ampicillin	AMP	100 g L^{-1} in HPLC Water	100 mg L^{-1}	Overexpression of *ampC* isolate selection
Cefotaxime	CTX	2 g L^{-1} in HPLC Water	2 mg L^{-1}	Dairy farm initial isolation selection (**Table 2.2, 2.3** and **2.4**) and CTX positive isolate selection
Kanamycin	KAN	50 g L^{-1} in HPLC Water	50 mg L^{-1}	CV601 recipient selection conjugation
Tetracycline	TET	10 g L^{-1} in 70 % ETOH	10 mg L^{-1}	TG2 recipient selection conjugation

Table 2.7: Antibiotic stocks for minimum inhibitory concentration agar dilution. Detailed are the potency (as defined by the supplier), stock concentration, solvent and concentration range tested. All stocks were prepared as a g L^{-1} concentration unless stated otherwise

Antibiotic	Abbrv	Supplier	Potency (µg/mg)	Stock Concentrations			Solvent	Concentration Range (mg L^{-1})
				Stock 1	Stock 2	Stock 3		
Ampicillin	AMP	Sigma	916	40	4	0.4	Water	4-512
Cloxacillin*	CLOX	Sigma	950	40	4	0.4	DMSO	4-512
Co-Amoxiclav (Amoxicillin and Clavulanic Acid) 4:1	AMC	Sigma	992	1	-	-	Water	4-512
Cefoxitin	FOX	Cayman Chemical Company	980	10	1	0.1	Water	0.032-512
Cefotaxime	CTX	Sigma	964	2	0.2	0.02	Water	0.25-512
Ceftazidime	CAZ	MedChem Express	990	2	0.2	0.02	Water	0.5-512

Cefpodoxime	CPD	Cayman Chemical Company	950	2	0.2	0.02	Ethanol	0.25-512
Cefquinome	CFQ	Chem Cruz	900	2	0.2	0.02	Water	0.125-512
Aztreonam	ATM	Cayman Chemical Company	950	2	0.2	0.02	Ethanol	0.25-512
Imipenem	IMP	Sigma	885	2	0.2	0.02	Water	0.5-8
Ertapenem	ERT	Sigma	843	2	0.1	0.01	Water	0.032-1
Meropenem	MER	Sigma	702	2	0.1	0.01	Water	0.064-2
Streptomycin	STREP	Sigma	732	40	4	0.4	Water	4-64
Gentamicin	GEN	Sigma	590	2	0.2	-	Water	1-8
Neomycin	NEO	Alfa Aesar	600	40	4	0.4	Water	4-32
Apramycin	APR	Sigma	450	40	4	0.4	Water	4-128
Tobramycin	TOB	Sigma	900	2	0.2	-	Water	1-8

Tetracycline	TET	Sigma	980	40	4	0.4	Ethanol	2-512
Tigecycline	TIG	Sigma	992	2	0.2	0.02	DMSO	0.25-2
Ciprofloxacin	CIP	Sigma	980	2	0.1	0.01	0.1M Acetic Acid	0.125-2
Enrofloxacin	ENR	Sigma	990	2	0.1	0.01	DMSO	0.032-64
Nalidixic Acid	NAL	Sigma	900	6	-	-	Water	0.032-512
SXT	SXT	Sigma	980	2	0.2	0.02	DMSO	0.5-16
Chloramphenicol	CHLOR	Sigma	980	2	0.2	0.02	Ethanol	4-32
Azithromycin	AZM	Chem Cruz	958	40	4	0.4	DMSO	8-64
Colistin	COL	Sigma	753	2	0.2	0.02	Water	1-8
Nitrofurantoin	NIT	Sigma	980	10	-	-	Ethanol	32-256

Footnote for Table 2.7: **Cloxacillin was only utilised when conducting MICs on the transposition transconjugants in Chapter 4*

Table 2.8: Enhanced transposition antibiotic stock and working concentrations

Antibiotic	Abbreviation	MIC	Stock Concentration and Solvent	1/2 MIC	1/4 MIC	1/10 MIC
Ampicillin	AMP	32 mg L^{-1}	10 g L^{-1} in HPLC Water	16 mg L^{-1}	8 mg L^{-1}	3.2 mg L^{-1}
Ceftazidime	CAZ	1 mg L^{-1}	0.1 g L^{-1} in HPLC Water	0.5 mg L^{-1}	0.25 mg L^{-1}	0.1 mg L^{-1}
Cloxacillin	CLOX	256 mg L^{-1}	25.6 g L^{-1} in HPLC water	128 mg L^{-1}	64 mg L^{-1}	25.6 mg L^{-1}

Footnote for Table 2.8*: MIC breakpoints were utilised from CLSI* (CLSI 2022) *for establishing antibiotic concentrations for enhanced transposition*

2.4. ANTIBIOTIC SUSCEPTIBILITY TESTING (AST)

AST assays were conducted according to CLSI Methods M07-A11 (CLSI 2018) and utilising principally EUCAST breakpoints (EUCAST 2022) (where available) or alternatively CLSI breakpoints (CLSI 2022). When no breakpoints were listed in either EUCAST or CLSI, the EUCAST guidance document "when there are no breakpoints" was followed (EUCAST 2021) and the epidemiological cut-off (ECOFF) value was utilised in addition to literature stated pharmacokinetic/pharmacodynamic (PK/PD) cut off values and wild type cut off values (CO_{WT}) with a reference to the literature included. An ECOFF is defined as the distribution of MIC values of an antimicrobial drug for a single bacterial species, that represent both the wild type population and those in the population that may have either acquired resistance or gained mutational resistance to the drug. Bacteria with MICs that are greater than the ECOFF are likely to have acquired or mutational resistance to the drug and those with an MIC equal to or lower than the ECOFF are most likely part of the wild type population (Espinel-Ingroff and Turnidge 2016; Tyson et al. 2018; Kahlmeter and Turnidge 2022).

EUCAST defines susceptibility breakpoints into the categories of susceptible, standard dosing regimen (S), susceptible, increase exposure (I) (with exposure defined as a function of how the drug administration, dose, dosing interval, infusion time, distribution and excretion may influence the bacteria during infection) and resistant (R) which are commonly termed S/I/R. Bacteria with results in the susceptible, standard dosing regimen category should have a

high likelihood of therapeutic success when the standard dosing regimen of the antibacterial agent is used. Bacteria with results in the susceptible, increase exposure (previously termed intermediate) should have a high likelihood of therapeutic success when the dosing regimen or concentration is adjusted and exposure to the antibacterial agent is therefore increased. Bacteria with resistant results have a high chance of therapeutic failure, even when exposure to the antimicrobial agent is increased (EUCAST 2019).

2.4.1. Minimum Inhibitory Concentration (MIC) Agar Dilution Assays

MIC assays were conducted using the agar dilution method with antibiotics tested listed in **Table 2.7**. Isolates were revived from frozen stocks along with the control strain ATCC25922 (listed in **Table 2.5**) on non-selective LB agar and incubated for between 18-20 hours at 37 °C. Antibiotic stocks listed in **Table 2.7**, were prepared according to the formula:

$$\frac{1000}{\mathrm{P}}\ x\ V\ x\ C = W$$

Where P = potency (µg/mg as specified by supplier), V = volume (ml), C = final concentration in multiples of 1000 (mg L^{-1}) and W = weight (mg). As specified in **Table 2.7**, appropriate solvents were added to each of the weighed antibiotics; antibiotics soluble in HPLC water were filter-sterilised using a 0.2 µM filter (Sartorius, Germany) prior to use. All antibiotics requiring a stock 2 or 3 were further diluted 1 in 10 using sterile HPLC water. Antibiotics soluble in alternative solvents including DMSO, ethanol and 0.1M acetic acid, were further diluted using sterile HPLC water for stocks 2 and 3.

Specified volumes of antibiotics as listed in **Table 2.7**, were added to a sterile 50 ml falcon tube, followed by the addition of 25 ml of MH agar, which had been cooled to 50 °C. The agar and antibiotic in the falcon tube were gently mixed by inverting the tube several times, before being poured into a 94x16 mm vented petri dish (Greiner Bio-One, UK) and allowed to set.

A MAST URI® DOT (**Figure 2.1**) machine was utilised for inoculating the agar plates using a 96 multi pin inoculator (MPI) head, where several of the pins were removed. The removal of pins from the 96 MPI head, allowed the remaining pins to fit into a 94x16 mm round petri dish as shown in **Figure 2.2**. This allowed for 52 inoculations per 94x16 mm plate, which included 50 isolates, the control ATCC25922 and a broth-only sterility control (SC). The MPI head was sterilised in 90% ethanol for 30 minutes and allowed to dry prior to use.

A direct colony suspension was made by picking 2-3 well isolated colonies from the revived isolates and the pan susceptible control ATCC25922 and dispersing them into 3 ml of sterile Dulbecco's PBS (Sigma-Aldrich) in a sterile bijou (**Section 2.1.2**) to achieve a turbidity of 0.5 McFarland standard (Oxoid, UK) when compared to a Wickerham card as seen in **Figure 2.3**, which should equate to around 10^8 CFU/ml. The McFarland standards consist of a chemical solution of sulfuric acid and barium chloride, which creates a fine precipitate when the two chemicals react in solution. McFarland standards are used to standardise an approximate bacterial number in a liquid suspension. The McFarland standards detailed in **Figure 2.3** show the McFarland standards for 0.5, 1.0, 2.0 and 3.0, however for the purposes of this study, only McFarland 0.5 was utilised. Of the McFarland standards shown in **Figure 2.3**, McFarland 0.5

equates to approximately 1.5 x 10^8, MacFarland 1.0 equates to approximately 3.0 x 10^8, McFarland 2.0 equates to approximately 6.0 x 10^8 and McFarland 3.0 equates to approximately 9.0 x 10^8.

A sterile round bottom 96 well plate (Corning™ Costar™, Fisher Scientific™, UK) was prepared with aliquots of 180 µl of sterile Dulbecco's PBS within each well. **Figure 2.4** details the 96-well microtiter plate layout, that was utilised for preparing the bacterial inoculum for each isolate, with the wells A3-6, B2-7, C1-8, D1-8, E1-8, F1-8, G2-7, H2-3 utilised for the isolates, well H5 utilised for the pan susceptible control ATCC25922 and a sterility control (SC) of Dulbecco's PBS only in well H6 with all remaining wells left empty. Each isolate was allocated to an individual well and 20 µl was added to the well from the direct colony suspension. Wells were then mixed by gently pipetting up and down. This addition of 20 µl from the direct colony suspension bijou to the 180 µl in the microtiter plate, resulted in a 1 in 10 dilution of cells within each well, with each well containing approximately 10^7 CFU/ml. The 96 well plate was placed underneath the MPI head, and each agar plate was inoculated, beginning with the non-selective plate (which acted as the growth control (GC)) and then followed by the lowest concentration of antibiotic. Between each set of antibiotic concentrations, a non-selective plate was inoculated, and the assay was completed with a non-selective plate. Plates were left to dry before being inverted and incubated overnight at 37 °C. Plates were read the following day to obtain the MIC, which was the lowest concentration of the antibiotic which resulted in no visible growth of the bacteria on the agar plates.

Figure 2.1: Mast URI dot machine, with the 96-multipin inoculator head fixed in place with 52 pins inserted. This image details the stage at which the MPI head was being sterilised in 90% ethanol prior to use

Figure 2.2: 96-multipin inoculator head detailing where pins were removed to fit the dimensions of the standard round 94x16 mm petri dish

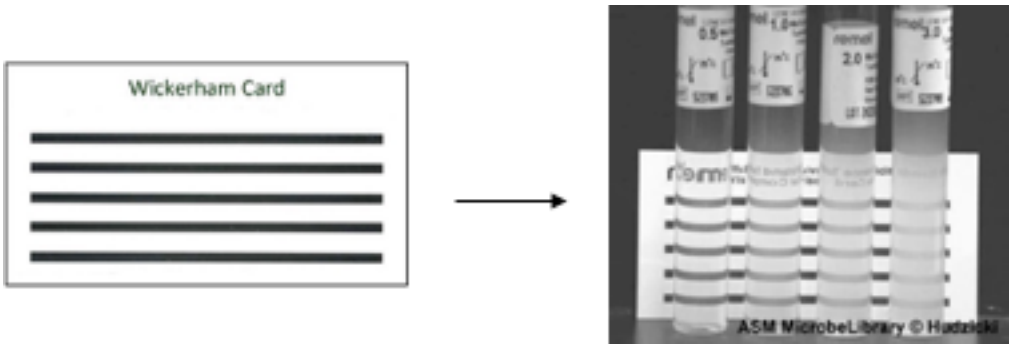


Figure 2.3: Wickerham card and McFarland standards 0.5, 1.0, 2.0 and 3.0 against the Wickerham card, showing the different and increasing turbidity, that equates to 1.5 x 10^8, 3.0 x 10^8, 6.0 x 10^8 and 9.0 x 10^8 respectively (adapted from: http://www.vetlab.com/McFarland%20Wickerham%20Method.pdf)

	1	2	3	4	5	6	7	8	9	10	11	12
A	EMPTY	EMPTY	1	2	3	4	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY
B	EMPTY	5	6	7	8	9	10	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY
C	11	12	13	14	15	16	17	18	EMPTY	EMPTY	EMPTY	EMPTY
D	19	20	21	22	23	24	25	26	EMPTY	EMPTY	EMPTY	EMPTY
E	27	28	29	30	31	32	33	34	EMPTY	EMPTY	EMPTY	EMPTY
F	35	36	37	38	39	40	41	42	EMPTY	EMPTY	EMPTY	EMPTY
G	EMPTY	43	44	45	46	47	48	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY
H	EMPTY	EMPTY	49	50	ATCC 25922	SC	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY

Figure 2.4: 96 well inoculation plate layout, containing the bacterial culture into which the multi-pin inoculator was inserted, which picked up approximately 1 µl of culture to be inoculated onto the surface of the agar plates. SC = sterility control

2.4.2. Heatmaps

Heatmaps were produced using Microsoft Excel conditional formatting, which detailed susceptible, intermediate and resistance results from

disc diffusion assay results provided by EVAL farms and MIC results from this study, as different shades of blue, for both *bla*$_{CTX-M}$ and overexpression of *ampC* isolates. **Appendix A** provides detail of the disc diffusion methods and **Appendix B** details disc concentrations, disc supplier and zone clearing breakpoints of the antibiotic discs used within disc diffusion assays by EVAL farms. The conditional formatting input parameters used within the heatmaps, is shown in **Table 2.9**. Percentages were calculated from each susceptibility total against total number of isolates. For example, in the heatmap example in **Table 2.9**, total resistance would be 33.3%, total intermediate would be 33.3% and total susceptible would be 33.3%.

Table 2.9: Conditional formatting input parameters for heatmaps

Cell Value	Cell Colour	Definition of Susceptibility
= 1		Resistance
= 2		Intermediate
= 3		Susceptible

2.5. PCR DNA PREPARATION

All DNA for PCR application was prepared from cultures of bacterial isolates freshly grown on LB agar at 37 °C for 18-20 hours.

2.5.1. Crude Preparation of DNA for PCR

For all PCR applications the crude DNA preparation method (Wei 2013) was utilised which uses boiling in TE buffer, to disrupt bacterial cell

membranes allowing access to the DNA. Heating is following by centrifugation to separate cellular contents from the cell wall debris. A single colony from an overnight agar plate culture was suspended into 100 µl of TE buffer within an Eppendorf tube and heated at 99 °C for 30 minutes. The sample was then centrifuged at 13,000 *x g* for 15 minutes. The supernatant was aspirated and transferred to a sterile 1.5 ml Eppendorf tube and used as the target DNA in PCR experiments. Any excess crude DNA preparation was stored at -20 °C for future use. Concentration and purity of DNA was measured using a NanoDrop as detailed in **Section 2.5.3**.

2.5.2. DNA Purification for Sequencing

PCR products requiring further analysis by sequencing were purified using a NEB T1030 Monarch® PCR & DNA Cleanup Kit (NEB, UK). Composition of NEB buffers are proprietary, but the following limited information was taken from NEB's website (available at: https://www.neb.com/faqs/2015/12/03/what-is-the-composition-of-each-buffer-provided-with-the-monarch-pcr-dna-cleanup-kit-5-g) as shown in **Table 2.10**.

Table 2.10: Composition of Buffers with NEB T1030 Kit

Buffer Name	Composition
DNA Binding Buffer	Guanidine and isopropanol-based
DNA Wash Buffer	Ethanol-based
Elution Buffer	10 mM Tris, 0.1 mM EDTA at pH 8.5

The kit produced high quality DNA through initial dilution of DNA at a ratio of binding buffer:DNA at 2:1 for fragments >2 kb and 5:1 for fragments <2 kb. Diluted DNA was then bound to the DNA silica membrane column with centrifugation at 16,000 *x g* for 1 minute, followed by two ethanol washes with centrifugation at 16,000 *x g* for 1 minute each and a final elution step in >6 µl of DNA elution buffer. Purity of DNA was measured using a NanoDrop as detailed in **Section 2.5.3**.

2.5.3. NanoDrop DNA Concentration and Purity

The concentration and purity of DNA was measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). When a 1 µl volume of the sample was applied to the base pedestal, the top pedestal was applied resulting in the formation of an aqueous column. The NanoDrop® used the surface tension of this aqueous column to shine UV light through it as seen in **Figure 2.5**, to determine concentration and purity of the DNA sample by absorbance of UV light at a wavelength of 260 nm. The concentration of the DNA was then determined according to the Beer Lambert Law equation to give a value in ng μl^{-1}. Purity of DNA was assessed by two values at 260/280 nm and 260/230 nm. A ratio of ~1.8 for 260/280 nm and ~2.0 for 260/230 nm is accepted as pure for DNA.

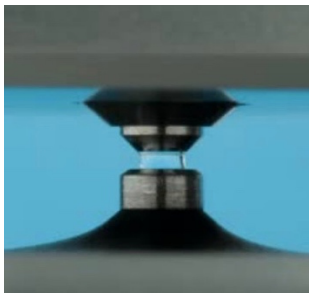


Figure 2.5: NanoDrop aqueous column (Adapted from: (Desjardins et al. 2009))

2.6. POLYMERASE CHAIN REACTION (PCR)

A C1000™ Thermal cycler (Bio-Rad, UK) was used for all PCR reactions. DreamTaq™ Green mastermix 2X (ThermoFisher Scientific, UK) (DreamTaq™ DNA polymerase, optimized DreamTaq Green buffer, 0.4 mM of each of the dNTPs and 4 mM $MgCl_2$) was used for all PCR experiments. Oligonucleotides were synthesised and obtained from Eurofins Genomics GmbH, Germany, and used at a final concentration of 10 pmol μl^{-1}. Oligonucleotides are listed in **Table 2.5**. Mastermix composition for all PCR experiments is listed in **Table 2.11**. Individual PCR conditions are given where each specific PCR reaction is described.

Table 2.11: Mastermix Composition for all PCR Experiments

Component	**Volume µl**
DreamTaq Green Mastermix (2X)	12.5 µl
Template DNA (10 pg – 1 µg)	2 µl
Forward Primer (0.1 – 1.0 µM)	1 µl
Reverse Primer (0.1 – 1.0 µM)	1 µl
HPLC water to 25 µl	8.5 µl
Total Reaction Volume	**25 µl**

2.6.1. Agarose Gel Electrophoresis

GelPilot® LE Agarose (Qiagen, UK) was added at the weight for the required percentage to TAE buffer at pH 8.0 and heated in a microwave oven until clear. The melted agarose was cooled to around 50 °C and then supplemented with ethidium bromide to a final concentration of 0.5 mg L^{-1} before being transferred to a plastic gel cast with a well comb to set. The set gel was then transferred to a gel tank containing 1 x TAE buffer and a DNA ladder loaded to the first well followed by PCR product to each proceeding well, with an optional additional ladder loaded to the final well. Gels were at a specified voltage according to each individual PCR section.

2.6.2. Gel Visualisation

Following completion of gel run time, products were visualised using a Bio-rad Universal Hood II- GelDoc System (Bio-Rad, USA).

2.6.3. *bla*$_{\text{CTX-M}}$

Isolates exhibiting a presumptive CTX type phenotype from studying the disc diffusion data supplied by EVAL farms, (including resistance to AMP, CAZ, CTX, CPD and ATM) were subject to genotypic analysis by PCR for *bla*$_{\text{CTX-M}}$. The isolates BCC2 (Ibrahim et al. 2016) and EcoSL3110-774 (encoding *bla*$_{\text{CTX-M-14}}$, and *bla*$_{\text{CTX-M-15}}$ respectively), were used as positive controls with HPLC water used as a negative control. The *bla*$_{\text{CTX-M}}$ primers utilised from a study by Dierikx et al. (2012) are listed in **Table 2.5** with the primer binding locations shown in **Figure 2.6**. The reaction conditions were as

follows: an initial denaturation at 94 °C for 5 minutes followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute with a final extension of 72 °C for 7 minutes. PCR products were resolved on a 1% TAE agarose gel at 85 V for 1 hour using a 100 bp Quick-Load® DNA Ladder (NEB, UK). DNA of isolates exhibiting positive bands on the gel with amplicons of the correct size were taken forward for WGS. All *bla*$_{CTX-M}$ gene typing was conducted from whole genome sequences, covering the entire gene.

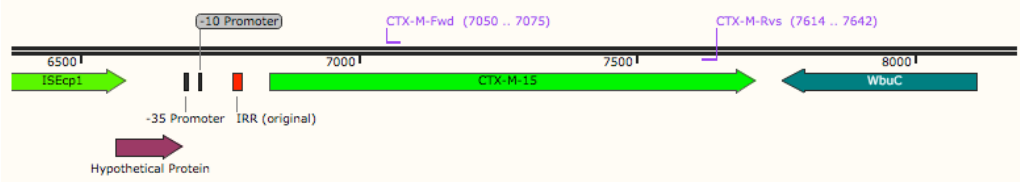


Figure 2.6. Primer binding sites for *bla*$_{CTX-M}$ denoted as CTX-M-Fwd and CTX-M-Rvs in purple

2.6.4. IS*Ecp1*

The primers designed as part of this study are given in **Table 2.5**. **Figure 2.7** details the primer binding locations for IS*Ecp1* denoted in purple as ISEcp1-Fwd and ISEcp1-Rvs resulting in the 846 bp PCR product.

EcoSL3110-774 was used as a positive control, with HPLC water used as a negative control. The reaction conditions were as follows: an initial denaturation at 95 °C for 5 minutes followed by 30 cycles of 94 °C for 1 minute, 54 °C for 1 minute, 72 °C for 2 minutes with a final extension of 72 °C for 10 minutes. PCR products were resolved on a 1% TAE agarose gel at 85 V for 1.5 hours using a 100 bp Quick-Load® DNA Ladder (NEB, UK).

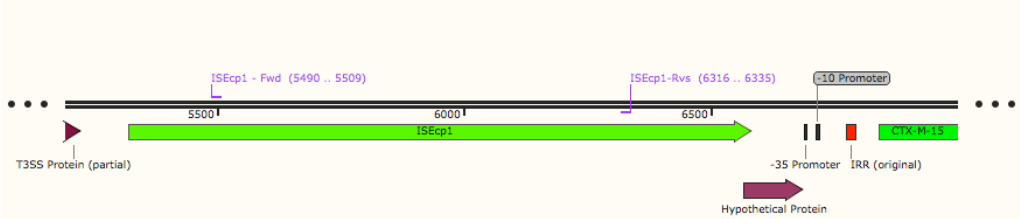


Figure 2.7. Primer binding sites for IS*Ecp1* denoted as ISEcp1-Fwd and ISEcp1-Rvs in purple

2.6.5. GFP

Isolates subject to enhanced transposition that produced transconjugants, were confirmed as true transconjugants by PCR confirmation of the presence of the GFP gene, that was present in the genome of the recipient strain, CV601. CV601 was used as a positive GFP PCR control with HPLC water used as a negative control. The GFP primers were utilised from a study by Andersen *et al*. (1998) and are listed in **Table 2.5**. The reaction conditions were as follows: an initial denaturation at 95 °C for 5 minutes followed by 30 cycles of 94 °C for 1 minute, 54 °C for 2 minutes, 72 °C for 1 minute with a final extension of 72 °C for 10 minutes. PCR products were resolved on a 1% TAE agarose gel at 85 V for 1.5 hours using a 100 bp Quick-Load® DNA Ladder (NEB, UK).

2.6.6. T3SS Lft-IS*Ecp1*

Primers spanning the left insertion point of IS*Ecp1* within the genome of the PacBio sequenced isolate: EcoSL3110-774, were designed as part of this study (listed in **Table 2.5**), to act as a control PCR following enhanced transposition, to look for absence of IS*Ecp1*. The primer binding locations for

the T3SS Lft-IS*Ecp1* PCR are shown in **Figure 2.8**. Isolates where the IS*Ecp1* had successfully mobilised from the chromosome to the resident plasmids, should fail to give a band during gel electrophoresis. The parent strains: EcoSL1010-687, EcoHS11212-876, EcoMHE1801-956 and EcoSS2501-961 acted as positive controls for the IS*Ecp1* PCR, with the recipient strain: CV601, and HPLC water, acting as negative controls. The reaction conditions were as follows: an initial denaturation at 95 °C for 5 minutes followed by 30 cycles of 94 °C for 1 minute, 52 °C for 1 minute, 72 °C for 2 minutes with a final extension of 72 °C for 10 minutes. PCR products were resolved on a 1% TAE agarose gel at 85 V for 1.5 hours using a 100 bp Quick-Load® DNA Ladder (NEB, UK).

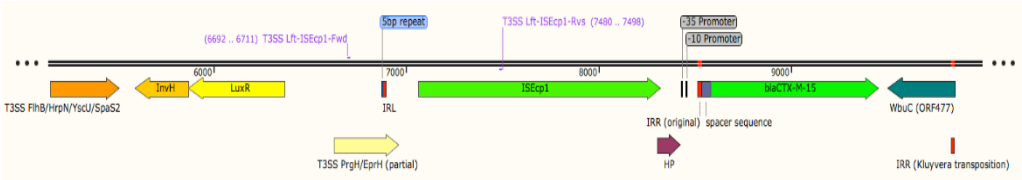


Figure 2.8: Primer binding sites for T3SS insertion point of IS*Ecp1* denoted as T3SS-ISEcp1-Fwd and T3SS-ISEcp1-Rvs in purple

2.6.7. Promoter and Attenuator Regions of *ampC*

Isolates exhibiting the overexpression of *ampC* phenotype in MIC assays (resistance to AMP, AMC, FOX and CTX) as described in **Section 2.4** were subject to PCR analysis to examine the promoter and attenuator regions of *ampC*. Primers from the study by Peter-Getzlaff et al. (2011b) were used and are listed in **Table 2.5**. The isolates EcoUR2111-869 and BCC2 (Ibrahim et al. 2016) were used as positive controls (as EcoUR2111-869 encoded an overexpression of *ampC* genotype, BCC2 encoded both the naturally occurring *ampC* but also $bla_{CTX\text{-}14}$ (Ibrahim et al. 2016)). ATCC25922 which encoded a

WT *ampC* was also utilised as a control and HPLC water was used as a negative control. The reaction conditions were as follows: an initial denaturation at 95 °C for 5 minutes followed by 30 cycles of 94 °C for 1 minute, 56 °C for 1 minute, 72 °C for 1 minute with a final extension of 72 °C for 10 minutes. PCR products were resolved on a 1% TAE agarose gel at 85 V for 45 minutes using a 100 bp Quick-Load® DNA Ladder (NEB, UK). PCR products were purified as per **Section 2.5.2** and sent for Sanger sequencing (**Section 2.7.1**). Sequenced products were visualised in Snapgene viewer to determine the *ampC* mutation type.

2.7. SEQUENCING

All whole genome sequencing (WGS) was completed using an Oxford Nanopore Technologies MinION device to generate long reads and an Illumina MiSeq to generate paired end short reads. All WGS was conducted by external organisations which are noted in the proceeding sections (**2.7.2** and **2.7.3**). Any downstream bioinformatics analysis which was conducted by external organisations is noted within each of the specific sections of **Section 2.8**. Short reads and long reads were hybrid assembled as described within the relevant sections. Sequences were supplied as FASTA, fastq, gbk and gff files.

2.7.1. Sanger Sequencing

The Sanger sequencing TubeSeq service (Eurofins Genomics, Germany) was utilised for sequencing of purified PCR products. DNA was

prepared as per Eurofins guidelines in a final volume of 15 µl and to a final concentration of 1 ng μl^{-1} for amplicon lengths of 150-300 bp, 5 ng μl^{-1} for amplicon lengths of 300-1,000 bp and 10 ng μl^{-1} for amplicon lengths of 1,000-3,000 bp. Prepared DNA was sent to Eurofins, in a 1.5 ml safety lock Eppendorf tube. Primers to a final volume of 15 µl were sent to Eurofins in a separate 1.5 ml safety lock Eppendorf tube at a final concentration of 10 pmol μl^{-1}.

PCR reactions were conducted as described in **Section 2.6**; with PCR products purified using a NEB T1030 PCR Clean-up Kit. Resultant purified amplicon DNA was checked for purity using a Nanodrop (**Section 2.5.3**) before being sent for sequencing. Sequencing results were visualised using Snapgene Viewer.

2.7.2. PacBio

Isolates EcoSL3110-774 and EcoUR2111-869 were sent for WGS as part of the EVAL farms study. PacBio sequencing was conducted by the Centre for Genomic Research (CGR) at the University of Liverpool using 10 kb libraries with 120 times coverage. Whole genome DNA (gDNA) preparation was conducted as part of the EVAL farms study, using the following methods: a Sigma GenElute kit (Sigma-Aldrich, UK) was used to purify gDNA. DNA was analysed with a Nanodrop to ensure the required final concentration of 10 µg in 150 µl TE buffer, with a 260:280 ratio of 1.8-2.0 and a 260:230 ratio of 2.0-2.2. For confirmation of sample integrity, the gDNA was also run on a 0.5 % agarose gel overnight for 17-18 hours at 30-35 V with a NEB 1 kb extend ladder to obtain true gDNA size. Samples were sent to the Centre for Genomic Research at the University of Liverpool in 1.5 ml safety lock Eppendorf tubes. Sequences were

assembled via the CANU assembler pipeline by University of Liverpool and supplied as FASTA, fastq, gbk and gff files.

2.7.3. Illumina Short Read and MinION Oxford Nanopore Technologies (ONT) Long Read Sequencing with Hybrid Assembly

Isolates were sent to Cardiff University on charcoal swabs for gDNA extraction using a Qiagen QIAamp DNA Mini QIAcube Kit via the automated platform QIAcube with an additional RNAse step. The gDNA was quantified using a Qubit v4.0. The gDNA extracted was used to generate sequences for both the Illumina short read and MinION (ONT) long read platforms.

Illumina sequencing library preparation was conducted using the Nextera XT v2 kit (Illumina) with bead-based normalisation for library quantification measurements and was sequenced via the Illumina MiSeq using a v3 300 cycle kit (Illumina). The read length was 300 bp, before trimming.

The gDNA for MinION sequencing was first subject to high performance isolation and purification via Solid Phase Reversible Immobilization (SPRI) bead clean up (Beckman-Coulter). The library preparation was conducted using the SQK-RBK110.96 rapid barcoding kit. Sequencing was conducted on R9.4 flow cells (Oxford Nanopore). The rapid barcoding library kit generated read lengths between 200 bp-60 kb.

2.8. BIOINFORMATICS

Sequences produced using both long read and short read platforms were further analysed using several bioinformatics programs detailed in the following sections.

2.8.1. Genome assemblies

Genomes were assembled by the sequencing service at University of Cardiff and supplied as FASTA, fastq, gbk and gff files. The total coverage (short and long reads) was between 50-100X. Short reads were trimmed using Trimgalore (--phred33 -q 25) v0.5.0. Long reads were basecalled using Guppy v5.0.11. Short reads were assembled in combination with long reads using Unicycler v0.4.7, using default parameters. Unicycler indicates circularisation in the contig name line with the "circular=true". Assembly statistics were calculated using the free desktop software Bandage v0.9.0 , available at: https://rrwick.github.io/Bandage/ and open reading frame numbers were calculated using the ORFinder online software available at: https://www.ncbi.nlm.nih.gov/orffinder/.

2.8.2. Geneious Prime

WGS were visualised using the bioinformatics platform Geneious Prime (Biomatters Ltd, New Zealand). This allowed coding regions (CDS), plasmids and other mobile genetic elements, resistance genes and virulence genes to be manually investigated, along with sequence assembly statistics such as overall %GC content, open reading frames (ORFs) and contig number and

size. In addition, Geneious was utilised for producing and annotating plasmid maps and cross sections of genomic regions to locate the insertion sites of mobile genetic elements.

2.8.2.1. Geneious Prime Alignments

Geneious Prime was utilised for aligning pairs of sequences from WGS for either sections of DNA containing a genetic environment of interest or whole plasmids. This method assessed the optimal relationship between the pair and provided nucleotide statistics of a mean length of sequence between the pair, number of identical sites and pairwise percentage identity. Geneious also produced an identity graphic that could be utilised for manual inspection of the paired sequences to identify areas of high and low identity.

2.8.3. BLAST

The BLAST database (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi#alnHdr_446416380) blastp search option was used to identify from translated amino acid sequences any CDS regions of interest. The query cover, E-value and percentage identity were all considered when deciding upon the correct gene name for annotation.

2.8.4. SnapGene Viewer

The SnapGene Viewer desktop program, was utilised for annotating regions of the sequence of interest by cutting and pasting the nucleotide sequence into a DNA Snapgene file, followed by blastp searches of CDS regions from the

Artemis file. Snapgene allowed for easy annotation of mobile genetic elements and primer design along with primer binding site annotation.

2.8.5. Centre for Genomic Epidemiology (CGE)

CGE is an online platform (available at: http://www.genomicepidemiology.org/) that allows genome sequence data to be analysed through a central database. CGE utilises rapid analysis algorithms and tools allowing data extraction from whole genome sequences (WGSs).

2.8.5.1. MLST 2.0 (Multi-Locus Sequence Typing)

The MLST 2.0 offered by CGE, allowed for the quick analysis of WGS FASTA files to identify the *E. coli* sequence type (ST). The online platform analysed the internal 450-500 bp of internal fragments of 7 housekeeping genes that are listed in **Table 2.12** against MLST stored allele sequences from five regularly updated online databases. For *E. coli* two MLST schemes were available but for the purposes of this thesis, Scheme 1 (Wirth et al. 2006) was utilised, as it has been demonstrated to have both the highest rate of match to alleles in the database and on average a larger number of alleles per locus (Larsen et al. 2012).

Table 2.12: Description of MLST 7 Housekeeping Genes

Gene	Full Name	Description	Ref
adk	Adenylate kinase	A phosphotransferase enzyme that catalyses phosphoryl transfer from adenosine triphosphate (ATP) to adenosine monophosphate resulting in adenosine diphosphate.	(Kim et al. 2005)
fumC	Fumarate hydratase/ Fumarase C	A class II fumarase isozyme involved in the tricarboxylic acid cycle (TCA) cycle. FumC is unaffected by oxidative stress and essentially acts as a backup to the FumAB enzymes in the event of oxidative damage.	(Flint 1994; Tseng 2006)
gyrB	DNA gyrase subunit B	An ATP-dependent type II topoisomerase involved in negative supercoiling of chromosomal double stranded DNA.	(Schoeffler et al. 2010)
icd	Isocitrate dehydrogenase	An $NADP^+$ dependent TCA cycle enzyme involved in producing α-ketoglutarate through oxidative decarboxylation of isocitrate.	(Jung et al. 2006)
mdh	Malate dehydrogenase	Catalytic enzyme involved in the TCA cycle, responsible for the oxidation of malate to oxaloacetate	(Bell et al. 2001)
purA	Adenylosuccinate synthetase	Catalytic enzyme involved in purine biosynthesis.	(Lee et al. 1999)

recA	Protein RecA	Essential to DNA maintenance and repair, genetic recombination and prophage induction.	(Horii et al. 1980)

2.8.5.2. ResFinder 4.1

ResFinder 4.1 from CGE is a web-based platform that includes two programs *ResFinder.py* and *PointFinder.py* that identified both resistance genes and chromosomal point mutations that may lead to resistance from WGSs by utilising BLAST for identification (Zankari et al. 2012a; Bortolaia et al. 2020).

2.8.5.3. VirulenceFinder 2.0

VirulenceFinder 2.0 from CGE analysed WGSs for potential virulence factors by matching to a database of 76 *E. coli* virulence genes within the CGE database (Joensen et al. 2014).

2.8.5.4. PlasmidFinder 2.1

PlasmidFinder 2.1 from CGE analysed WGS FASTA files against an online database of 116 replicon sequences taken from 559 fully sequenced *Enterobacteriaceae* (Carattoli et al. 2014).

2.8.6. Phylogenetics

All phylogenetic tree files were kindly produced and supplied by Dr Kirsty Sands of University of Oxford, with all phylogenetic data analysis and tree annotation performed as part of this study. In total, n=98 *E. coli* isolates were included into the species wide whole genome tree (following quality filtering) and further smaller trees were produced from this, for individual groups of isolates explored in the results **Chapters 3** and **5** respectively. Genomes were annotated by Dr Kirsty Sands using Prokka v1.14.5 and the resulting .gff files

were input into Panaroo v.1.2.10 to create a core genome alignment. A maximum likelihood tree was generated using IQtree v2.0 and annotated using iTOL v.5.7.

2.8.6.1. Enterobase Comparison

All ST2325 genomes (n=110) and metadata were downloaded from Enterobase on 2nd October 2021, with the final total included in analysis being n=105 (following quality filtering and removal of genomes with no assembly).

2.8.7. SNP distance Comparison

All SNP based data generation, were kindly produced and supplied by Dr Kirsty Sands of University of Oxford, with all SNP data analysis performed as part of this study. A representative reference of each dominant sequence type (ST) was selected from the hybrid genomes for variant calling using Snippy version 4.6.0 with the --ctgs flag for the Enterobase genomes v4.4.5, Gubbins v2.3.4 snp-sites v2.5.1 and IQ-tree v2.0. SNP pairwise distances were generated using snp-dists v0.6. Phylogenetic trees were mid-rooted or outgroup rooted where appropriate (a clear single distinct branch) and annotated using iTOL v5.7.

2.9. TRANSPOSITION OF IS*Ecp1*

The protocol for enhanced transposition was adapted from Lartigue *et al.* (2006b) and Nordmann *et al.* (2008a). A baseline rate of transposition was

calculated within non-selective media, with CAZ, AMP and CLOX used as selective agents to examine enhancement of transposition. Concentrations used for each of these antibiotics can be seen in **Table 2.7.** The MIC breakpoints for establishing antibiotic concentrations for enhanced transposition as listed in **Table 2.7**, were utilised from CLSI (CLSI 2022) as opposed to the EUCAST breakpoints (EUCAST 2022) utilised for MIC assays, with the main difference noted being AMP which has a EUCAST breakpoint of 8 mg L^{-1} and CLSI that has a susceptible breakpoint of 8 mg L^{-1} but a resistant breakpoint of 32 mg L^{-1}.

2.9.1. Antibiotic Induced Transposition of IS*Ecp1*

IS*Ecp1* positive isolates (donors) were selected from the $bla_{\text{CTX-M-15}}$ confirmed isolates. The donors were revived from frozen stocks along with the recipient CV601 on non-selective LB agar and incubated at 37 °C for 18-20 hours. A single colony suspension was made into 5 ml LB broth with and without sub-inhibitory levels of the antibiotics (**Table 2.7)** the following day from the donor revive plates. Cultures were grown at 37 °C and 180 RPM (Medline Scientific™ ISF-7100 Floor Standing Incubator Shaker, Fisher Scientific, UK) for 18 hours. A single colony suspension was also made of CV601 into 5 ml LB broth plus 50 mg L^{-1} kanamycin and incubated at 37 °C and 180 RPM (Medline Scientific™ ISF-7100 Floor Standing Incubator Shaker, Fisher Scientific, UK) for 18 hours. The donor cultures were diluted the following day 1 in 100 into 5 ml LB broth non-selective and grown for 3 hours at 37 °C with weak agitation of 100 RPM (Medline Scientific™ ISF-7100 Floor Standing Incubator Shaker, Fisher Scientific, UK). The CV601 culture was centrifuged in 1 ml aliquots at 3, 000 RPM (Sigma 2-7 Compact Centrifuge, Sigma-Aldrich, Germany) for 5

minutes and washed in 1 ml of MRD twice with a final resuspension in 1 ml of MRD. An optical density reading was then taken using a Cecil CE 2021 UV VIS Spectrophotometer at 600 nm and cultures adjusted using MRD to around 0.5-07 OD_{600} to achieve a culture containing around $1x10^8$ CFU ml^{-1}. The adjusted CV601 culture was then used as the recipient component of matings and for plate counts.

2.9.2. Plate Counts

Plate counts were carried out for both the donors and recipient cultures and were taken from the initial 3 hour cultures for the donors and from the OD_{600} adjusted culture of CV601. Serial dilutions from 10^0 – 10^{-7} were prepared for each culture in MRD and plated in duplicate as 100 µl spread plates onto LB plus 100 mg L^{-1} AMP for the donors and LB plus 50 mg L^{-1} kanamycin for CV601. Plates were incubated overnight for 18 hours at 37 °C. Colonies were counted and CFU ml^{-1} calculations conducted the following day using **Equation 2.1**.

$$CFU/ml = (\frac{Number\ of\ colonies}{Amount\ plated})/Dilution\ Plated$$

(Eq 2.1)

2.9.3. Conjugation Mating

A conjugation mating between an IS*Ecp1* positive donor and the recipient CV601 strain was carried out at a 1:4 ratio by adding 200 µl of donor culture to 800 µl of CV601. The mixed culture was gently vortexed and then incubated for 3 hours at 37 °C without agitation. After 3 hours the mating was

stopped by vigorous vortexing and then placing on ice. A serial dilution from 10^0 – 10^{-4} was prepared in MRD and transconjugants selected for by plating 0.1 ml volumes in duplicate on LB plus 100 mg L^{-1} AMP (IS*Ecp1* selective marker) and 50 mg L^{-1} kanamycin (CV601 selective marker). Plates were incubated overnight for an initial 18 hours at 37 °C with an additional incubation of 18 hours if colonies were very small or absent. Transposition transconjugant (TT) (a TT is defined as the IS*Ecp1* having mobilised by transposition into a resident plasmid, followed by conjugation into the recipient strain CV601) colonies were confirmed as positive via UV illumination of the GFP CV601 marker using a UVGL58 UVP Dual Tube Handheld UV Lamp (FisherScientific, UK) at a wavelength of 365 nm. Colonies that were positive for GFP were then counted and the CFU ml^{-1} calculated using **Equation 2.1**. Single TT colonies were re-streaked onto LB plus 100 mg L^{-1} AMP and 50 mg L^{-1} kanamycin to obtain a pure culture and incubated over night for 18 hours at 37 °C. The transposition rate was calculated using **Equation 2.2**.

$$Transposition\ Rate = \frac{CFU/ml\ of\ Transconjugants}{CFU/ml\ of\ Donors}$$

(Eq 2.2)

2.9.4. Conjugation Mating of TTs into the New Recipient TG2

TT isolates were revived on LB agar from frozen stocks and grown overnight at 37 °C for 20 hours. A single colony suspension was made the following day into 5 ml LB broth supplemented with 100 mg L^{-1} AMP. Cultures were grown at 37 °C and 180 RPM (Medline Scientific™ ISF-7100 Floor

Standing Incubator Shaker, Fisher Scientific, UK) for 18 hours. A single colony suspension was also made of TG2 into 5 ml LB broth supplemented with 10 mg L^{-1} tetracycline and incubated at 37 °C and 180 RPM (Medline Scientific™ ISF-7100 Floor Standing Incubator Shaker, Fisher Scientific, UK) for 18 hours. The TT cultures were sub-cultured the following day 1 in 100 into 5 ml LB broth n/s and grown for 3 hours at 37 °C with weak agitation of 100 RPM (Medline Scientific™ ISF-7100 Floor Standing Incubator Shaker, Fisher Scientific, UK). The TG2 culture was spun down in 1 ml aliquots at 3, 000 RPM (Sigma 2-7 Compact Centrifuge, Sigma-Aldrich, Germany) for 5 minutes and washed in 1 ml MRD twice with a final resuspension in 1 ml MRD. An optical density reading was then taken using a Cecil CE 2021 UV VIS Spectrophotometer at 600 nm and cultures adjusted using MRD to around 0.5-07 OD_{600} to achieve a culture containing around 1 x 10^8 CFU/ml. The adjusted TG2 culture was then utilised as the recipient component of matings and for plate counts. Plate counts were conducted according to **Section 2.10.2**, using 100 mg L^{-1} AMP for TT selection and 10 mg L^{-1} tetracycline for TG2 selection. Conjugation matings were conducted according to **Section 2.10.3** with 100 mg L^{-1} AMP and 10 mg L^{-1} tetracycline used as double selective agents. Conjugation rate was calculated using **Equation 2.3**.

$$Conjugation\ Rate = \frac{CFU/ml\ of\ Transconjugants}{CFU/ml\ of\ Recipients}$$

(Eq 2.3)

Chapter 3

bla$_{\text{CTX-M}}$ AND MOBILITY

3.1. INTRODUCTION

Increased reports of widespread *bla*$_{\text{CTX-M}}$ type ESBLs within *Enterobacteriaceae* are being made worldwide, not only in humans, but also animals (Shiraki et al., 2004; Kojima et al., 2005; Pitout et al., 2005; Cantón and Coque, 2006; Li et al., 2007; Girlich et al., 2007; Livermore et al., 2007; Schmid et al., 2013; Ramadan et al., 2019; Dantas Palmeira and Ferreira, 2020). *Enterobacteriaceae* encoding *bla*$_{\text{CTX-M}}$ have not only been cited as being present in food producing animals such as chickens, swine and cattle (Zurfluh et al. 2014b; Cormier et al. 2019; Balázs et al. 2021) but also companion animals such as horses (Shnaiderman-Torban et al. 2020a; Shnaiderman-Torban et al. 2020b) and dogs (Cormier et al., 2019; Abbas et al., 2019; Balázs et al., 2021), wild animals such as deer (Ballash et al. 2022), birds (Haenni et al. 2020; Islam et al. 2021), small mammals such as hedgehogs (Garcias et al. 2021) and aquatic environments (Zurfluh et al. 2014b; Herrig et al. 2020; Ejaz et al. 2021). Beta-lactam antibiotics are a commonly used therapy in cattle especially for the treatment of mastitis and are also used during the dry cow period (Weber et al. 2021). Several species of both Gram negative and Gram positive bacteria are known etiological agents of bovine mastitis. These mastitis-causing agents may be classed based on the bacterial origin, as either contagious or environmental (Shaheen M et al. 2016; Lakew et al. 2019; Cheng and Han 2020). The major bacterial reservoir of contagious agents is the udder, with colonisation and growth in the teat canal (Cheng and Han 2020). Infection is spread from cow-to-cow, with the primary infection hotspot being the milking parlour and from handling of the udder during milking (Schreiner and Ruegg 2002; Cheng and Han 2020). Contagious bacterial agents have the capability to establish sub-

clinical infections, which may be chronic, persistent and hard-to-treat. Examples of contagious agents include *Corynebacterium bovis*, *Mycoplasma* spp. *Streptococcus agalactiae* and *Staphylococcus aureus* (Shaheen M et al. 2016; Kibebew 2017; Cheng and Han 2020).

The primary reservoir of environmental mastitis-causing pathogens is the housing and general environment of the cattle such as the bedding, manure, faeces, wastewater and soil (Smith and Hogan 1993; Bradley 2002; Hogan and Smith 2012; Shaheen M et al. 2016). The environmental bacteria are often described as opportunistic, causing chance infection, and are highly influenced by farm management practice (Smith and Hogan 1993; Hogan and Smith 2012; Cheng and Han 2020). Environmental agents colonise and multiply in the udder, inducing a host immune response, resulting in clinical mastitis and this is followed by rapid elimination from the udder (Bradley 2002; Cheng and Han 2020). Examples of environmental agents include *Enterococcus* spp., *E. coli*, *Klebsiella* spp. *Streptococcus uberis*, *Streptococcus dysgalactiae* and coagulase negative *Staphylococcus* (Smith and Hogan 1993; Hogan and Smith 2012; Klaas and Zadoks 2018; Cheng and Han 2020).

E. coli is a commonly associated pathogen causing bovine mastitis and beta-lactam antibiotics such as penicillin G, ceftiofur (EFT) and cefquinome (CFQ) are often used as therapy for these types of infections (Blum et al. 2014; Kempf et al. 2016). The use of beta-lactams in food-producing animals (FPAs), may select for resistance to human critical antibiotics (van Boeckel et al. 2019; Ogunrinu et al. 2020; Tiseo et al. 2020). As already discussed in **Section 1.2**, with the continued usage of beta-lactam antibiotics in animals, the emergence of multi-drug resistance determinants such as broad spectrum *bla*$_{\text{CTX-M,}}$ which

hydrolyses many beta-lactam antibiotics, continues to be of worldwide concern (Rossolini et al. 2008a; Lynch et al. 2013; McDanel et al. 2017; Afema et al. 2018a; Dantas Palmeira and Ferreira 2020). The association of *bla*$_{\text{CTX-M}}$ with mobile genetic elements, such as plasmids and transposable elements, allows for transmission and potentially expression of *bla*$_{\text{CTX-M}}$ within environments such as dairy farms (Eckert et al. 2004; Liebana et al. 2013; Irrgang et al. 2017a). Therefore, using initial phenotypic data, this chapter explored the resistance mechanisms that were present within the dairy farm, how that resistance might mobilise and disseminate but also persist throughout the dairy farm environment. This was achieved by firstly studying the disc diffusion assay data that were kindly supplied by EVAL farms, then by PCR analysis searching for *bla*$_{\text{CTX-M}}$ and then further phenotypic characterisation via MICs. Lastly detailed genetic analysis via WGS with phylogenetic and SNP distance analysis was conducted to look at clonality.

For the purposes of this study only *E. coli* was investigated. The rationale for choosing *E. coli* was, to look at intraspecies diversity over interspecies diversity. By looking at a smaller number of a single bacterial species, they could be studied in greater detail rather than looking at a larger number of different species of bacteria but in only minor detail. In addition, *E. coli* is classed as a model organism (Cronan 2014; Blount 2015) and is often a sentinel species targeted in antimicrobial resistance surveillance (Anjum et al. 2021). *E. coli* is also an extremely important bacterium in terms of resistance carriage, with huge diversity within its accessory genome through the acquisition of resistance determinants (Tenaillon et al. 2010; Yang et al. 2019; Montealegre et al. 2020). *E. coli* is also a significant pathogen within human clinical samples

(Kaper et al. 2004; Percival and Williams 2014) and was also a causative agent of bovine mastitis on this particular dairy farm for which beta-lactam antibiotics were given as therapy. Benefits of using *E. coli* are that it grows quickly under aerobic conditions at 37 °C, on simple commercially available media with an overnight culture of around 18 hours producing a CFU/ml of approximately $10^9/10^{10}$.

The focus of this study was to investigate different types of beta-lactamase resistance, and this was chosen due to the high usage of beta-lactam type antibiotics within the dairy industry. Therefore, only beta-lactamase type phenotypes were selected from the EVAL farms disc diffusion assay data and subsequently grouped into either the *bla*$_{\text{CTX-M}}$ (which is explored in this Chapter) or the *ampC* group (which is explored in **Chapter 5**).

3.2. SELECTION OF DAIRY FARM *E. coli* ISOLATES FOR THE *bla*$_{\text{CTX}}$ GROUP

A collection of 1,000 *E. coli* dairy farm isolates was available, which had been collected between May 2017 and June 2018 and characterised phenotypically as part of the EVAL Farms research study (with full details described in **Section 2.2.1**). The phenotypic antibiotic resistance profiles of each isolate were initially characterised via the disc diffusion assay method, by EVAL Farms (Baker et al. 2022a) (with methods detailed in **Appendix A**). This initial phenotypic characterisation included an antibiotic panel of 16 antibiotics that are listed in **Appendix B**. From the EVAL farms collection of 1,000 *E. coli* isolates,

47 isolates were initially chosen to be included in the *bla*$_{CTX}$ group according to the following selection criteria:

i) Resistance to the penicillin ampicillin (AMP) and the 3rd generation cephalosporin cefotaxime (CTX)

ii) Susceptibility to the beta-lactam/beta-lactamase inhibitor combination amoxicillin/clavulanic acid (AMC) and the cephamycin cefoxitin (FOX)

It was hoped that these selection criteria would provide an initial discriminatory method to differentiate between possible *bla*$_{CTX-M}$ and overexpression of *ampC* genotypes. Unfortunately, EVAL farms did not include CFQ within their panel of 16 antibiotics and CFQ is a good indicator for distinguishing between *bla*$_{CTX-M,}$ which should be resistant, and overexpression of *ampC,* which should be susceptible. Seven of the isolates included in the initial selection, exhibited resistance to both AMC and FOX. These seven were included for two reasons, firstly they were sampled at similar time points to the remaining 40 in the group and secondly, they were included to identify if multiple resistance mechanisms such as *bla*$_{CTX-M}$ and overexpression of *ampC* might be present in the same bacteria. Typical phenotypic profiles of *bla*$_{CTX-M}$ and overexpression of *ampC* are:

- *bla*$_{CTX-M}$: resistance to AMP, CTX and CFQ and susceptibility to AMC and FOX
- *ampC*: resistance to AMP, AMC, FOX, and reduced susceptibility to CTX and susceptibility to CFQ

Eight of the *E. coli* that included isolates 854, 855, 856, 870, 871, 872 968 and 969 had originally been isolated on Tryptone Bile X-glucuronide (TBX)

supplemented with 2 µg ml $^{-1}$ CTX. TBX is a chromogenic agar, that contains 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-glucuronide), which is designed to detect the enzyme activity of the *E. coli* highly specific enzyme glucuronidase. The X-glucuronide complex is absorbed by *E. coli* cells and the chromophore and glucuronide bond is split by intracellular glucuronidase, releasing the chromophore which then builds up inside the *E. coli* cells, giving glucuronidase positive colonies a blue/green colour on the agar plate (Hansen and Yourassowsky 1984). Two *E. coli* strains 726 and 774 had been isolated on MacConkey agar supplemented with 2 µg ml $^{-1}$ CTX, whilst the remaining *E. coli* (listed in **Table 2.2**) had been isolated on CHROMagar ESBL (an orientation medium with a supplement designed to detect ESBL-producing Gram negative bacteria). Following the *E. coli* initial isolation on the two selective media (MacConkey agar supplemented with 2 µg ml $^{-1}$ CTX and CHROMagar ESBL), all isolates were additionally subcultured by EVAL farms onto the chromogenic selective medium TBX supplemented with 2 µg ml $^{-1}$ CTX. Subculturing onto TBX medium was conducted to ensure only *E. coli* were included in the panel of bacteria. However, some *E. coli* strains namely O157:H7 are glucuronidase negative and therefore appear as white colonies on TBX (Ratnam et al. 1988). As O157:H7 should be handled under containment level (CL) 3 (HSE 2018) and the EVAL farms study was working under CL2, using TBX enabled the prevention of O157:H7 from entering the study by avoiding the white colonies from being selected from the TBX agar plates. The EVAL farms study was interested in investigating a large variety of different resistance mechanisms on the dairy farm and therefore had chosen to utilise different isolation procedures, so that a greater amount of the whole population could be

isolated, rather than only a sub-population by utilising for example only CHROMagar ESBL, which would likely only detect ESBL encoding bacteria. The purposes of the investigation in this chapter however, was specifically concerned with detecting *bla*$_{\text{CTX-M}}$ ESBLs.

The 47 *E. coli* isolates selected for the *bla*$_{\text{CTX}}$ group, were collected from nine locations on a UK dairy farm over a four month period from October 2017 – January 2018 by the EVAL farms study. Dairy farm sampling locations are listed in **Table 2.1**.

All 47 isolates are listed in **Table 2.2** and **2.3**, which detail the full isolate codes, isolation date, location and isolation media.

3.2.1. Heatmap of Disc Diffusion Assay Results from EVAL Farms

Figure 3.1 shows the disc diffusion assay results from EVAL farms with the phenotypic resistances displayed in a heatmap, as susceptible/intermediate/resistant (S/I/R) results for the 47 isolates. The number of resistant results (RES), intermediate results (INT) and susceptible results (SUS), along with the representative percentages are listed along the bottom. Only S/I/R data was supplied by EVAL farms and no zone sizes were available.

The antibiogram profiles of the selected isolates are shown in the heatmap in **Figure 3.1,** which showed 100 % of the isolates were resistant to AMP and CTX with high frequency of resistance to cefpodoxime (CPD), CAZ, aztreonam (ATM) and tetracycline (TET) at 97.9 %, 93.6 %, 91.5 % and 76.6 % respectively. Resistance to azithromycin (AZM) was moderate at 31.9 %, with

only a low frequency of isolates displaying resistance to AMC, streptomycin (STREP), FOX and sulfamethoxazole/trimethoprim (SXT) at 14.9 %, 12.8 %, 12.8 % and 8.5 % respectively. Chloramphenicol (CHLOR), ciprofloxacin (CIP) and nitrofurantoin (NIT) resistance was only seen in 3 isolates (each isolate displaying resistance to one antibiotic each) resulting in a percentage resistance amongst the selected *E. coli* strains of 2.1 % per antibiotic respectively.

Isolates exhibited a high frequency of intermediate resistance to CIP and STREP at 80.9 % and 63.8 % with a moderate frequency of resistance to nalidixic acid (NAL) at 40.4 %. Low frequency intermediate resistance was seen to CAZ and ATM at 6.4 % and 4.3 % with FOX, imipenem (IMP), SXT, CHLOR and NIT all at 2.1 %. As all isolates exhibited resistance to the extended spectrum aminopenicillin AMP, the 3rd generation cephalosporins and the monobactam ATM, the presence of *bla*$_{CTX-M}$ was suspected. However, the seven isolates: 854, 855, 856, 870, 871, 872 and 968 (outlined in red on the heatmap of **Figure 3.1**), in addition to the penicillin, cephalosporin and the monobactam cluster, also exhibited resistance to AMC (clavulanic acid, a beta-lactamase inhibitor), with only intermediate resistance to CAZ seen in isolates 855, 871 and 968 and all but 968 of the seven showing resistance to FOX. Resistance to a beta-lactamase inhibitor AMC and FOX was possibly indicative of another beta-lactamase mechanism such as *ampC*. The seven isolates exhibiting resistance to AMC were also the only *E. coli* which were initially isolated on TBX medium supplemented with 2 µg ml $^{-1}$ CTX.

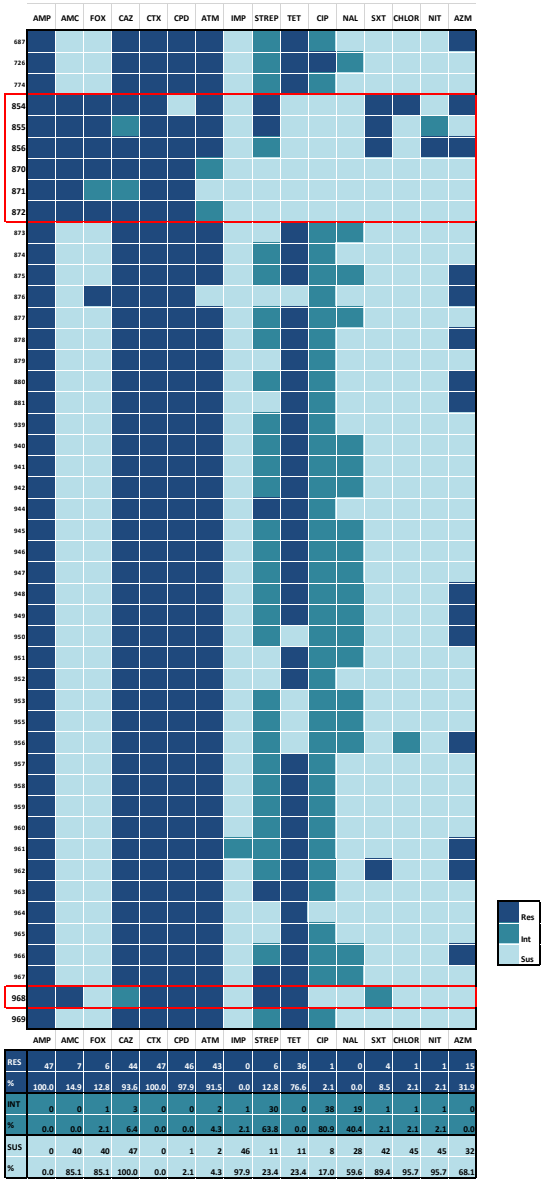


Figure 3.1: Heatmap detailing antibiograms of disc assay data supplied by EVAL farms of the 47 *E. coli* isolates placed into the *bla*$_{CTX}$ group. Isolate number is listed along the vertical axis and antibiotics are along the horizontal axis. Number and percentage resistant (RES), intermediate (INT) and susceptible (SUS) are included along the bottom of the heatmap. Antibiotic acronyms: AMP (ampicillin), AMC (amoxicillin/clavulanic acid), FOX (cefoxitin), CAZ (ceftazidime), CTX (cefotaxime), CPD (cefpodoxime), ATM (aztreonam), IMP (imipenem), STREP (streptomycin), TET (tetracycline), CIP (ciprofloxacin), NAL (nalidixic acid), SXT (Trimethoprim/sulfamethoxazole, CHLOR (chloramphenicol), NIT (nitrofurantoin) and AZM (azithromycin)

3.2.2. PCR confirmation of *bla*CTX-M and IS*Ecp1*

As part of the EVAL farms study, isolate 774 was sent for PacBio sequencing as per **Section 2.7.2** with assembly via SPAdes version 2.0.0. Analysis of the PacBio sequence of 774 as part of this study identified the mobile element IS*Ecp1* in association with *bla*CTX-M-15 and therefore all preliminary analysis of *bla*CTX-M and IS*Ecp1* was conducted in isolate 774. A previous study by Ibrahim et al. (2016) had also identified IS*Ecp1* in association with *bla*CTX-M-14, within the isolates BCC2 and BCE3 which were sampled in March 2014 from the same dairy farm as the EVAL farms isolates. To further characterise the remaining 46 isolates in the *bla*CTX group, a PCR analysis was conducted to search for both *bla*CTX-M as detailed in **Section 2.6.3;** and IS*Ecp1* as detailed in **Section 2.6.4**. Both PCRs for *bla*CTX-M and IS*Ecp1* were conducted at the same time, with the same DNA obtained from the same colony and on the 46 isolates that included the 39 detailed in **Table 2.2** and the 8 isolates 854, 855, 856, 870, 871, 872, 968 and 969 detailed in **Table 2.3**, with isolate 774 acting as the positive control.

Figure 2.3 details the primer binding locations for *bla*CTX-M denoted as CTX-M-Fwd and CTX-M-Rvs in purple producing the 593 bp PCR product. The *bla*CTX-M primers were utilised from a study by Dierikx et al. (2012) and are listed in **Table 2.6**. From the IS*Ecp1* sequence of 774, IS*Ecp1* primers were designed as part of this study (as detailed in **Section 2.3.2**). **Figure 2.5** details the primer binding locations for IS*Ecp1* denoted as ISEcp1-Fwd and ISEcp1-Rvs in purple resulting in the 846 bp PCR product.

The gels following electrophoresis of PCR products to search for *bla*CTX-M and IS*Ecp1* in the 46 isolates with BCC2 (Ibrahim et al. 2016) which encoded a

bla$_{\text{CTX-M-14}}$ in association with IS*Ecp1* and 774 acting as the positive controls, are shown in **Figures 3.2** and **Figure 3.3** respectively.

The 39 isolates listed in **Table 2.2** tested positive for both *bla*$_{\text{CTX-M}}$ and ISE*cp1* by PCR, but the eight listed in **Table 2.3** were negative for both as shown in **Figures 3.2** and **Figure 3.3**. As was concluded with the phenotypic heatmap analysis in **Section 3.2.1** with the seven isolates 854, 855, 856, 870, 871, 872 and 968 all showing resistance to AMC and FOX, these seven isolates plus the additional isolate of 969, that were PCR negative for *bla*$_{\text{CTX-M}}$, were the only *E. coli* of the 47 that were isolated on TBX medium supplemented with 2 µg ml $^{-1}$ CTX (as detailed in **Table 2.3**).

From the combination of the disc diffusion assay data and the PCR results for *bla*$_{\text{CTX-M,}}$ these eight isolates were suspected of encoding an overexpression of the *ampC* genotype and were therefore transferred to the *ampC* group, which is explored in more detail in **Chapter 5**. This brought the final number in the *bla*$_{\text{CTX}}$ group to 39.

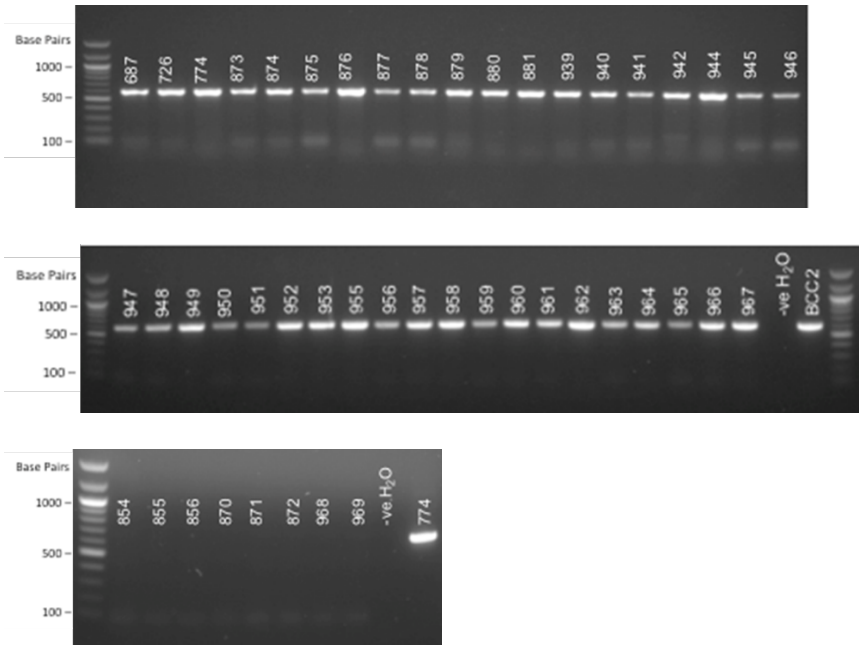


Figure 3.2: Gel electrophoresis images of *bla*$_{CTX-M}$ PCR of putative ESBL *E. coli*. The gels show 593 bp *bla*$_{CTX-M}$ amplimers. PCR products were resolved on a 1% TAE agarose gel with a 100 bp NEB quickload ladder (New England Biolabs, (UK) Ltd) at 85 V for 1 hour. Isolates BCC2 (encoding *bla*$_{CTX-M-14}$) (Ibrahim et al. 2016) and 774 (encoding *bla*$_{CTX-M-15}$) were used as positive controls with water used as a negative control

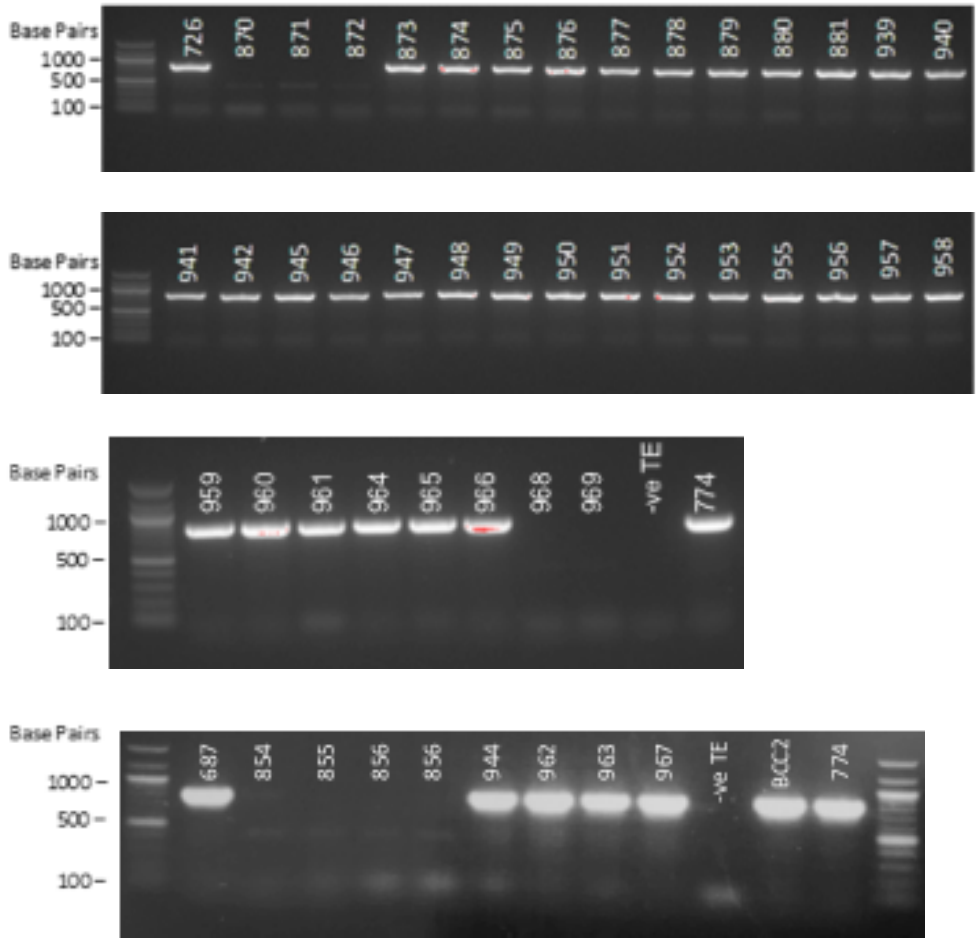


Figure 3.3: Gel electrophoresis images of IS*Ecp1* PCR of putative ESBL *E. coli*. The gels show 846 bp IS*Ecp1* amplimers. PCR products were resolved on a 1% TAE agarose gel with a 100 bp NEB quickload ladder (New England Biolabs, (UK) Ltd) at 85 V for 1.5 hours. Isolates BCC2 (IS*Ecp1* positive) (Ibrahim et al. 2016) and 774 (IS*Ecp1* positive) were used as positive controls, with TE buffer at pH 8.0 used as a negative control. Primers were designed from isolate 774 as per Section 2.3.2

3.3. MIC PHENOTYPIC CHARACTERISATION OF *bla*CTX ISOLATES WITH AN EXTENDED PANEL OF ANTIBIOTICS: RATIONALE

Further phenotypic characterisation as part of this study was conducted via MIC determination as per **Section 2.4.1** with an extended panel of antibiotics. The panel was extended from the 16 utilised by EVAL farms (listed in **Appendix B**), to include a total of 25 antibiotics and these are listed in **Table 2.8**. The rationale for the choices for the extended panel were made with several ideal outcomes in mind. These outcomes included (1) choosing antibiotics that could act as a screen for several important antibiotic resistance families, (2) antibiotics that could select for different sublineages within resistance groups and (3) for distinguishing between resistance mechanisms that may give similar phenotypic resistances.

Inclusion of the three carbapenems ertapenem (ERT), IMP and meropenem (MER) provided a carbapenemase screen to cover the main carbapenem type resistance genes including *bla*KPC type Ambler class A carbapenemase, *bla*IMP, *bla*VIM and *bla*NDM type Ambler class B beta-lactamases and *bla*OXA-48 and *bla*OXA-181 Ambler class D enzymes. **Table 3.1** shows an example of the likely phenotypes (S/I/R) arising from the carbapenemase genes with and without the addition of an ESBL (adapted from (Nordmann et al. 2012)).

The rationale for choosing agar dilution over broth dilution was made, due to the large number of bacteria to be tested. Both methods have pros and cons, with broth dilution suitable for testing a wide range of dilutions and

multiple antibiotics on one 96-well microtiter plate. However, broth dilution is difficult to read by eye and testing large numbers of bacteria requires large volumes of microtiter plates to be prepared. Agar dilution in comparison allows for large numbers of bacteria to be tested on one agar plate and a physical growth of the bacteria to be read on the agar plate. However, agar dilution can be time consuming, if large numbers of antibiotic dilutions are required, as each dilution need to be mixed into a separate agar plate. In addition, agar dilution relies on the migration of the antibiotic through the agar plate, which can be problematic with large molecules with colistin a good example.

Table 3.1: The likely phenotypes arising from individual and combinations of carbapenemase genes and from carbapenemases in combination with ESBLs. Adapted from (Nordmann et al. 2012)

Carbapenemase Genes ↓	**Antibiotics** → AMC	CTX	CAZ	IMP	ERT	MER	ATM
KPC	S/I	R	R	S/I/R	I/R	S/I/R	R
KPC + ESBL	I/R	R	R	I/R	I/R	I/R	R
IMP/VIM/NDM	R	R	I/R	S/I/R	I/R	S/I/R	S
IMP/VIM/NDM + ESBL	R	R	R	I/R	R	S/I/R	R
OXA-48/OXA-181	R	S/I	S	S/I	S/I	S/I	S
OXA-48/OXA-181 + ESBL	R	R	R	I/R	I/R	I/R	R

***Footnote for Table 3.1**: AMC – amoxicillin/clavulanic acid, CTX – cefotaxime, CAZ – ceftazidime, IMP – imipenem, ERT – ertapenem, MER – meropenem and ATM – aztreonam*

The aminoglycosides streptomycin, gentamicin, neomycin, apramycin and tobramycin were chosen to cover the main aminoglycoside modifying enzymes including N-Acetyltransferases (AAC) which catalyse acetyl CoA-dependent acetylation of an amino group with most variants conferring gentamicin resistance and some conferring tobramycin or apramycin resistance; the O-Adenyltransferases (ANT) which catalyse ATP-dependent adenylation of a hydroxyl group with many variants conferring tobramycin and streptomycin resistance; and the O-Phosphotransferases (APH) which catalyse ATP-dependent phosphorylation of a hydroxyl group with variants conferring resistance mostly to streptomycin and neomycin.

The three 3rd generation cephalosporins were chosen to identify ESBL type resistances including *bla*CTX-M types. Most *bla*CTX-M enzymes have weak catalytic activity against CAZ, however, amino acid changes resulting in *bla*CTX-M variants are associated with increased activity against CAZ. Examples within three of the five CTX phylogroups include seven variants with increased activity against CAZ including CTX-M-15, CTX-M -23 and CTX-M -42 from Group 1, CTX-M-16, CTX-M -19 and CTX-M -27 from group 9 and CTX-M-25 (Karisik et al. 2006; Novais et al. 2008). The cephamycin FOX and 4th generation cephalosporin CFQ were included, so the definitive parameters for distinguishing between overexpression of *ampC* and *bla*CTX-M were included (Caroff et al. 1999a; Drawz and Bonomo 2010a; Peter-Getzlaff et al. 2011a; Haenni et al. 2014a). Finally, CPD was chosen as it is a good substrate for most ESBLs (Potz et al. 2004).

The inclusion of tigecycline was decided based on the increased reports in the literature of the appearance of *tetX* and its variants, which result in

resistance to 3rd and 4th generation tetracyclines (Gasparrini et al. 2020). Finally, colistin was included to cover *mcr* variants (Wang et al. 2018). As was discussed in **Section 1.2.1.1**, the use of human critical antibiotics in agriculture is believed to have promoted resistance in humans via *tetX* (He et al. 2019b) and *mcr* variants and these are often associated with plasmids (Wang et al. 2018; Wang et al. 2020b).

As was stated in **Section 2.4**, the majority of breakpoints for the extended panel were taken from EUCAST v12.0 (EUCAST 2022). The rationale for choosing EUCAST over CLSI, was that EUCAST is not so biased towards human only testing and EUCAST also has a large amount of WT distribution data available. In addition, EUCAST also produce documents that are specific to veterinary AMR testing. In addition, the EUCAST VetCAST subcommittee which was formed in 2015, was set up to deal specifically with antimicrobial susceptibility testing in bacteria of pathogens of animal origin (VetCAST 2019). However, when EUCAST breakpoints were not available CLSI breakpoints (CLSI 2022) were firstly utilised (where available), followed by literature-stated breakpoints in combination with ECOFF values and PK/PD cut off values. In addition, the EUCAST guidance document "When there are no breakpoints" (EUCAST, 2021) was also followed for advice on alternatives for establishing a breakpoint. A good example of an antibiotic with no breakpoint is CFQ, which is solely for veterinary use as it is not approved for human usage (El-Hewaity et al. 2014) and therefore it has no clinical breakpoint listed within either EUCAST or CLSI (El-Hewaity et al. 2014; Teale and Borriello 2021). There is however an ECOFF value of 0.125 mg L^{-1} for cefquinome listed by EUCAST (available at: https://mic.eucast.org/search/) (with ECOFF defined in **Section 2.4**) and a

literature-stated breakpoint of 0.25 mg L^{-1} (Zhang et al. 2021). Other examples of antibiotics which lacked a clinical breakpoint for *Enterobacteriaceae*, included enrofloxacin with an ECOFF of 0.125 mg L^{-1} and literature-stated breakpoint of 2 mg L^{-1} (Temmerman et al. 2020), azithromycin with an ECOFF of 8 mg L^{-1} and literature-stated breakpoint of 32 mg L^{-1} (Gomes et al. 2019) and nalidixic acid also with an ECOFF of 8 mg L^{-1} and literature-stated breakpoint of 32 mg L^{-1} (Ruiz et al. 2002). Therefore, with cefquinome, enrofloxacin, azithromycin and nalidixic acid, the ECOFF and any literature- stated breakpoints were utilised. It is a well-known problem amongst veterinary-specific antibiotics, that breakpoints stated by either EUCAST or CLSI are lacking (Toutain et al. 2017).

3.3.1. Antibiotic MIC Determination of the 39 *bla*CTX Isolates

MIC assays were conducted using the agar dilution method as detailed in **Section 2.4.1** using a panel of 26 antibiotics (listed in **Table 2.8**), to determine the level of resistance of the 39 *E. coli* isolates in the *bla*CTX group. Of most interest was the level of resistance to the aminopenicillin AMP, the 3rd generation cephalosporins CTX, CAZ and CPD, the 4th generation cephalosporin CFQ and the monobactam ATM.

Resistant results were obtained for seven of the antibiotics that included AMP, CAZ, CTX, CPD, CFQ, ATM and TET, with the MIC results detailed in **Table 3.2.** In **Table 3.2** any literature utilised breakpoints are written in red and any susceptible results within this table are highlighted in green. Only susceptible results were obtained for the remaining 19 antibiotics, which included AMC, FOX, IMP, ERT, MER, STREP, GENT, NEO, APR, TOB, TIG,

NAL, CIP, ENR, NIT, CHLOR, SXT, COL and AZM, with the MIC results detailed in **Table 3.3**. In **Table 3.3**, any literature utilised breakpoints are written in red and the references to the literature used for obtaining these breakpoints are detailed in **Section 3.3**.

All isolates showed high level resistance to AMP, CTX, CPD, CFQ and ATM which would be expected as all were encoding *bla*$_{\text{CTX-M}}$. Additionally, all isolates were resistant to CAZ, but the MIC result was a much lower concentration at 16 mg L^{-1} to the other 3rd generation cephalosporins which had results of >512 mg L^{-1} for CTX and 512 mg L^{-1} for CPD. The rationale for choosing CAZ was outlined in **Section 3.3** and described how amino acid changes resulting in *bla*$_{\text{CTX-M}}$ variants, creates increased activity against CAZ and the MIC of 16 mg L^{-1} is in line with literature stated MIC results for *bla*$_{\text{CTX-M-15}}$ types (Dhanji et al. 2011a; Williamson et al. 2012). With an MIC of ≤2 mg L^{-1}, 950, 953, 955 and 956 were the only isolates that returned a susceptible result for TET, with the rest returning a resistant result which included seven isolates with a result of 128 mg L^{-1} and the rest with a result of 64 mg L^{-1}.

Additionally, it was noted following the MICs, that some of the resistant results supplied by EVAL farms from the disc assays, were no longer present. These included: resistance to SXT seen within isolate 962, intermediate resistance to STREP seen within isolates 687, 726, 774, 939, 947, 956, 961 and 962 and intermediate CHLOR resistance seen within isolate 956. Subsequent WGS of isolates in the *bla*$_{\text{CTX}}$ group which is explored later in this Chapter in **Section 3.5** allowed for manual investigation of the genome sequences, but this also failed to identify any mechanism that could account for these resistances, and they could have been the result of errors within the disc diffusion assay. As

EVAL farms only conducted the disc diffusion assays once, it cannot be confirmed for certain whether this was the result of user error, subjective result reading of the zone diameter, faulty discs or as a result of that particular batch of media affecting the result on that day. Alternatively, the loss of a resistance plasmid, following recovery from frozen culture (Wright and Crease 1996; Koenig 2003), could have occurred but as plasmids were not analysed at the sampling stage by EVAL farms, this could not be determined at this later stage.

Table 3.2: Antibiotics for which resistant results were obtained from the MIC assays of the 39 isolates in *bla*$_{CTX}$ group that included AMP, CAZ, CTX, CPD, CFQ, ATM and TET

Antibiotics →	**AMP**	**CAZ**	**CTX**	**CPD**	**CFQ**	**ATM**	**TET**
Breakpoints ↓	**Sensitive ≤ / Resistant > (mg L^{-1})**						
EUCAST	8	1/4	1/2	1	-	1/4	4/16
Literature Stated	-	-	-	-	0.25	-	-
ECOFF	8	0.5	0.25	1	0.125	0.25	-
Isolates ↓	**MIC →**						
687	>512	16	>512	512	128	32	64
726	>512	16	>512	512	128	32	64
774	>512	16	>512	512	128	32	64
873	>512	16	>512	512	128	32	64
874	>512	16	>512	512	128	32	128
875	>512	16	>512	512	128	32	128
876	>512	16	>512	512	128	32	128
877	>512	16	>512	512	128	32	64
878	>512	16	>512	512	128	32	64
879	>512	16	>512	512	128	32	64

880	>512	16	>512	512	128	32	128
881	>512	16	>512	512	128	32	128
939	>512	16	>512	512	128	32	64
940	>512	16	>512	512	128	32	64
941	>512	16	>512	512	128	32	64
942	>512	16	>512	512	128	32	64
944	>512	16	>512	512	128	32	64
945	>512	16	>512	512	128	32	64
946	>512	16	>512	512	128	32	64
947	>512	16	>512	512	128	32	64
948	>512	16	>512	512	128	32	64
949	>512	16	>512	512	128	32	64
950	>512	16	>512	512	128	16	2
951	>512	16	>512	512	128	16	64
952	>512	16	>512	512	128	16	64
953	>512	16	>512	512	128	32	≤2
955	>512	16	>512	512	128	16	≤2
956	>512	16	>512	512	128	32	≤2

957	>512	16	>512	512	128	16	64
958	>512	16	>512	512	128	32	64
959	>512	16	>512	512	128	32	64
960	>512	16	>512	512	128	32	64
961	>512	16	>512	512	128	32	64
962	>512	16	>512	512	128	32	64
963	>512	16	>512	512	128	32	64
964	>512	16	>512	512	128	32	128
965	>512	16	>512	512	128	32	128
966	>512	16	>512	512	128	32	64
967	>512	16	>512	512	128	32	64
ATCC25922	≤4	≤0.5	≤0.25	0.5	≤0.25	≤0.25	≤2

Footnote for Table 3.2*: Any literature utilised breakpoints are written in red with the references to the literature used for obtaining these breakpoints detailed in* ***Section 3.3.*** *Any susceptible results are highlighted in green.*

Table 3.3: Antibiotics for which only susceptible results were obtained from the MIC assays of the 39 isolates in the *bla*CTX group. As all 39 isolates returned identical MIC results, only the antibiotics, the EUCAST or literature-stated breakpoints (in red text) and the MIC result are listed

Antibiotic	EUCAST Breakpoints Sensitive ≤ / Resistant > (mg/L)	Result
AMC	8	≤4
FOX	8/32	2
IMP	2/4	≤0.5
ERT	0.5	≤0.032
MER	2/8	≤0.064
STREP	8/64	8
GENT	2	≤1
NEO	8/16	≤4
APR	8/64	≤4
TOB	2	≤1
TIG	0.5	≤0.25
NAL	32	32
CIP	0.25/0.5	0.25
ENR	2	1
NIT	64	≤32

CHLOR	8	≤4
SXT	2/4	≤0.5
COL	2	≤1
AZM	32	≤8

Footnote for Table 3.3**: Any breakpoints in red text were literature-stated breakpoints with the references to the literature used for obtaining these breakpoints detailed in **Section 3.3

3.4. GENOTYPIC DATA OF CEFOTAXIME RESISTANT DAIRY FARM *E. coli* ISOLATES

Phenotypic disc diffusion assay resistance profiles and subsequent MICs (as shown in **Section 3.3)**, showed high level resistance to antibiotics often associated with the ESBL gene *bla*$_{\text{CTX-M}}$ and all the isolates were shown to carry this gene by PCR (**Section 3.2.2**). WGS offered further understanding of the genotype, mobile elements and how the genotype may drive the phenotypic resistance profiles seen in **Table 3.2**. The genetic environment of each of the IS*Ecp1* elements could also be analysed and compared to the principle isolate of 774.

Initial sequence analysis conducted on the PacBio sequenced isolate 774 had discovered an IS*Ecp1* element in association with *bla*$_{\text{CTX-M-15}}$. As was stated in **Section 3.2.2**, isolate 774 served as the isolate for all preliminary analysis of *bla*$_{\text{CTX-M}}$ and IS*Ecp1* due to the availability of the PacBio sequence supplied by EVAL farms. The PacBio sequence of isolate 774 resulted in only 2 contigs of 14, 682 bp and 4,712,267 bp and no plasmids were found. As PacBio

sequencing results in fewer contigs than short read sequencing and Hifi reads give both length and accuracy, there was a good level of confidence when confirming the location, size and genes of the genetic environment of IS*Ecp1*. However, whilst long read sequencing produces less contigs, it has its limitations, in that long read sacrifices base call accuracy for less contigs. Some of the benefits to hybrid over *de novo* assembly are, downstream analysis is made easier, including mapping and genome annotation, there is greater resolution of plasmids and also gene location (whether chromosomally-encoded or encoded on a plasmid). These improvements are due to the base accuracy gained from the short read combined with the lower contig number from the long read. In this the two sequencing platforms complement each other (Miller et al. 2017). Therefore, it was decided that any further sequencing conducted as part of this study, should be conducted via both Illumina short read and MinION Oxford Nanopore Technologies (ONT) long read as per **Section 2.7.3** with hybrid assembly as per **Section 2.8.1**. As 774 only had a long-read assembly, it was included together with the remaining 38 isolates in the *bla*CTX group and all were sequenced via both long and short read with hybrid assembly. Performing a hybrid assembly would result in greater confidence in the location of the IS*Ecp1* elements.

3.4.1. Illumina Short Read and MinION (ONT) Long Read Sequencing with Hybrid Assembly of 39 *bla*CTX Isolates

Sequencing via both the Illumina short read and MinION (ONT) long read platforms (**Section 2.7.3**) was conducted by University of Cardiff on all 39 IS*Ecp1* and *bla*CTX-M positive isolates listed in **Table 2.2**. Furthermore, it was

also possible to analyse the degree of genetic relatedness within this group of isolates through phylogenetic analysis (**Section 2.8.6**) and SNP distance comparison (**Section 2.8.7**) and determine whether the spread of the *bla*$_{\text{CTX-M-15}}$ within the dairy farm was due to clonal expansion or transfer of the mobile element IS*Ecp1* in association with *bla*$_{\text{CTX-M-15}}$.

In addition, WGS also allowed for *bla*$_{\text{CTX-M}}$ typing through blastp searches of the amino acid sequence (**Section 2.8.3**), MLST analysis using MLST 2.0 (**Section 2.8.6.1**), the identification of additional resistance genes using ResFinder 4.1 (**Section 4.1**), virulence factors using VirulenceFinder 2.0 (**Section 2.8.6.3**) and the characterisation of plasmids using PlasmidFinder 2.1 (**Section 2.8.6.4**). In addition, the production of high-quality genomes (through a hybrid assembly) enabled greater resolution in the locality of where genes were encoded.

3.4.2. Assembly Statistics and Contigs with Plasmids of *bla*$_{\text{CTX}}$ Isolates

The assembly statistics and open reading frame (ORF) numbers were calculated for all the 39 sequenced isolates in the *bla*$_{\text{CTX}}$ group as described in **Section 2.8.1**. The free software Bandage (available at: https://rrwick.github.io/Bandage) was utilised to calculate contig number, locate contigs containing plasmids, overall genome size, and the N50 number. N50 is described as the median length L where 50 % of the nucleotides should lie within at least L as defined by the International Human Genome Sequencing Consortium (2001). The online ORFinder software (available at: https://www.ncbi.nlm.nih.gov/orffinder/) was utilised to calculate ORF

numbers. Geneious Prime and Bandage were utilised to assess whether chromosomes and plasmids were complete, which contigs contained plasmids through manual inspection of the WGS and %GC content. The %GC content was on average 50.7% (range of 50.6% - 50.8%) in all isolates, which is typical of other *E. coli* genome %GC content within the literature as noted by Wang and Reeves (2000) and Mann and Chen (2010).

The hybrid assembly resulted in mostly complete chromosomes and plasmids and allowed good confidence for identifying where resistance genes were encoded and good accuracy for gauging plasmid sizes. PlasmidFinder 2.1 was utilised to locate contigs containing plasmids as per **Section 2.8.6.4**. All plasmids were found to be cryptic. All the assembly statistics are detailed in **Table 3.4** which also includes the ORF number, contig number, contig numbers containing a plasmid, whether the plasmids were complete, overall genome size, overall %GC content, N50 number and whether the chromosome was complete. The majority of N50 numbers were around 4.7 million, demonstrating long complete contigs and a good assembly, whereas 880 which had a poor assembly had an N50 of 1.5 million. This lower N50 in isolate 880, could signify a larger number of small fragmented contigs in the assembly, that lowered the N50 number. Two examples of the output images from Bandage are shown in **Figure 3.4** and **Figure 3.5** for isolates 880 and 948 respectively, which demonstrate an incomplete chromosome and plasmid from the 880 assembly and a complete chromosome and complete plasmid from the 948 assembly. Detailed on **Figures 3.4** and **3.5** are the contig numbers and sizes in (bp). In addition, it was noted that contigs 10 of 4,237 bp, 11 of 4,018 bp, 12 of 3,959 bp and 17 of 2,080 bp were also circular in the 880 assembly and there were two circular contigs in

948, contig 3 of 4,018 bp and contig 4 of 3,959 bp. Circular contigs can represent MGEs such as small plasmids and subsequent blastn searches of the nucleotide sequence of each of the circular contigs in 880 and 948, returned results detailing them as plasmid DNA but referred to as “unnamed plasmid” in the database.

Table 3.4 Assembly statistics* for the 39 sequenced isolates in the *bla*$_{CTX}$ group

Isolate	Number of ORFs	Total Number of Contigs	Contig Number Containing a Plasmid (replicon and plasmid size (bp))	Complete Plasmid	Overall Genome Size (bp)	Overall %GC Content	N50 Number	Complete Chromosome
687	4,552	11	2 (IncFIC 61,878 bp) 3 (IncI2 59,595 bp)	Yes Yes	4,845,732 bp	50.6%	4,695,066 bp	Yes
726	4,596	7	2 (IncI1 105,560 bp) 3 (IncF1C 61,868 bp)	Yes Yes	4,898,234 bp	50.8%	4,711,679 bp	Yes
774	4,381	3	None	n/a	4,707,327 bp	50.8%	4,699,350 bp	Yes
873	4,612	9	2 (IncI1 69,389 bp) 3 (IncFIC 61,867 bp) 4 (IncX4 32,452 bp)	Yes Yes Yes	4,895,634 bp	50.7%	4,699,571 bp	Yes
874	4,639	8	2 (IncI1 105,558 bp) 3 (IncFIC 62,755 bp)	Yes Yes	4,919,530 bp	50.7%	4,699,609 bp	Yes

			4 (IncX4 32,450 bp)	Yes				
875	4,626	8	2 (IncI1 105,566 bp) 3 (IncFIC 61,867 bp) 4 (IncX4 32,451 bp)	Yes Yes Yes	4,914,272 bp	50.7%	4,700,406 bp	Yes
876	4,633	7	2 (IncI1 105,561 bp) 3 (IncFII 64,447 bp) 4 (IncX4 32,452 bp)	Yes Yes Yes	4,914,853 bp	50.7%	4,700,179 bp	Yes
877	4,623	9	2 (IncI1 87,563 bp) 3 (IncFIC 61,864 bp) 4 (IncX4 32,450 bp)	Yes Yes Yes	4,910,607 bp	50.7%	4,709,599 bp	Yes
878	4,619	17	None	n/a	4,806,936 bp	50.8%	3,900,948 bp	No
879	4,338	8	2 (IncI1 84,267 bp) 3 (IncFIC 61,866 bp) 4 (IncX4 32,452 bp)	Yes Yes Yes	4,855,001 bp	50.7%	4,658,078 bp	Yes

880	4,601	24	5 (IncFIC/I1 50,117 bp) 6 (IncFIC/I1 47,504 bp) 7 (IncFIC/I1 42,967 bp) 8 (IncX4 32,451 bp)	No – IncFIC and IncI1 plasmids spread across contigs 5, 6 and 7 Yes	4,890,733 bp	50.7%	1,545,096 bp	No
881	4,622	8	2 (IncI1 102,050 bp) 3 (IncFIC 61,864 bp) 4 (IncX4 32,406 bp)	Yes Yes Yes	4,910,094 bp	50.7%	4,910,094 bp	Yes
939	4,595	5	2 (IncI1 105,564 bp) 3 (IncFIC 61,867 bp)	Yes Yes	4,896,868 bp	50.7%	4,721,460 bp	Yes
940	4,584	5	2 (IncI1 105,565 bp) 3 (IncFIC 61,867 bp)	Yes Yes	4,886,824 bp	50.7%	4,711,415 bp	Yes
941	4,597	5	2 (IncI1 105,564 bp) 3 (IncFIC 61,867 bp)	Yes Yes	4,897,401 bp	50.7%	4,721,993 bp	Yes
942	4,514	4	None	n/a	4,751,314 bp	50.7%	4,656,624 bp	Yes
944	4,625	7	2 (IncI1 105,565 bp)	Yes	4,912,049 bp	50.7%	4,699,951 bp	Yes

			3 (IncFIC 61,867 bp)	Yes				
			4 (IncX4 32,452 bp)	Yes				
945	4,612	8	2 (IncI1 105,565 bp)	Yes	4,911,418 bp	50.7%	4,721,662 bp	Yes
			3 (IncFIC 61,867 bp)	Yes				
946	4,612	8	2 (IncI1 105,563 bp)	Yes	4,911,410 bp	50.7%	4,721,657 bp	Yes
			3 (IncFIC 61,867 bp)	Yes				
947	4,625	7	2 (IncI1 105,564 bp)	Yes	4,910,848 bp	50.7%	4,699,875 bp	Yes
			3 (IncFIC 61,867 bp)	Yes				
			4 (IncX4 32,451 bp)	Yes				
948	4,478	4	2 (IncFIC 61,866 bp)	Yes	4,791,830 bp	50.8%	4,721,987 bp	Yes
949	4,613	7	2 (IncI1 105,565 bp)	Yes	4,915,453 bp	50.7%	4,732,465 bp	Yes
			3 (IncFIC 61,867 bp)	Yes				
950	4,593	5	2 (IncI1 105,565 bp)	Yes	4,891,432 bp	50.7%	4,716,023 bp	Yes
			3 (IncFIC 61,867 bp)	Yes				
951	4,606	5	2 (IncI1 105,566 bp)	Yes	4,907,351 bp	50.7%	4,731,941 bp	Yes

			3 (IncFIC 61,867 bp)	Yes				
952	4,602	6	2 (IncI1 105,563 bp) 3 (IncFIC 61,866 bp)	Yes Yes	4,902,828 bp	50.7%	4,722,079 bp	Yes
953	4,587	6	2 (IncI1 105,557 bp) 3 (IncFIC 61,850 bp)	Yes Yes	4,881,647 bp	50.7%	4,695,262 bp	Yes
955	4,594	5	2 (IncI1 105,559 bp) 3 (IncFIC 61,867 bp)	Yes Yes	4,892,035 bp	50.7%	4,716,632 bp	Yes
956	4,589	6	2 (IncI1 105,562 bp) 3 (IncFIC 61,867 bp)	Yes Yes	4,893,273 bp	50.7%	4,716,660 bp	Yes
957	4,599	6	2 (IncI1 105,562 bp) 3 (IncFIC 61,866 bp)	Yes Yes	4,899,970 bp	50.7%	4,722,983 bp	Yes
958	4,412	5	2 (IncFIC 61,866 bp)	Yes	4,735,971 bp	50.8%	4,702,390 bp	Yes
959	4,647	6	2 (IncI1 105,558 bp) 3 (IncFIC 61,863 bp) 4 (IncX4 39,090 bp)	Yes Yes Yes	4,928,079 bp	50.7%	4,713,591 bp	Yes

960	4,386	8	2 (IncI1 105,562 bp) 3 (IncFIC 61,865 bp) 4 (IncX4 39,095 bp)	Yes Yes Yes	4,929,909 bp	50.7%	4,712,431 bp	Yes
961	4,629	5	2 (158,269 bp) 3 (IncX4 39,095 bp)	Yes but IncI1 and IncFIC both in contig 2 Yes	4,919,354 bp	50.7%	4,714,013 bp	Yes
962	4,628	9	2 (75,590 bp) 3 (51,134 bp) 5 (24,952 bp) 4 (IncX4 39,064 bp)	No IncI1 and IncFIC spread across contigs 2, 3 and 5 Yes	4,915,154 bp	50.7%	4,708,700 bp	Yes
963	4,518	7	2 (IncFIC 61,865 bp) 3 (IncI1 40,261 bp)	Yes Yes	4,825,533 bp	50.7%	4,711,319 bp	Yes
964	4,590	7	2 (IncFIC 61,864 bp)	Yes	4,869,976 bp	50.7%	4,723,307 bp	Yes

			3 (IncX4 39,095 bp)	Yes				
965	4,458	6	2 (IncFIC 45,492 bp)	Yes	4,769,443 bp	50.7%	4,711,498 bp	Yes
966	4,558	7	2 (IncI1 105,560 bp)	Yes	4,860,469 bp	50.7%	4,720,358 bp	Yes
967	4,592	9	2 (IncFIC 61,865 bp)	Yes	4,874,260 bp	50.7%	4,704,796 bp	Yes
			3 (IncI1 51,220 bp)	Yes				
			4 (IncX4 39,094 bp)	Yes				

Footnote for Table 3.4*: The assembly statistics included the number of ORFS, total number of contigs, contig number containing a plasmid with plasmid replicon and size (bp), whether the plasmid was complete, overall genome size (bp), %GC content, N50 number and whether chromosome was complete*

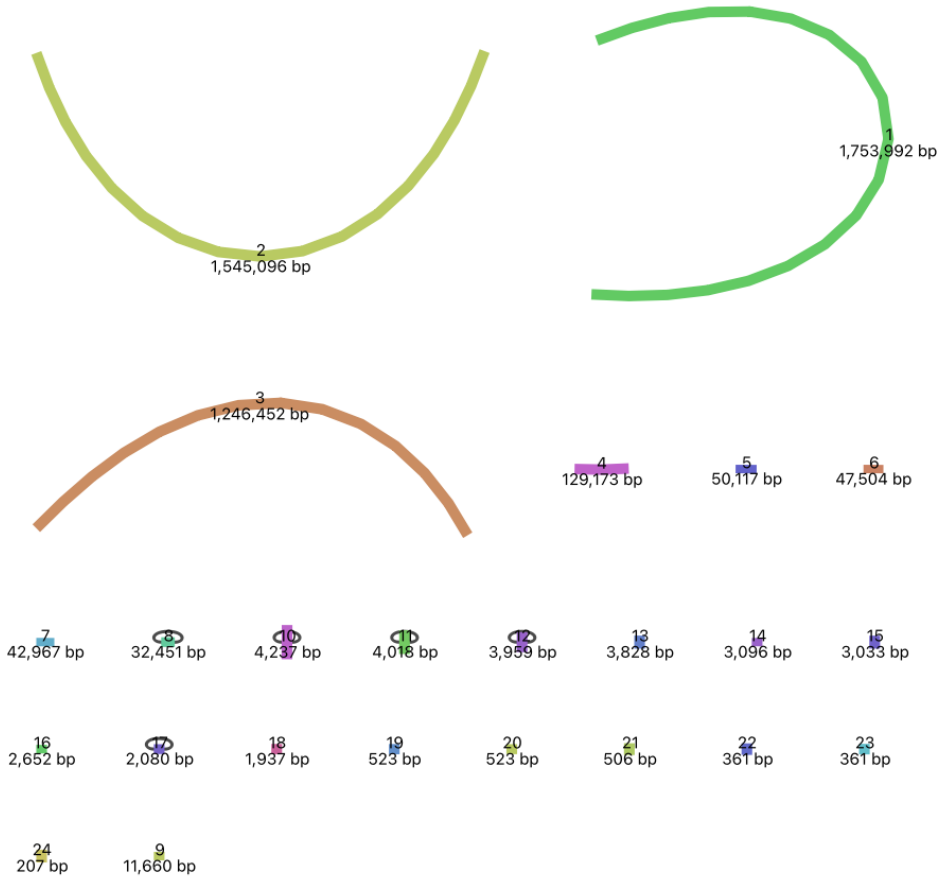


Figure 3.4: Output image from Bandage for the assembly of isolate 880 showing the incomplete chromosome located in contigs 1 of 1,753,992, contig 2 of 1,545,096 bp, contig 3 of 1,246,452 bp and contig 4 of 129,173 bp. The IncFIC and IncI1 plasmids were spread across contigs 5, 6 and 7 of 50,117 bp, 47,504 bp and 42,967 bp respectively. Contig 8 of 32,451 bp which is circular and complete contained the IncX4 plasmid. Contigs 10, 11, 12 and 17 were also circular and returned blastn results of plasmid DNA but referred to as "unnamed plasmid"

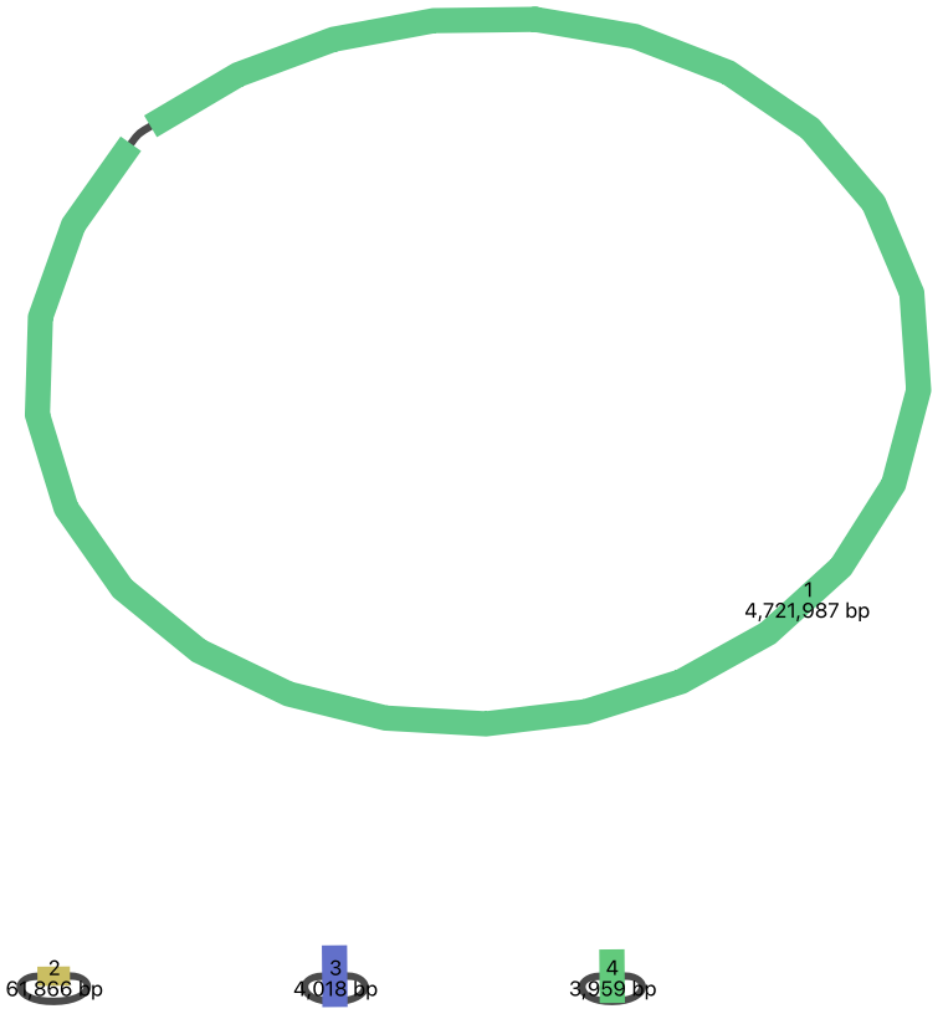
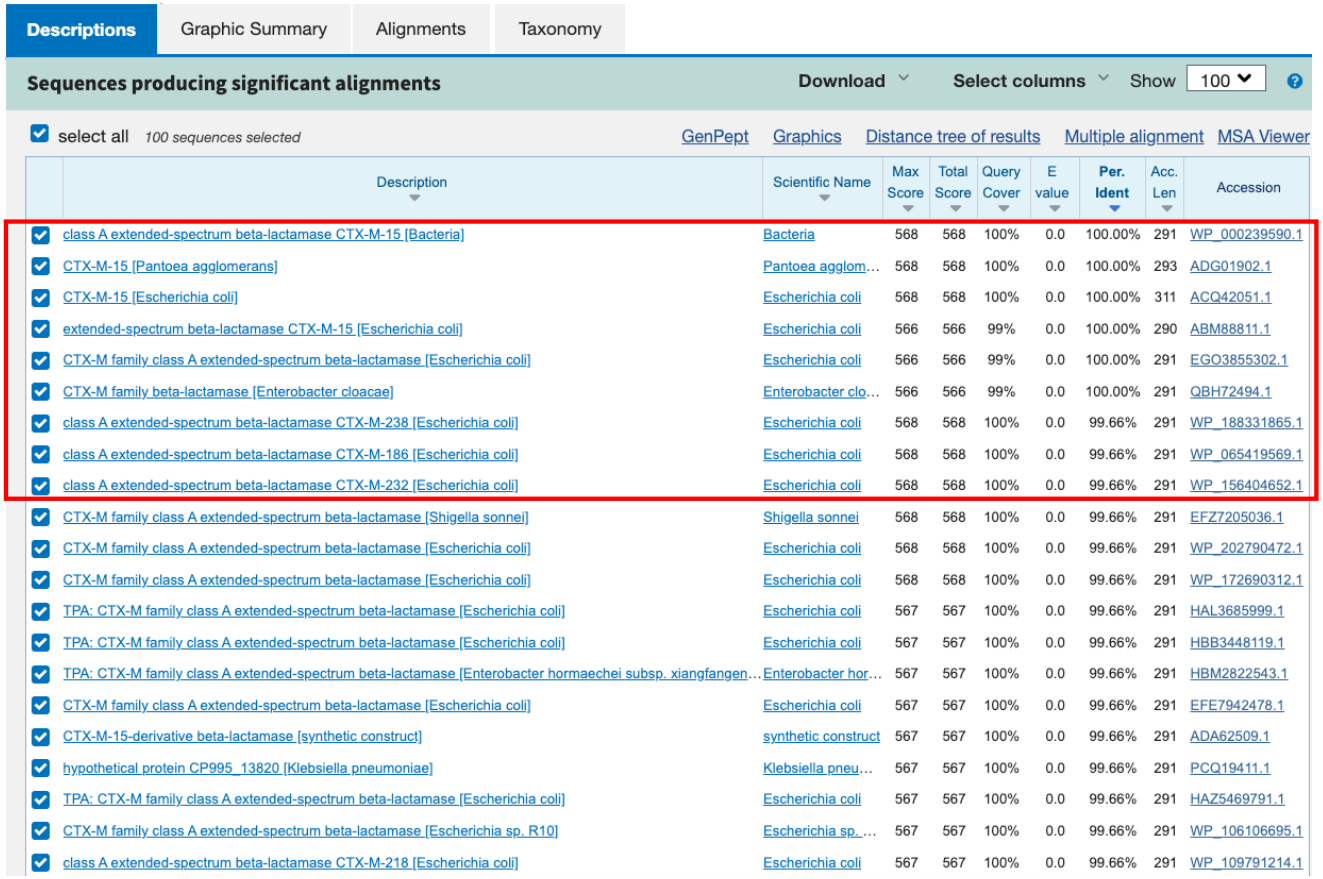


Figure 3.5: Output image from Bandage for the assembly of isolate 948 showing the circular and complete chromosome located in contig 1 of 4,721,987 bp and the circular and complete contig 2 which contained the IncFIC plasmid of 61,866 bp. Contigs 3 and 4 were also circular and returned blastn results of plasmid DNA but referred to as "unnamed plasmid"

3.4.3. *bla*$_{\text{CTX-M}}$ Typing

All *bla*$_{\text{CTX-M}}$ variant typing was achieved via analysis of the WGS, using blastp searches of the FASTA sequence of the entire amino acid sequence (as described in **Section 2.6.3.1**). An example of the blastp search conducted on isolate 774 can be seen in **Figure 3.6,** with the first nine results highlighted within the red box as an example of how single amino acid substitutions can alter *bla*$_{\text{CTX-M}}$ type. The remaining 38 isolates in the *bla*$_{\text{CTX}}$ group returned identical blastp results to isolate 774. In **Figures 3.7, 3.8** and **3.9** are the alignments of the first nine hits in the blastp search list shown in **Figure 3.6** (when sorted in order of Per. Identity on https://blast.ncbi.nlm.nih.gov/), which detail the location of amino acid changes that determined the *bla*$_{\text{CTX-M}}$ type. The alignments of the first three hits in the blastp search list for 774 shown in **Figure 3.7** were all *bla*$_{\text{CTX-M-15}}$ with a query cover result of 100%, an E-value of 0.0 and a percentage identity of 100% and the remaining 38 in the *bla*$_{\text{CTX}}$ group all returned identical results to 774. The alignments of the results for hits 4-6 in the blastp search list for 774 shown in **Figure 3.8**, returned a percentage identity of 100% but with a query cover of only 99%. Hit 4 returned a *bla*$_{\text{CTX-M-15}}$ result but hit 5 and 6 results were of an unspecificed type. The lower query cover was likely the result of these 3 hits being 291 amino acids as opposed to the 290 amino acids utlised in the original search. The alignments of the results for hits 7-9 in the blastp search list for 774 shown in **Figure 3.9** returned a percentage identity of 99.66% and a query cover of 100% with hit 7 returning a result of *bla*$_{\text{CTX-M-238}}$ with an amino acid substitution at T10A, hit 8 returning a result of *bla*$_{\text{CTX-M-186}}$ with an amino acid substitution at L20S and hit 9 returning a result of *bla*$_{\text{CTX-M-232}}$ with an amino acid substitution at A15T. The results of the blastp analysis demonstrated

the importance of conducting searches with the entire amino acid sequence, as single amino acid substitutions can result in different *bla*$_{\text{CTX-M}}$ types as was evident with hits 7, 8 and 9 that returned results of *bla*$_{\text{CTX-M-238}}$, *bla*$_{\text{CTX-M-186}}$ and *bla*$_{\text{CTX-M-232}}$ respectively. The *bla*$_{\text{CTX-M}}$ typing concluded all 39 isolates in the *bla*$_{\text{CTX}}$ group were of *bla*$_{\text{CTX-M-15}}$ type, indicated from the query cover, E value and per. Ident values.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenPept Graphics Distance tree of results Multiple alignment MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
class A extended-spectrum beta-lactamase CTX-M-15 [Bacteria]	Bacteria	568	568	100%	0.0	100.00%	291	WP_000239590.1
CTX-M-15 [Pantoea agglomerans]	Pantoea agglom…	568	568	100%	0.0	100.00%	293	ADG01902.1
CTX-M-15 [Escherichia coli]	Escherichia coli	568	568	100%	0.0	100.00%	311	ACQ42051.1
extended-spectrum beta-lactamase CTX-M-15 [Escherichia coli]	Escherichia coli	566	566	99%	0.0	100.00%	290	ABM88811.1
CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]	Escherichia coli	566	566	99%	0.0	100.00%	291	EGQ3855302.1
CTX-M family beta-lactamase [Enterobacter cloacae]	Enterobacter clo…	566	566	99%	0.0	100.00%	291	QBH72494.1
class A extended-spectrum beta-lactamase CTX-M-238 [Escherichia coli]	Escherichia coli	568	568	100%	0.0	99.66%	291	WP_188331865.1
class A extended-spectrum beta-lactamase CTX-M-186 [Escherichia coli]	Escherichia coli	568	568	100%	0.0	99.66%	291	WP_065419569.1
class A extended-spectrum beta-lactamase CTX-M-232 [Escherichia coli]	Escherichia coli	568	568	100%	0.0	99.66%	291	WP_156404652.1
CTX-M family class A extended-spectrum beta-lactamase [Shigella sonnei]	Shigella sonnei	568	568	100%	0.0	99.66%	291	EFZ7205036.1
CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]	Escherichia coli	568	568	100%	0.0	99.66%	291	WP_202790472.1
CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]	Escherichia coli	568	568	100%	0.0	99.66%	291	WP_172690312.1
TPA: CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]	Escherichia coli	567	567	100%	0.0	99.66%	291	HAL3685999.1
TPA: CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]	Escherichia coli	567	567	100%	0.0	99.66%	291	HBB3448119.1
TPA: CTX-M family class A extended-spectrum beta-lactamase [Enterobacter hormaechei subsp. xiangfangen…	Enterobacter hor…	567	567	100%	0.0	99.66%	291	HBM2822543.1
CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]	Escherichia coli	567	567	100%	0.0	99.66%	291	EFE7942478.1
CTX-M-15-derivative beta-lactamase [synthetic construct]	synthetic construct	567	567	100%	0.0	99.66%	291	ADA62509.1
hypothetical protein CP995_13820 [Klebsiella pneumoniae]	Klebsiella pneu…	567	567	100%	0.0	99.66%	291	PCQ19411.1
TPA: CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]	Escherichia coli	567	567	100%	0.0	99.66%	291	HAZ5469791.1
CTX-M family class A extended-spectrum beta-lactamase [Escherichia sp. R10]	Escherichia sp. …	567	567	100%	0.0	99.66%	291	WP_106106695.1
class A extended-spectrum beta-lactamase CTX-M-218 [Escherichia coli]	Escherichia coli	567	567	100%	0.0	99.66%	291	WP_109791214.1

Figure 3.6: The blastp search conducted on the CTX-M gene from isolate 774 with the first 9 results highlighted within the red box as an example of how single amino acid substitutions can alter *bla*$_{\text{CTX-M}}$ type as indicated by the percentage query cover and per. Identity results

MULTISPECIES: class A extended-spectrum beta-lactamase CTX-M-15 [Bacteria]

Sequence ID: WP_000239590.1 Length: 291 Number of Matches: 1

See 75 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 291 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
568 bits(1464)	0.0	Compositional matrix adjust.	291/291(100%)	291/291(100%)	0/291(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  1    MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
Sbjct  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
```

Blast Hit 1

CTX-M-15, partial [Pantoea agglomerans]

Sequence ID: ADG01902.1 Length: 293 Number of Matches: 1

Range 1: 3 to 293 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
568 bits(1464)	0.0	Compositional matrix adjust.	291/291(100%)	291/291(100%)	0/291(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  3    MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  62

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  63   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  122

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  123  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  182

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  183  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  242

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
Sbjct  243  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  293
```

Blast Hit 2

CTX-M-15 [Escherichia coli]

Sequence ID: ACQ42051.1 Length: 311 Number of Matches: 1

See 4 more title(s) See all Identical Proteins(IPG)

Range 1: 21 to 311 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
568 bits(1463)	0.0	Compositional matrix adjust.	291/291(100%)	291/291(100%)	0/291(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  21   MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  80

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  81   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  140

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  141  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  200

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  201  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  260

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
Sbjct  261  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  311
```

Blast Hit 3

Figure 3.7: blastp results from NCBI returning a Per. Identity and query cover results of 100% confirming all to be of *bla*CTX-M-15 type

extended-spectrum beta-lactamase CTX-M-15, partial [Escherichia coli]

Sequence ID: ABM88811.1 Length: 290 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 290 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
566 bits(1460)	0.0	Compositional matrix adjust.	290/290(100%)	290/290(100%)	0/290(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  1    MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG  290
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG
Sbjct  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG  290
```

Blast Hit 4

CTX-M family class A extended-spectrum beta-lactamase [Escherichia coli]

Sequence ID: EGQ3855302.1 Length: 291 Number of Matches: 1

Range 1: 1 to 290 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
566 bits(1460)	0.0	Compositional matrix adjust.	290/290(100%)	290/290(100%)	0/290(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  1    MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG  290
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG
Sbjct  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG  290
```

Blast Hit 5

CTX-M family beta-lactamase, partial [Enterobacter cloacae]

Sequence ID: QBH72494.1 Length: 291 Number of Matches: 1

See 4 more title(s) See all Identical Proteins(IPG)

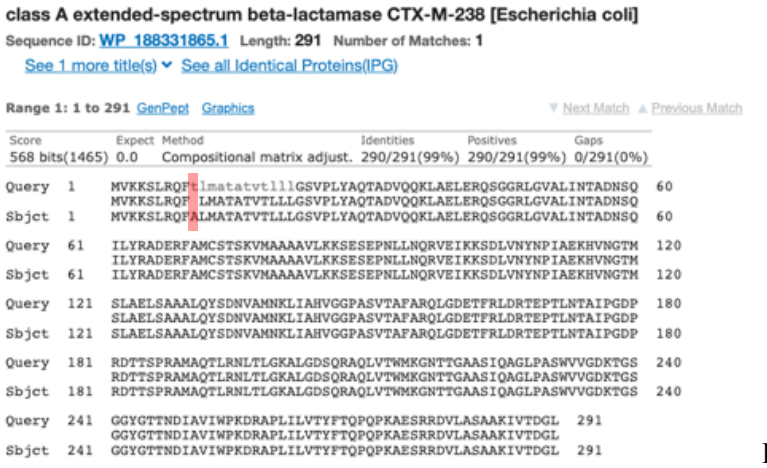
Range 1: 2 to 291 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
566 bits(1459)	0.0	Compositional matrix adjust.	290/290(100%)	290/290(100%)	0/290(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  2    MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  61

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  62   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  121

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  122  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  181

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  182  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  241

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG  290
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG
Sbjct  242  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDG  291
```

Blast Hit 6

Figure 3.8: blastp results from NCBI returning a Per. Identity of 100% but with a query cover result of 99%. Hit 4 returned a *bla*$_{\text{CTX-M-15}}$ result but hit 5 and 6 results were of an unspecificed type. The lower query cover was likely the result of these 3 hits being 291 amino acids as opposed to the 290 amino acids utlised in the original search



class A extended-spectrum beta-lactamase CTX-M-238 [Escherichia coli]

Sequence ID: WP_188331865.1 Length: 291 Number of Matches: 1

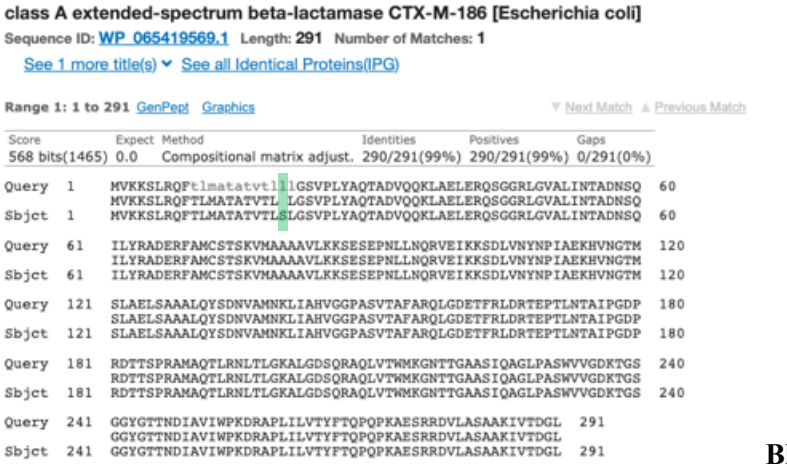
See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 291 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
568 bits(1465)	0.0	Compositional matrix adjust.	290/291(99%)	290/291(99%)	0/291(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  1    MVKKSLRQFALMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
Sbjct  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
```

Blast Hit 7



class A extended-spectrum beta-lactamase CTX-M-186 [Escherichia coli]

Sequence ID: WP_065419569.1 Length: 291 Number of Matches: 1

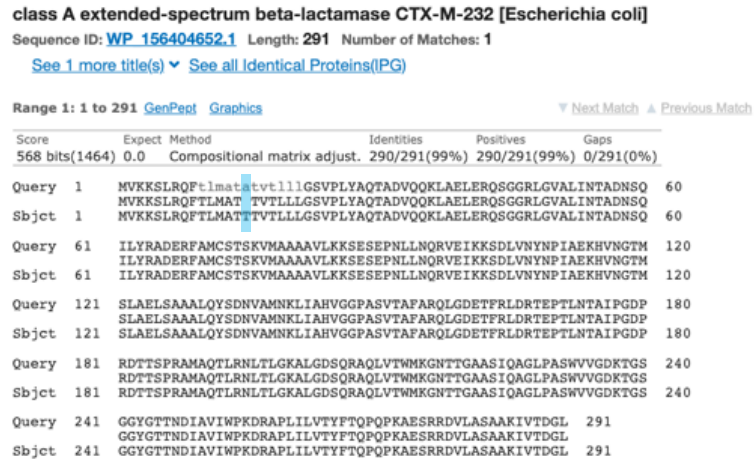
See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 291 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
568 bits(1465)	0.0	Compositional matrix adjust.	290/291(99%)	290/291(99%)	0/291(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  1    MVKKSLRQFTLMATATVTLSLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
Sbjct  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
```

Blast Hit 8



class A extended-spectrum beta-lactamase CTX-M-232 [Escherichia coli]

Sequence ID: WP_156404652.1 Length: 291 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 1 to 291 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
568 bits(1464)	0.0	Compositional matrix adjust.	290/291(99%)	290/291(99%)	0/291(0%)

```
Query  1    MVKKSLRQFtlmatatvtlllGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60
            MVKKSLRQFTLMATTVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ
Sbjct  1    MVKKSLRQFTLMATTVTLLLGSVPLYAQTADVQQKLAELERQSGGRLGVALINTADNSQ  60

Query  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120
            ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM
Sbjct  61   ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM  120

Query  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180
            SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP
Sbjct  121  SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP  180

Query  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240
            RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS
Sbjct  181  RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS  240

Query  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
            GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL
Sbjct  241  GGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESRRDVLASAAKIVTDGL  291
```

Blast Hit 9

Figure 3.9: blastp results from NCBI returning a query cover result of 100% but with a Per. Identity of 99.66%. Hit 7 returned a result of *bla*CTX-M-238 with an amino acid substitution at T10A (highlighted in red). Hit 8 returned a result of *bla*CTX-M-186 with an amino acid substitution at L20S (highlighted in green). Hit 9 returned a result of *bla*CTX-M-232 with an amino acid substitution at A15T (highlighted in blue)

3.4.4. ResFinder and PointFinder Analysis of Sequence Data

Further sequence analysis using both the ResFinder (that also incorporates PointFinder) (Zankari et al. 2012b) from CGE (**Section 2.9.5.2**) revealed resistance gene carriage in addition to *bla*$_{\text{CTX-M-15}}$ that included *tetAR* conferring tetracycline resistance and *qnrS1* providing low level quinolone resistance. It is known that QnrS1 confers weak resistance to fluoroquinolones when it is present alone as noted within the literature by both Allou et al. (2009) and Porse et al. (2020). All isolates within the *bla*$_{\text{CTX}}$ group were susceptible to both the fluoroquinolones enrofloxacin and ciprofloxacin and the quinolone nalidixic acid. Clinically relevant, high-level resistance to both fluoroquinolones such as enrofloxacin and ciprofloxacin and quinolones such as nalidixic acid, has been shown mainly to be associated with point mutations within the region denoted as the quinolone resistance determining region (QRDR) of DNA gyrase (GyrA) and topoisomerase IV (ParC). Resistance to quinolones and fluoroquinolones from mutations introduced into the QRDR, arises as a result of alterations to the target enzymes. The QRDR occurs on the DNA binding surface of the enzyme and mutations in this region are believed to reduce drug binding of quinolones to the enzyme-DNA complex (Yoshida et al. 1991; Ruiz 2003; Jacoby 2005; Fàbrega et al. 2009). In *E. coli* these regions associated with resistance mutations are defined as codons 67-106 for *gyrA* and codons 56-108 for *parC* (Johnning et al. 2015). The most frequently reported "known mutations" within both *gyrA* and *parC*, which have been shown to result in high level resistance, are the amino acids substitutions D87N and S83L in *gyrA* and S80I within *parC* (Chen et al. 2001; Sáenz et al. 2003; Onseedaeng and Ratthawongjirakul 2016; Yu et al. 2020). However, none of the known

mutations in either *gyrA* or *parC* that are commonly associated with quinolone resistance, were located in any of the strains.

The results of the ResFinder would appear to match the phenotypic findings of the MICs. All but isolates 950, 953, 954 and 956 of the 39 *bla*CTX isolates showed resistance to tetracycline following phenotypic profiling as described in **Section 3.3.1**, and the absence of *tetAR* within the WGS of these four isolates, would appear to confirm this.

In all but isolate 962, the resistance genes that were detected from the ResFinder searches, were found within contig 1 and therefore were encoded on the bacterial chromosome, in 962 the *tetAR* were found in a separate contig and this was further confirmed by manual investigation of the WGS using Geneious Prime. In all isolates, both the *qnrS1* and *bla*CTX-M-15 resistance genes were found within the IS*Ecp1* genetic environment region and in all but isolate 962, *tetAR* were also found with the IS*Ecp1* genetic environment region, which is discussed in more depth in **Section 3.6.1.1**.

3.4.5. Virulence Genes

Virulence genes located using VirulenceFinder 2.0 (**Section 2.8.6.3**) included the EHEC-associated glutamate decarboxylase gene *gad* involved in acid resistance, which provides an oral route colonising bacterium with resistance to stomach acid (de Biase and Pennacchietti 2012); the ExPEC associated *iss*, which confers increased serum survival, complement resistance and protection from host defences (Biran et al. 2021); *sitA* a peri-plasmic iron binding protein which mediates the transport of iron for iron acquisition (Sabri et al. 2006; Schouler et al. 2012; Ibrahim et al. 2019); and *traT* a conjugal

transfer surface exclusion protein involved in complement resistance (Al-Janabi et al. 2018; Sarowska et al. 2019b). Most of the virulence genes were found to be chromosomally-encoded including *gad*, *iss* and *sitA* and the occurrence of the only plasmid-encoded virulence gene *traT*, coincided with carriage of the IncFIC and IncFII plasmids in each isolate, as listed in **Table 3.2**. Isolates 774, 878 and 942 with no plasmids and isolate 966 which only carried the IncI1 plasmid, were the only isolates not encoding *traT*.

The significance of both chromosomally and plasmid encoded virulence genes and their association with specific pathotypes are explored in more detail in **Chapter 5**, **Section 5.4.5**. As only a few individual virulence genes were found, the 39 isolates were not matched to a specific pathotype, as usually a combination of specific virulence factors is required for determination of a pathotype (Kaper et al. 2004). However, the presence of virulence genes could serve to provide pathogenic potential for these *E. coli*. In addition, the finding of identical virulence genes in the chromosome and plasmid type of each isolate, signified another possible indication of clonality in these 39 isolates.

3.4.6. MLST

The MLST provided by the CGE (Larsen et al. 2012), showed all 39 isolates within this group to be of the same sequence type ST2325. However, whilst MLST is a good method for suggesting clonality between the core genomes of bacteria, consisting of genes present within all strains of a species, there remains an important variable part of the genome that can be quite different known as the accessory genome (Medini et al. 2005; Tettelin et al. 2008). This makes MLST less reliable at identifying true clonality between a subset of

isolates of the same species. The accessory genome is often where the gene repertoire that makes an isolate unique, such as plasmids, transposons, insertion sequences and point mutations creating frameshifts, are found. These genes are often important to survival by providing selective advantages including host colonisation, niche adaptation, increased virulence, and antimicrobial resistance (Medini et al. 2005; Tettelin et al. 2008). Therefore, a more in-depth approach was required to utilise the wealth of data produced by the WGS and this was achieved by producing a whole genome phylogeny and SNP distance comparison of the 39 isolates in the *bla*CTX group.

3.5. PHYLOGENETICS

A whole genome phylogeny and SNP distance comparison was conducted on 37 isolates from the *bla*CTX group (the two isolates 878 and 880 were removed during quality filtering due to poor sequencing coverage and assembly) and 105 ST2325 genomes downloaded from Enterobase (**Section 2.8.6.1**). EcoMHE1212-939 from the *bla*CTX group, was utilised as the reference genome and was selected as it had the best coverage and assembly. This whole genome phylogeny and SNP distance comparison was conducted firstly to investigate if the 37 isolates in the *bla*CTX group were clonally related and therefore to determine whether the spread of *bla*CTX-M-15 within those 37 isolates from this particular farm, was truly through clonal expansion. Secondly with the addition of all ST2325 isolates downloaded from the database Enterobase for the whole genome phylogeny and SNP distance comparison, an investigation was conducted into whether the 37 isolates were related to other ST2325 from the

database and whether there was an association of IS*Ecp1* and/or *bla*$_{\text{CTX-M}}$. The geographical sampling locations and niches of the Enterobase isolates were also investigated to discover where the majority of ST2325 were located and from which niche they were most commonly found. This final investigation of niche hoped to answer the question of whether ST2325 appeared to be bovine-associated.

The whole genome phylogeny maximum likelihood tree shown in **Figure 3.10** was produced using IQtree v2.0, with annotation achieved using the iTOL v.5.7 (**Section 2.8.7**), for the 37 isolates in the *bla*$_{\text{CTX}}$ group and 105 ST2325 genomes downloaded from Enterobase. As all the *bla*$_{\text{CTX}}$ isolates were encoding *qnrS1* and *bla*$_{\text{CTX-M-15}}$ and all but 4 (950, 953, 955 and 956) were encoding *tetAR*, a search for *tetAR*, *qnrS1* and any *bla*$_{\text{CTX-M}}$ variants was conducted using ResFinder 4.1 as per **Section 2.8.6.2**. In addition, a search was conducted on all the ST2325 genomes in Enterobase for IS*Ecp1*. It was hoped this search for the resistance genes *tetAR*, *qnrS1* and the *bla*$_{\text{CTX-M}}$ variants along with IS*Ecp1*, would help to identify any genomes in Enterobase that had a similar IS*Ecp1* genetic environment to the 37 *bla*$_{\text{CTX}}$ isolates. Any *tetAR*, *qnrS1* and *bla*$_{\text{CTX-M}}$ variants found were annotated around the outside of the tree in **Figure 3.10** using a binary data input from iTOL v.5.7 and are displayed as a filled blue square when the gene was present. Any Enterobase isolates on the tree in **Figure 3.10** that were positive for IS*Ecp1* had the tree label shown in red and all of the 37 *bla*$_{\text{CTX}}$ isolates were identified on the tree by a green highlight across the tree label.

The tree revealed the overall level of diversity between the ST2325 isolates and there did appear to be some clustering of isolates on the tree in

Figure 3.10. The *bla*$_{CTX}$ EVAL farms isolates formed what appeared to be a clonal cluster which is coloured green on the tree in **Figure 3.10**. A few other additional groups appeared to form genetic clusters, which were separate from the *bla*$_{CTX}$ EVAL farms isolates. As all reads from the isolates which encompassed both the 37 *bla*$_{CTX}$ isolates and the 105 Enterobase isolates were mapped to the reference genome EcoMHE1212-939 which came from the *bla*$_{CTX}$ group, divergence within the ST2325 isolates could therefore largely be due to the accessory genome.

However, only so much information could be inferred from analysing the phylogenetic tree alone and therefore to understand the relationship between the 105 Enterobase isolates and the 37 EVAL farms isolates in the *bla*$_{CTX}$ group, SNP distance comparison was conducted which is explored in **Section 3.6.3.** For ease of comparison between the tree in **Figure 3.10** and the SNP distance comparison in **Section 3.6.3**, any groups that were identified by SNP distance comparison as appearing to be either clonal or closely related, were annotated onto the tree in **Figure 3.10** by shading the leaves, branches and clade of that group and are identified by the coloured ranges key.

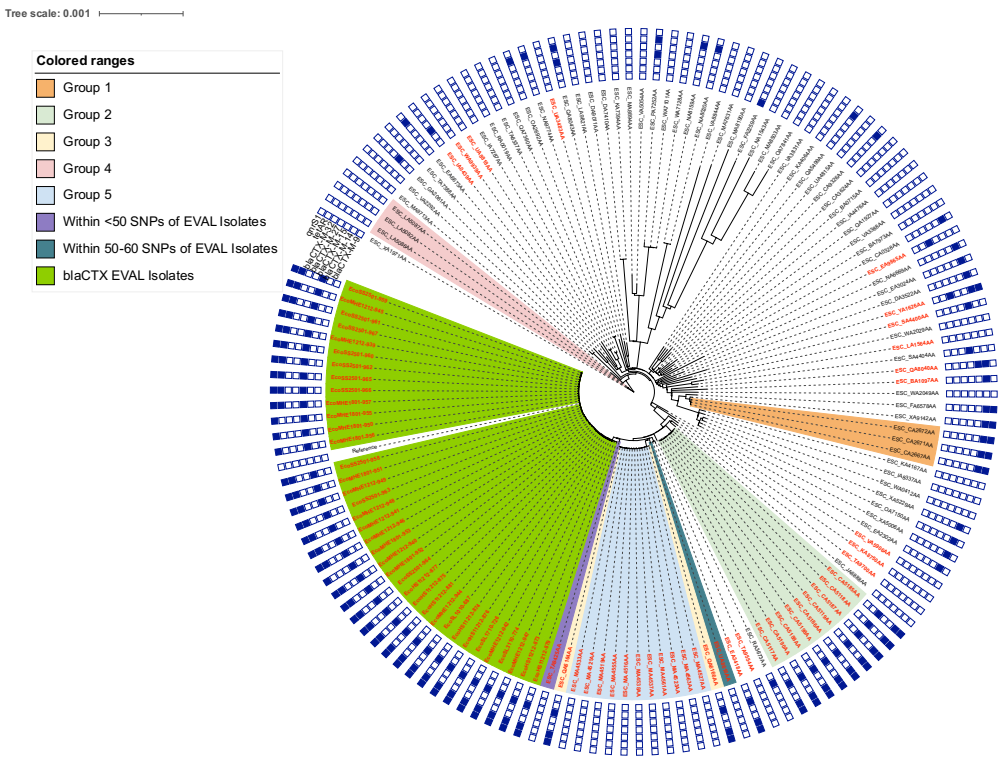


Figure 3.10 The whole genome phylogeny maximum likelihood tree showing the 37 *bla*$_{CTX}$ isolates in combination with the 105 ST2325 genomes downloaded from Enterobase. The resistance gene carriage of each is annotated around the outside of the tree with positive carriage denoted as a blue square. Any isolates positive for IS*Ecp1* had the tree label shown in red and the 37 *bla*$_{CTX}$ isolates were highlighted in green. The colour range key and shaded clades on the tree, relates to groups of isolates that were identified as possible clonal groups from the SNP distance comparison

3.5.1. IS*Ecp1* and *bla*$_{CTX-M}$ from the 105 ST2325 *E. coli* Genomes from Enterobase

The genome screening conducted on the 105 Enterobase ST2325 *E. coli* isolates to detect *bla*$_{CTX-M}$ variants and the resistance genes *tetAR* and *qnrS1*, which as stated in **Section 3.6**, were indicated on **Figure 3.10** as the binary data

around the outside of the tree, with a blue square confirming positive carriage of a resistance gene. Of the 105 ST2325 isolates downloaded from Enterobase, a total of 38 were found to be encoding IS*Ecp1* with 17 found to also be encoding a *bla*CTX-M. All but two of the 17 were of *bla*CTX-M-15 type with the remaining two of *bla*CTX-M-32 and *bla*CTX-M-27 type. The assemblies of the genomes downloaded from Enterobase were largely derived from short read only sequencing data (which is indicative of limited quality) and therefore it was difficult to be certain of whether the IS*Ecp1* genetic environments were located chromosomally or in a plasmid. Manual inspection of the genome in all isolates positive for both IS*Ecp1* and *bla*CTX-M was conducted to confirm where IS*Ecp1* and *bla*CTX-M were in relation to one another. Therefore, it was possible to determine that the IS*Ecp1* was in the same region as *bla*CTX-M in 16 of the 17 IS*Ecp1* and *bla*CTX-M positive Enterobase isolates, as the IS*Ecp1* transposase was located directly upstream of the *bla*CTX-M gene and was located in the same contig. In the isolate found to be encoding both an IS*Ecp1* and *bla*CTX-M-32, the *bla*CTX-M-32 was found in the middle of a large contig surrounded by what appeared to be chromosomal DNA which was different to the contig containing IS*Ecp1*.

In addition, 13 of the 15 IS*Ecp1* and *bla*CTX-M-15 positive Enterobase isolates were found to be encoding both *tetAR* and *qnrS1* with the remaining 2 of the 15 encoding either *qnrS1* or *tetAR* alone. The IS*Ecp1* was located to the same region as *tetAR* in 2 isolates and to the same region as *qnrS1* in 4 isolates. In only 1 isolate however did both *tetAR* and *qnrS1* appear to be located to the same region as IS*Ecp1*. However as stated above, due to the poor assemblies of the genomes downloaded from Enterobase, it was difficult to be certain which

contigs made up the entirety of the IS*Ecp1* genetic environment. Therefore, the locations of the *tetAR*, *qnrS1* and IS*Ecp1* genes in relation to each other was merely an observation from the sequence data that was available.

This analysis looking at IS*Ecp1* in association with ST2325 appeared to show that IS*Ecp1* was quite widespread throughout the 105 isolates being found in 37.1% of the isolates. In addition, the most commonly found *bla*$_{\text{CTX-M}}$ variant in association with IS*Ecp1* was *bla*$_{\text{CTX-M-15}}$, which was the same as the *bla*$_{\text{CTX}}$ isolates in this study This could suggest that ST2325 may have an association with IS*Ecp1* and *bla*$_{\text{CTX-M-15}}$ but considering the sample size available from Enterobase was small, it is difficult to be certain how widespread IS*Ecp1* and *bla*$_{\text{CTX-M-15}}$ are throughout ST2325 isolates that are not represented in the database. It was also interesting to find that *tetAR* and *qnrS1* was found in the same isolates as IS*Ecp1*. With better assemblies available from Enterobase, it would have been possible to construct the genetic environments with more accuracy and therefore assess how similar these were to the *bla*$_{\text{CTX}}$ EVAL isolates. However, as many of the genomes in Enterobase were produced from short read sequencing only, with many having >100 contigs, it was difficult to contextualise any MGE information from this. Therefore, the findings of these genes alongside IS*Ecp1* were merely an observation but considering the frequency at which IS*Ecp1*, *bla*$_{\text{CTX-M-15}}$, *tetAR* and *qnrS1* was found within this small sample, it would appear that there may be some association between these resistance genes and IS*Ecp1* elements found in ST2325. A larger dataset would allow this hypothesis to be examined more thoroughly, with the next step also being to look at how associated IS*Ecp1* is to other STs and how similar these are to ST2325.

3.5.1.1. Geographical Location and Sampling Niches

The information in relation to sampling location and sample type was limited for some isolates downloaded from Enterobase and therefore additional manual searches of both the Bio Project ID and Accession numbers were conducted to try and fill the gaps in the information provided by Enterobase. **Appendix C** lists the full metadata for all the isolates downloaded from Enterobase and includes details of the genes of interest, sampling information and dates, Bio Project ID, accession numbers and country of origin. Inclusion of the metadata is useful for understanding the epidemiological patterns of AMR in relation to sample source, geographical location and association with specific resistance genes, as this can infer areas of clusters of potential important clones. A good example of this is the pandemic clone ST131, which was discussed in **Section 1.5.2** which has also been found associated with IS*Ecp1* and *bla*CTX-M-15 (Hirai et al. 2013a; Stoesser et al. 2016; Ludden et al. 2020) which was discussed in **Section 1.3.2.5.2**. **Appendix D** lists the full results for the ResFinder search, which includes the Enterobase file name, node location of the gene, sequence location, gene name, coverage and accession number. **Appendix E** lists the full output from the IS*Ecp1* search that includes the Enterobase fie name, node location of the gene, sequence location, which DNA strand IS*Ecp1* was encoded on, %coverage and %identity.

Table 3.5 lists the number of isolates from Enterobase within each niche (livestock, companion animal, wild animal, human, food, environment and non-defined (ND)), followed by the subtypes of samples within that niche as bullet points and the total number of isolates within the niche with source within the niche totals as bullet points.

The highest represented niche was livestock which made up 60% (64 isolates) of the total isolates downloaded from Enterobase. Within the livestock niche the highest numbers were bovine samples, which made up 31.4% (33 isolates) and this was followed by poultry/avian 14.3% (15 isolates) and ovine/goat 9.5% (10 isolates). This could suggest that there is an association between ST2325 and bovine, however bovine and poultry are more intensely farmed than sheep or goats (Pandey and Upadhyay 2022b) and therefore, it could just be that there are more bovine and poultry isolates represented in the database. But again, as stated in **Section 3.6.1.1**, due to the low sample size this cannot be determined for certain, but it does offer an interesting insight into how ST2325 is represented in the database.

The geographical location of isolates from Enterobase encompassed a total of 19 countries. Most of the isolates were from the United States which totalled 49 out of the 105 Enterobase isolates, this was followed by the UK which totalled 19 and Germany which totalled 7. The remaining isolates were represented across 16 different countries with between 1-4 isolates from each which included the countries (with number of isolates in brackets) Australia (1), Canada (1), Chile (1), China (2), Croatia (1), France (2), Kenya (4), Luxembourg (2), Nepal (1), Netherlands (3), Nigeria (3), Pakistan (2), Singapore (1), South Africa (2), Spain (1 and Vietnam (1) with 2 that were ND. Even though ST2325 is widespread across 19 countries, many only returned results for a few isolates. Even though the US and UK had the largest majority, the low numbers of ST2325 in the database as a whole and the small numbers of ST2325 in other countries, this does not seem to suggest ST2325 represents a dominant widespread clone. Also, of note were ST2325 isolates that were identified in

human samples. The 3 isolates from Nigeria included 2 from humans sampled in August 2015 and 1 from poultry sampled in February 2019 and were therefore part of two different Bio Project ID's. From the Netherlands there was one human isolate and one bovine isolate represented in the database, but these were from different Bio Project IDs and were sampled during 2014 and 2016. There were 4 isolates from Kenya which included 1 human and 3 poultry/avian and these were part of the same Bio Project ID and sampled between October 2015 and June 2016. These interesting findings between human and animal isolates were investigated further for evidence of clonality between them, by looking at the SNP distance comparison, which is investigated in **Section 3.6.2**.

Table 3.5: The number of isolates downloaded from Enterobase within each niche

Niche (and source as bullet points)	Total in Niche (source totals as bullet points)
Livestock ▪ Bovine ▪ Poultry/Avian ▪ Ovine/Goat ▪ Swine ▪ ND/Others	64 ▪ 33 ▪ 15 ▪ 10 ▪ 5 ▪ 1
Companion Animals ▪ Canine (Military Dog) ▪ Canine (Domestic Dog) ▪ Equine ▪ Feline	8 ▪ 3 ▪ 3 ▪ 1 ▪ 1
Wild Animal ▪ Wild Boar ▪ White Tailed Deer ▪ ND/Others	6 ▪ 3 ▪ 1 ▪ 2
Human	7
Food	1
Environment	5
Non-defined (ND)	14

3.5.2. SNP Distance Comparison

A pairwise SNP distance matrix containing a total of 142 genomes and 1 reference genome, was produced from the 105 ST2325 Enterobase genomes and the 37 EVAL farms *bla*CTX isolates. The best quality assembly from the 37 EVAL farms *bla*CTX isolates was selected as the reference genome. The entire SNP matrix of 142 genomes is extremely large as a figure, therefore it is placed into **Appendix F**. The maximum number of SNPs between all 142 isolates in the comparison was 1,730. A SNP cut off of >80 SNPs was established for this dataset based on the distribution of SNP values. Groups of isolates that appeared to be closely related were all <80 SNPs apart and most other isolates that did not form closely related groups were >100 SNPs apart with many >300 SNPs apart.

3.5.2.1. Small Separate Clonal Groups Within the 142 Enterobase and *bla*CTX Genomes

Within the 105 Enterobase genomes there were some small, isolated groups that looked to each form a separate clonal cluster (denoted as clonal groups) and therefore a trimmed SNP matrix was produced. **Figure 3.11** shows the trimmed SNP distance matrix of the 5 separate clonal groups from the 105 Enterobase genomes, two isolates that were within <60 SNPs of the *bla*CTX isolates (denoted as <50 SNPs of EVAL isolates and 50-60 SNPs of EVAL isolates on the tree in **Figure 3.10** and the SNP matrix in **Figure 3.11**) and the 37 *bla*CTX EVAL farms isolates. In **Figure 3.11** any SNP value <10 was highlighted in yellow, SNP values between 11-50 were highlighted orange and

SNP values between 51-80 were highlighted green with any SNP values >80 left white. What is demonstrated in **Figure 3.11**, was that the *bla*$_{CTX}$ EVAL farms isolates were within 0-6 SNPs of each other and therefore appeared to be clonal, which was evident on the tree in **Figure 3.10** as they clustered together. Within the clonal groups from Enterobase, Group 1 were within 0-2 SNPs of each other, Group 2 were within 0-6 SNPs of each other, Group 3 were within 0-2 SNPs of each other, Group 4 were within 0-2 SNPs of each other and Group 5 were within 0-4 SNPs of each other. All but Group 3 clustered together on the tree in **Figure 3.10** (although they were part of the same clade, they were not side by side). The *bla*$_{CTX}$ EVAL farms isolates and each group alone appeared to be clonal but unrelated to any other groups or isolates. Only Groups 3 and 5 looked to be clonally related to each other, as all isolates within Groups 3 and 5 combined were within 1-4 SNPs of each other and group 3 and 5 also clustered as one group on the tree in **Figure 3.10**. However no other groups appeared to be closely related to each other with the closest being the combined Group 3 and 5 which were within 58-72 SNPs of the *bla*$_{CTX}$ EVAL farms isolates. The two separate isolates ESC_TA9425AA (denoted as <50 SNPs of EVAL isolates on **Figure 3.11**) and ESC_UA8616AA (denoted as 50-60 SNPs of EVAL isolates on **Figure 3.11**) were within 36-45 and 49-58 SNPs of the *bla*$_{CTX}$ EVAL isolates respectively and therefore were the most closely related from the Enterobase isolates to the *bla*$_{CTX}$ EVAL farms isolates. What the SNP distance analysis clearly showed however, was there were multiple sets of evidence for clustering of ST2325 isolates, but at the same time there was diversity as *E. coli* itself is diverse.

Information from Enterobase in relation to Bio Project IDs and sampling location for the clonal groups, indicated Group 1 were all from Bio Project ID PRJEB8774 and sampled in the UK, from wild boar faeces in the wild animal niche, Group 2 were all from Bio Project ID PRJEB8776 and sampled in the UK, from bovine faeces in the livestock niche, Group 3 were all from Bio Project ID PRJNA293225 and sampled in the US, from mixed locations with one sample detailed as animal related lairage swab and one as river water, Group 4 were all from Bio Project ID PRJNA433857 and sampled in Germany, from military dog faeces, Group 5 were all from Bio Project ID PRJNA293225 and sampled in the US, mostly from goat carcass swabs in the livestock niche with two from river water in the environment niche. Finally, the two separate isolates ESC_TA9425AA from Bio Project ID PRJEB33169 was taken from a Spanish bovine faecal sample and ESC_UA8616AA which had no information in relation to Bio Project ID, sample type or location.

The information gained from looking at the SNP distance comparison of the ST2325 genomes from Enterobase together with the 37 *bla*$_{\text{CTX}}$ EVAL farms isolates, showed that ST2325 appears to form small clonal groups and this was clear from the genomic data available from Enterobase that came from studies reporting clear evidence of clonality in their isolates and the isolates of the *bla*$_{\text{CTX}}$ group analysed as part of this study. However, these clonal groups appeared to be isolated in all but Groups 3 and 5 and therefore it would appear for the majority, the clonality was related to samples from the same studies and geographical area. It would not appear that ST2325 is a particularly widespread clone within the genomes available from the database and in comparison, to a significant dominant clone like ST131 (which was described in **Chapter 1**,

Section 1.5.2), which is represented with >14,000 genomes in Enterobase, the ST2325 numbers were relatively small. However, many of the isolates downloaded from Enterobase were still only within 300-400 SNPs of each other. As the majority of the ST2325 isolates were identified from animals with only a few from humans, this could possibly indicate a potential route ST2325 has taken from animals into the human population. However, as was stated in **Section 3.6.1.2**, a greater number of samples would be required for this hypothesis to be looked at more rigorously.

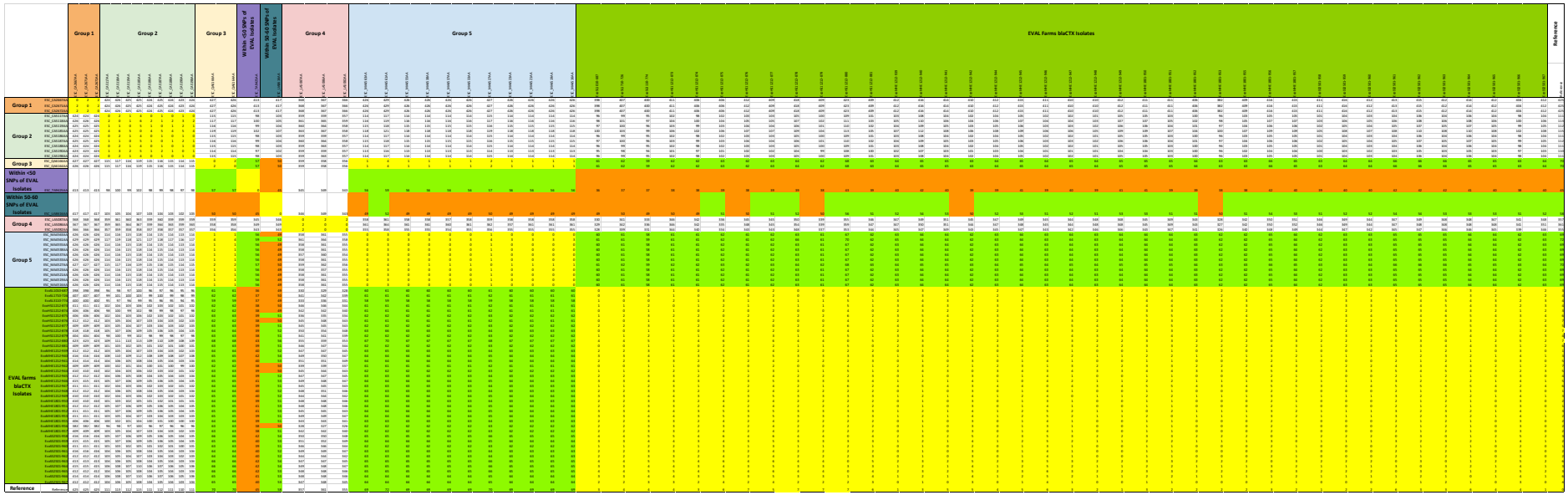


Figure 3.11: SNP distance matrix of the 5 separate clonal groups from Enterobase and the two separate isolates from Enterobase denoted as <50 SNPs of EVAL isolates and 50-60 SNPs of EVAL isolates, along with the *bla*CTX EVAL farms isolates. Any SNP values <10 are highlighted in yellow, any between 11-50 are highlighted in orange, any between 51-80 are highlighted in green and any >80 were left white

3.6. IS*Ecp1*

As detailed in **Section 3.3.2,** IS*Ecp1* was shown as being present within all 39 of the EVAL farms isolates from the *bla*$_{CTX}$ group. From these 39 IS*Ecp1* positive isolates, the WGS allowed for IS*Ecp1* genetic environments to be constructed and compared.

It has been frequently reported in studies, including those by Humeniuk et al. (2002b) , Poirel et al. (2002), Bonnet (2004a), Rodríguez et al. (2004), Lartigue et al. (2006a), Rossolini et al. (2008a), Literacka et al. (2009) and Bevan et al. (2017a), that the progenitors of *bla*$_{CTX-M}$ were possibly genes from a *Kluyvera* spp. chromosome and were likely then mobilised by IS*Ecp1*. IS*Ecp1* was first described by Stapleton (1999) within the plasmid pST01 of *E. coli* (Accession number: AJ242809).

IS*Ecp1* is able to mobilise a downstream-located *bla*$_{CTX-M}$ via a one-ended transposition mechanism and is flanked by inverted repeats (IRs) denoted as left and right (IR_L and IR_R). IS*Ecp1* often utilises IR_L alongside an imperfect IR_R that resembles the original IR_R, allowing for the capture of adjacent genes further downstream. This mechanism results in 5 bp repeats flanking the left of IR_L and the right of IR_R. IS*Ecp1* also brings the promoter sequences -35 and -10 for high level expression of *bla*$_{CTX-M}$ (Nordmann and Poirel 2005; Zong et al. 2010a). Searches for the IR_L and IR_R along with the 5 bp repeats, give a good indication of the likely size of the IS*Ecp1* element.

3.6.1. IS*Ecp1* Genetic Environments of 39 Isolates in the *bla*CTX Group

IS*Ecp1* was initially identified and analysed in the PacBio sequenced isolate 774. To investigate the presence of IS*Ecp1* within the remaining EVAL farms isolates, further sequencing of the remaining 38 isolates in the *bla*CTX group and 774 was conducted via Illumina short read and MinION (ONT) long read with hybrid assembly. This hybrid assembly of all of the isolates, gave a good level of accuracy for construction of the IS*Ecp1* genetic environment and for confirming whether it was chromosomal or plasmid encoded.

To size the IS*Ecp1* elements, the IR_L and the suspected new IR_R were located along with the 5 bp repeats either side of the IRs (which are explored in more detail in **Section 3.7.1.1**). The size was calculated from 5 bp to the left of IR_L to the 5 bp to the right of $IR_{R(new)}$. The IS*Ecp1* element within most of the EVAL farms isolates in the *bla*CTX-M group, were of the same size at 23,612 bp and contained the resistance genes *bla*CTX-M-15, *qnrS1* and *tetAR* along with a variety of other MGEs including transposons and insertion sequences, which are explored in detail in **Section 3.7.1.1**. **Figure 3.12** shows the 23,612 bp IS*Ecp1* element denoted as +*tetAR* and details the outer edges of the surrounding chromosome where the IS*Ecp1* had inserted. As can be seen in **Figure 3.12**, the IS*Ecp1* had interrupted a Type III Secretion System (T3SS) *prgH*/*eprH* (denoted PrgH/EprH (partial) within **Figure 3.12**), effectively splitting it in half. PrgH/EprH is part of the needle complex of the T3SS involved in the delivery of effector proteins to host cells (Zhou et al. 2014a). The consequences of this chromosomal insertion event that resulted in two truncated halves of *prgH*/*eprH*, could be a reduction in pathogenicity fitness to this bacterium. **Figures 3.13** and

3.14 detail either end of the element and the surrounding chromosome, showing the two partial pieces of the T3SS gene at the beginning and end of the IS*Ecp1* element.

In a few of the EVAL farms isolates (950, 953, 955 and 956), that were susceptible to TET following MIC testing (**Section 3.3.1**) and found not to be carrying the *tetAR* genes, the IS*Ecp1* element was smaller in size by 5,587 bp at 18,025 bp but still identical within these isolates and is shown in **Figure 3.15** and denoted as Δ*tetAR*. On studying the genetic environment of these four isolates, the smaller size was found to be due to the absence of the region encoding the tetracycline resistance that included a relaxase, respectively *tetAR*, *yedA* and Tn*AS1*. However, the remaining region of the IS*Ecp1* genetic environments and insertion points into the chromosome, in these four TET -ve isolates were identical to the isolates containing the larger IS*Ecp1* genetic environment shown in **Figure 3.12**. As all but 950, 953, 955 and 956 were identical, only two figures, one representing the majority of the isolates, designated as IS*Ecp1 +tetAR* and one representing the 4 isolates 950, 953, 955 and 956 designated as IS*Ecp1* Δ*tetAR* were constructed and shown in **Figures 3.12** and **3.15** Only the insertion point of the interrupted T3SS *prgH*/*eprH* annotated as (partial) of the surrounding chromosome is shown in **Figure 3.15**, however the insertion points and surrounding chromosome within Δ*tetAR*, were identical to +*tetAR* and therefore when referring to where the IS*Ecp1* inserted into the chromosome, only **Figure 3.12** will be referenced.. The 5,587 bp region that was missing from the Δ*tetAR* IS*Ecp1* element is shown in **Figure 3.16**.

The hybrid assembly gave good evidence and confidence that IS*Ecp1* and its genetic environment containing $bla_{\text{CTX-M}}$, *tetAR* and *qnrS1* were

chromosomally encoded, as they were present on contig 1 and the surrounding genetic environment abutting the 5 bp repeats and IRs was chromosomal as shown in **Figure 3.12**.

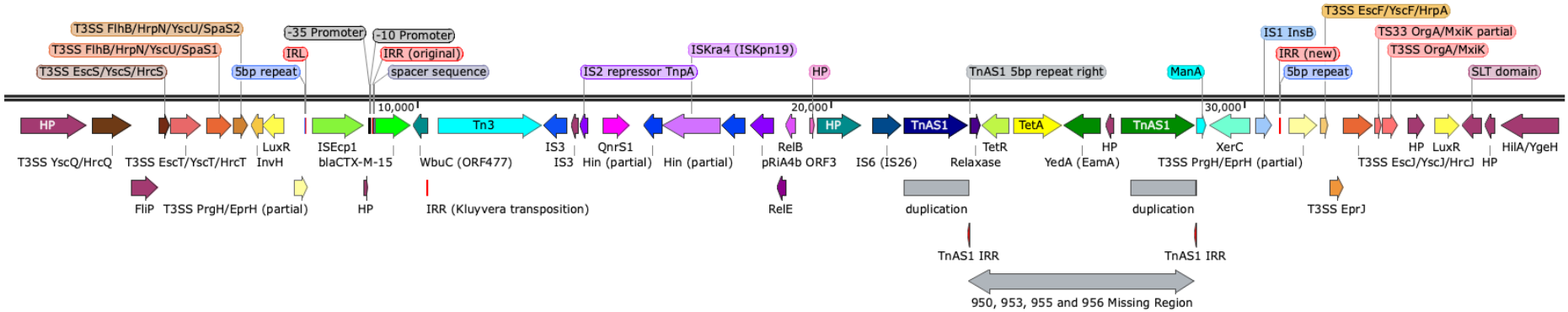


Figure 3.12: The +*tetAR* IS*Ecp1* element of 23,612 bp within the chromosome of the majority of IS*Ecp1* positive EVAL farms *E. coli* isolates and details the surrounding chromosome around the insertion point of the IS*Ecp1* element

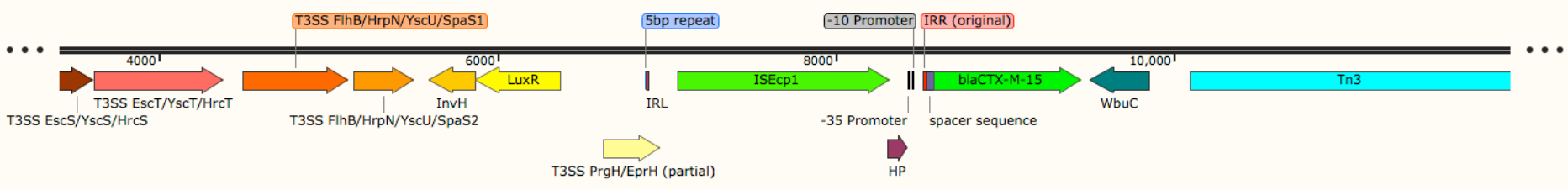


Figure 3.13: The left hand insertion point of IS*Ecp1* in *E. coli* isolate 774, showing the left truncated half of the T3SS *prgH/eprH*. Also detailed is the left 5 bp repeat and both the IR_L and $IR_{R(original)}$ along with the -35 and -10 promoter locations

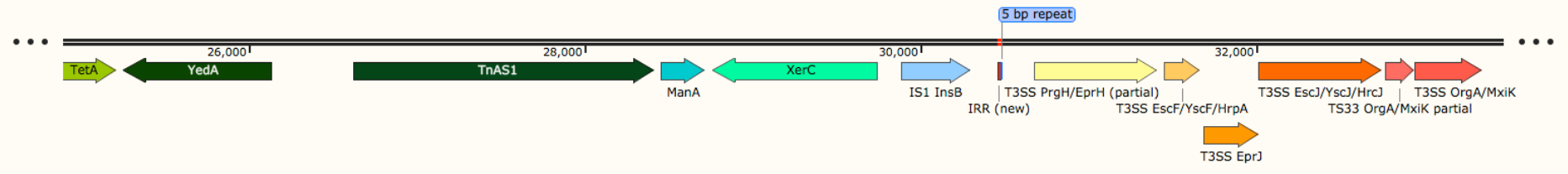


Figure 3.14: The right hand insertion point of IS*Ecp1* in *E. coli* isolate 774, showing the right 5 bp repeat, the new IR_R and the right truncated half of the T3SS *prgH/eprH*

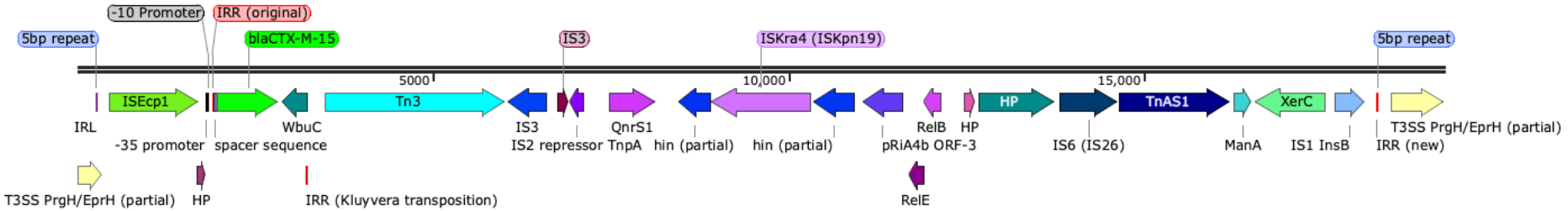


Figure 3.15: The Δ*tetAR* IS*Ecp1* element within the chromosome of the *E. coli* isolates 950, 953, 955 and 956, of 18,025 bp which details only the immediate surrounding chromosome of the insertion point of the IS*Ecp1* element which is the interrupted T3SS *prgH*/*eprH*

Figure 3.16: The region which was missing from the Δ*tetAR* IS*Ecp1* element within *E. coli* isolates 950, 953, 955 and 956 but which was present within all other IS*Ecp1* positive EVAL farms *E. coli* isolates, which includes the relaxase, *tetAR*, *yedA* and Tn*3*

3.6.1.1. The IS*Ecp1* Inverted Repeats (IRs) and Individual Genes of the IS*Ecp1* Genetic Environment

As the IR_L has not been reported to change across IS*Ecp1* elements, this was identified through comparison with IR_L sequences mentioned within previous studies by Poirel et al. (2003), Lartigue et al. (2006a) and Bae et al. (2006). All the EVAL farms isolates appeared to have used an 18 bp IR_L (CCTAGATTCTACGTCAGT) (shown in **Figure 3.17**) and IS*Ecp1* elements using an 18 bp IR_L have been mentioned previously within the literature by Bae et al. (2006). Locating the 5 bp repeats at either side of the IR_L and IR_R was key to both locating the alternative IR_R and also gauging the size of the IS*Ecp1* element. The 18 bp $IR_{R(original)}$ (ACACACGTGGAATTTAGG) shown in **Figure 3.17,** located directly to the right of IS*Ecp1* had 14 bp complementary to the IR_L (bases complementary to the IR_L are underlined). The 18 bp $IR_{R(original)}$ was identified by referencing a listed sequence associated as being an IR_R within previous studies by Bae et al. (2006), Lartigue et al. (2006a) and Wachino et al. (2006). The $IR_{R(original)}$ was denoted as original, as this would have been the IR_R which would have originally been used to mobilise IS*Ecp1* alone without any accompanying genes. The presence of the 5 bp repeats TAGTA to the left of IR_L and right of IR_R, indicated the possible location of the imperfect $IR_{R(new)}$ (GCTCCCATCGCTTCGCGA) shown in **Figure 3.17**, used to mobilise *bla*CTX-M-15 within isolate 774 that had 8 bases complementary to the IR_L (bases complementary to the IR_L are underlined).

It was believed that IS*Ecp1* mobilised *bla*CTX-M-15 using the imperfect $IR_{R(new)}$ due to both the interrupted T3SS gene *prgH*/*eprH* and the presence of the 5 bp duplications at either end of the element. With this in mind, the

transposable element size would be 23,612 bp, which is unlikely to be the original size of the element, as previously reported IS*Ecp1* elements have been in the region of around 2-6 kb in size (Lartigue et al. 2006a; Zong et al. 2010a).

Between the 5 bp and IR_L and the alternative IR_R and 5 bp repeat of the IS*Ecp1* element shown in **Figure 3.12**, were several transposons, insertion sequences and other genes including *wbuC,* a cupin fold metalloprotein often denoted as ORF477. Within **Figure 3.12** the IR_R denoted as $IR_{R(}$*Kluyvera* transposition) (GCGCACGTAGGTCCCAGG) that had 11 bases complementary to the IR_L (bases complementary to IR_L are underlined), was the likely IR_R originally used by IS*Ecp1* to mobilise a beta-lactamase gene away from a *Kluyvera* spp. chromosome, resulting in a truncated *wbuC* from 477 bp to 348 bp (Gołębiewski et al. 2007; Nagano et al. 2009). Also present within the IS*Ecp1* element were genes for a Tn*3* transposase, an IS*3* transposase, a *qnrS1* quinolone resistance gene, IS*Kra4*-like element IS*Kpn19* family transposase which had interrupted the DNA invertase *hin*, an ORF-3 family protein from plasmid pRiA4b, the type 2 toxin-antitoxin system *relEB*, an IS*6*-like element IS*26* family transposase, the putative inner membrane transporter *yedA*, two variant Tn*AS1* transposases with one appearing to be a truncated version, a relaxase, the mannose-6-phosphate isomerase *manA* and a tyrosine recombinase *xerC*. Each Tn*AS1* was adjacent to a 38 bp IR_R which showed perfect homology with two other IR_R sequences of Tn*1721* listed in the database under the accession numbers NC_019062.1 and KY007017.1. The *tnpA* of Tn*1721* and Tn*AS1* also showed high levels of sequence homology and searches of the literature would appear to suggest they both mobilise by a one-ended transposition mechanism (Mötsch et al. 1985; Nicolas et al. 2015). Tn*1721* has also been noted in association with *tetAR*

(Wiebauer et al. 1981; Pasquali et al. 2005). A study by Sadek et al. (2021) identified Tn*AS1* on an IncHI2 plasmid pEGYMCR isolated within *E. coli*. The Tn*AS1* transposition unit with two variant Tn*AS1* transposases, was found to be flanking a relaxase, *tetAR* and *yedA* (annotated as *eamA*) and is detailed in **Figure 3.18**. The first Tn*AS1* to the left of *tetR* is 1,707 bp and the second Tn*AS1* is 1,848 bp. The two Tn*AS1* transposons are almost identical, however, the second Tn*AS1* transposon has an additional 141 bp at the start of the sequence followed by 1,707 bp, which is identical to the first Tn*AS1* sequence. The IncHI2 pEGYMCR can be found within the database under the accession number MT499884. pEGYMCR was found to be very similar but not identical to the Tn*AS1* transposition unit associated with the IS*Ecp1* element of isolate 774. Within 774 Tn*AS1* to the left of *tetR* was 1,566 bp and the Tn*AS1* to the right of *yedA* was 1,797 bp with the first 1,566 bp an identical duplication of the Tn*AS1* to the left of *tetR* (duplication regions are denoted on **Figure 3.18** as grey boxes annotated as duplication). Tn*AS1* is described within the database as "Tn*3*-like" and this presents an unusual scenario, in that transposons of the Tn*3* family are known to exhibit what has been termed "target immunity", meaning there should not be multiple insertions of a Tn*3* type transposon within the same piece of target DNA (Nicolas et al. 2010; Grindley 2014). It could be surmised however that due to the small size of Tn*AS1*, it is different enough from Tn*3* not to be affected by Tn*3* insertion immunity. Kieffer et al. (2019b) demonstrated mobilisation of a plasmid-mediated *mcr-5* cassette by Tn*AS1*, through the use of a recombinant pBAD-Tn*AS1* plasmid and a plasmid pACYC-*mcr-5* encoding colistin resistance. Both were transformed into an *E. coli* strain, RZ211 carrying a conjugative plasmid pOX38 encoding gentamicin resistance. This was able to

demonstrate mobilisation of *mcr-5* through Tn*AS1* transposition, similar to mobilisation by miniature inverted-repeat transposable elements, in that the original gene cassette contained no transposase gene but had the inverted repeats present, that can be recognised by Tn*AS1* in *trans*.

The Tn*AS1* transposition unit therefore could be a possible mechanism for tetracycline resistance mobilisation through transposition and could be evidence of modular assembly of the IS*Ecp1* element. However, it was not certain whether what was seen in the ISE*cp1* genetic environments shown in **Figure 3.10** and **3.11** was indeed due to a modular assembly or from one transposition event. Several recombination events could have occurred due to the presence of both transposons and insertion sequences between the IRs, indicating a possible hotspot for insertion within this part of the chromosome. Alternatively, it could be due to the acquisition of genetic components from subsequent transposition events, that collected genes following the recognition of a new imperfect IR_R. The IR_L, $IR_{R(original)}$, $IR_{R(new)}$ and $IR_{R(\textit{Kluyvera}\ transposition)}$ are all shown in **Figure 3.15** (bases complementary to the IR_L are shown in red).

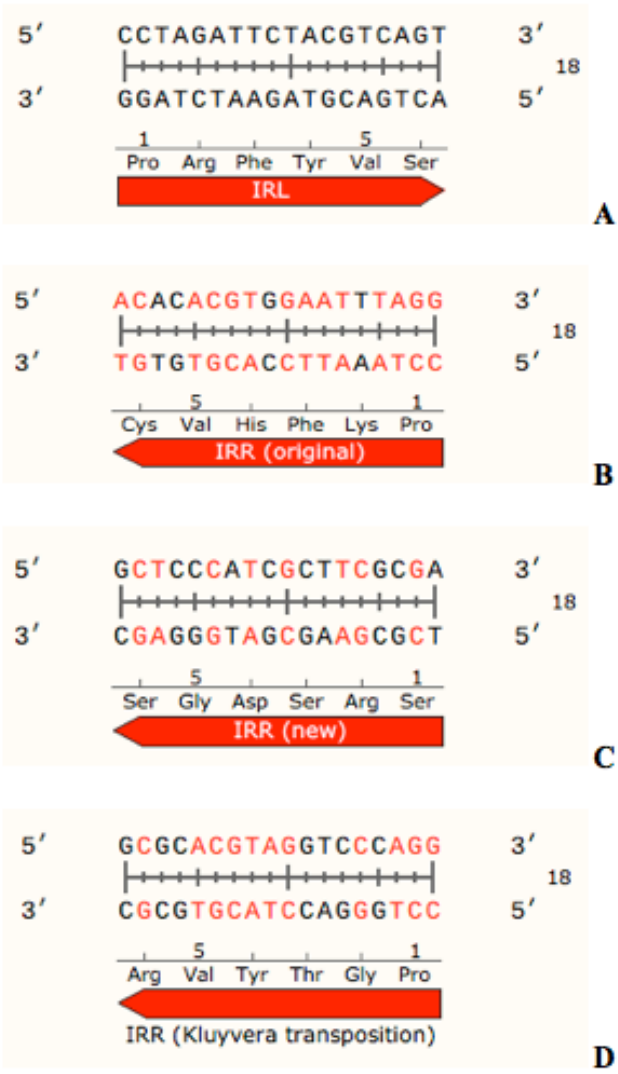


Figure 3.17: The IRs found within the proximity of IS*Ecp1* of isolate 774, with A showing the 18 bp IR_L, B showing the 28 bp $IR_{R(original)}$, C showing the 18 bp $IR_{R(new)}$ and D showing the 18 bp $IR_{R(Kluyvera\ transposition)}$. Bases complementary to IR_L are shown in red

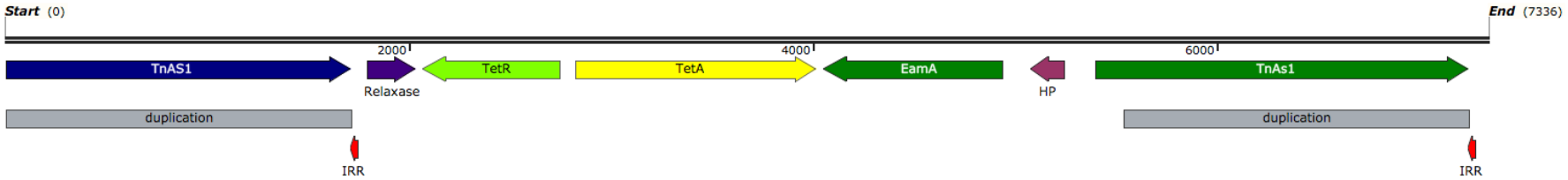


Figure 3.18: Tn*AS1* transposition unit of pEGYMCR from the study by Sadek et al. (2021)

3.6.2. ISE*cp1* Genetic Environment Geneious Pairwise Alignment

To compare exactly how similar the IS*Ecp1* elements were, a Geneious pairwise alignment was conducted that incorporated genes from the surrounding chromosome and the IS*Ecp1* element. The alignment was conducted from *yscQ*/*hrcQ* to the *hilA*/*ygeH* (with genes shown in **Figure 3.11**) with a mean length of 28,538 bp. Pairwise identity was 95.8% with 80.2% identical sites across 23,880 bp. A percentage identity matrix was produced in Geneious that showed most of the isolates were 95.328% to 100% identical to each other. Five isolates had lower percentage identity from 80.578% and this was due to four of these isolates being 950, 953, 955 and 956 which were all missing the *tetAR* region and isolate 962 where the *tetAR* region in association with Tn*AS1* was found in contig 6 in the assembly and therefore had not been included in the original alignment. The finding of Tn*AS1* in association with *tetAR* in contig 6 of isolate 962, could have been due to a couple of reasons. Either it could have simply been an assembly error or alternatively the transposable element Tn*AS1* was in the process of mobilising away from the chromosome. Interestingly, when this assembly was viewed in Bandage, contig 6 of 5,488 bp was circularised as can be seen in **Figure 3.14**. The 5,488 bp annotated genomic region of contig 6 is shown in **Figure 3.15** and was found to be identical to the Tn*AS1* region of all the other +*tetAR* IS*Ecp1* isolates. From annotating the Tn*AS1* from contig 6, it was found that the two separate Tn*AS1* annotations and duplications that had been found when constructing the IS*Ecp1* genetic environment shown in **Figure 3.10**, were actually one single transposase. The finding of this circular Tn*AS1*, shows the potential for *tetAR* to mobilise independently from the IS*Ecp1* element and suggests the Tn*AS1* possibly

mobilises via either a copy out and paste in or cut out and paste in mechanism (Bouuaert and Chalmers 2010; Skipper et al. 2013).

	ISEcp1 Region 687	ISEcp1 Region 726	ISEcp1 Region 774	ISEcp1 Region 873	ISEcp1 Region 874	ISEcp1 Region 875	ISEcp1 Region 876	ISEcp1 Region 877	ISEcp1 Region 878	ISEcp1 Region 879	ISEcp1 Region 880	ISEcp1 Region 881	ISEcp1 Region 939	ISEcp1 Region 940	ISEcp1 Region 941	ISEcp1 Region 942	ISEcp1 Region 944	ISEcp1 Region 945	ISEcp1 Region 946	ISEcp1 Region 947	ISEcp1 Region 948	ISEcp1 Region 949	ISEcp1 Region 950	ISEcp1 Region 951	ISEcp1 Region 952	ISEcp1 Region 953	ISEcp1 Region 955	ISEcp1 Region 956	ISEcp1 Region 957	ISEcp1 Region 958	ISEcp1 Region 959	ISEcp1 Region 960	ISEcp1 Region 961	ISEcp1 Region 962	ISEcp1 Region 963	ISEcp1 Region 964	ISEcp1 Region 965	ISEcp1 Region 966	ISEcp1 Region 967
ISEcp1 Region 687		99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 726	99.993		99.993	99.993	99.993	99.993	99.993	99.993	95.328	98.541	99.993	99.993	99.993	99.993	99.993	99.993	99.993	99.993	99.993	99.993	99.993	99.993	83.505	99.993	99.993	83.505	83.505	83.492	99.993	99.993	99.993	99.993	99.993	83.505	99.993	99.993	99.993	99.993	99.993
ISEcp1 Region 774	100	99.993		100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 873	100	99.993	100		100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 874	100	99.993	100	100		100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 875	100	99.993	100	100	100		100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 876	100	99.993	100	100	100	100		100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 877	100	99.993	100	100	100	100	100		95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 878	95.335	95.328	95.335	95.335	95.335	95.335	95.335	95.335		96.619	95.335	95.335	95.335	95.335	95.335	95.335	95.335	95.335	95.335	95.335	95.335	95.335	80.578	95.335	95.335	80.578	80.578	80.571	95.335	95.335	95.335	95.335	95.335	80.578	95.335	95.335	95.335	95.335	95.335
ISEcp1 Region 879	98.547	98.541	98.547	98.547	98.547	98.547	98.547	98.547	96.619		98.547	98.547	98.547	98.547	98.547	98.547	98.547	98.547	98.547	98.547	98.547	98.547	82.139	98.547	98.547	82.139	82.139	82.132	98.547	98.547	98.547	98.547	98.547	82.139	98.547	98.547	98.547	98.547	98.547
ISEcp1 Region 880	100	99.993	100	100	100	100	100	100	95.335	98.547		100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 881	100	99.993	100	100	100	100	100	100	95.335	98.547	100		100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 939	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100		100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 940	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100		100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 941	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100		100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 942	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100		100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 944	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100		100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 945	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100		100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 946	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100		100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 947	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100		100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 948	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100		100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 949	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100		83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 950	83.512	83.505	83.512	83.512	83.512	83.512	83.512	83.512	80.578	82.139	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512		83.512	83.512	100	100	99.984	83.512	83.512	83.512	83.512	83.512	100	83.512	83.512	83.512	83.512	83.512
ISEcp1 Region 951	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512		100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 952	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100		83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 953	83.512	83.505	83.512	83.512	83.512	83.512	83.512	83.512	80.578	82.139	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	100	83.512	83.512		100	99.984	83.512	83.512	83.512	83.512	83.512	100	83.512	83.512	83.512	83.512	83.512
ISEcp1 Region 955	83.512	83.505	83.512	83.512	83.512	83.512	83.512	83.512	80.578	82.139	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	100	83.512	83.512	100		99.984	83.512	83.512	83.512	83.512	83.512	100	83.512	83.512	83.512	83.512	83.512
ISEcp1 Region 956	83.498	83.492	83.498	83.498	83.498	83.498	83.498	83.498	80.571	82.132	83.498	83.498	83.498	83.498	83.498	83.498	83.498	83.498	83.498	83.498	83.498	83.498	99.984	83.498	83.498	99.984	99.984		83.498	83.498	83.498	83.498	83.498	99.984	83.498	83.498	83.498	83.498	83.498
ISEcp1 Region 957	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498		100	100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 958	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100		100	100	100	83.512	100	100	100	100	100
ISEcp1 Region 959	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100		100	100	83.512	100	100	100	100	100
ISEcp1 Region 960	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100		100	83.512	100	100	100	100	100
ISEcp1 Region 961	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100		83.512	100	100	100	100	100
ISEcp1 Region 962	83.512	83.505	83.512	83.512	83.512	83.512	83.512	83.512	80.578	82.139	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	83.512	100	83.512	83.512	100	100	99.984	83.512	83.512	83.512	83.512	83.512		83.512	83.512	83.512	83.512	83.512
ISEcp1 Region 963	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512		100	100	100	100
ISEcp1 Region 964	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100		100	100	100
ISEcp1 Region 965	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100		100	100
ISEcp1 Region 966	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100		100
ISEcp1 Region 967	100	99.993	100	100	100	100	100	100	95.335	98.547	100	100	100	100	100	100	100	100	100	100	100	100	83.512	100	100	83.512	83.512	83.498	100	100	100	100	100	83.512	100	100	100	100	

Figure 3.19: The percentage identity alignment of the IS*Ecp1* genetic environment region of the 39 *bla*CTX isolates produced in Geneious Prime. Isolates highlighted in yellow and with lower percentage identities indicate isolates that had the Δ*tetAR* IS*Ecp1* element and the isolate highlighted in pink, indicates 962 where the Tn*AS1* in association with *tetAR* was found in contig 6 separate from the IS*Ecp1* region

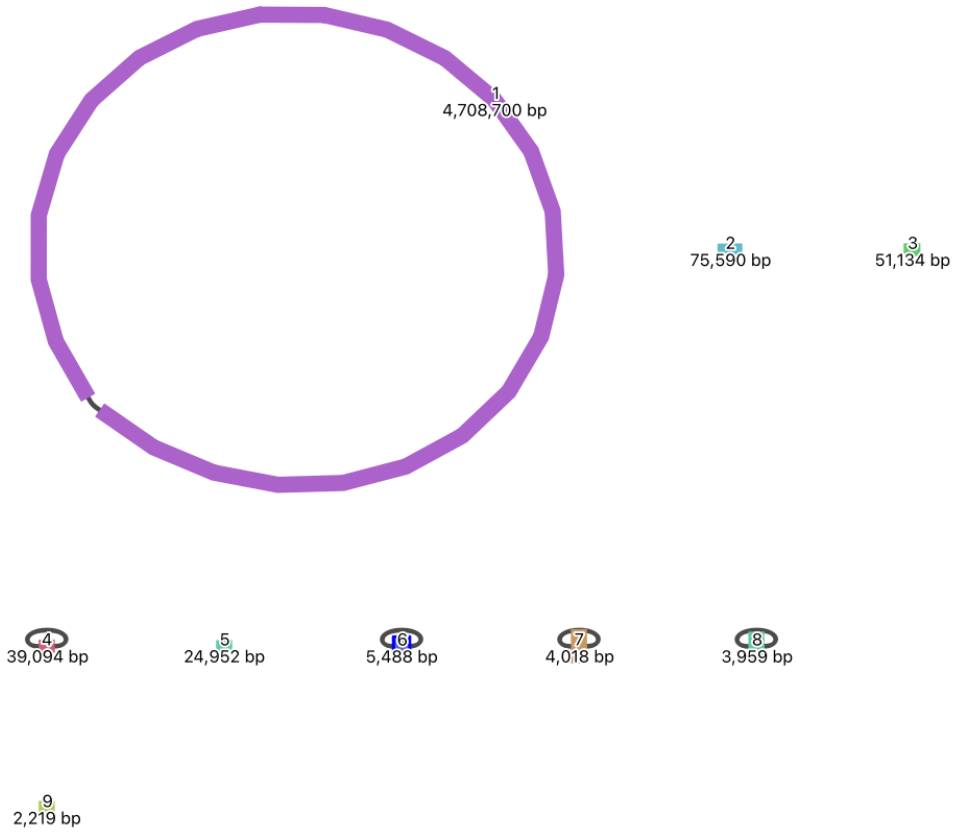


Figure 3.20: The output image from Bandage for the assembly of isolate 962 showing nodes 1-9 with the complete 4,708,700 bp chromosome in contig 1 and the circularised contig 6 of 5,488 bp that contained Tn*AS1* in association with *tetAR*

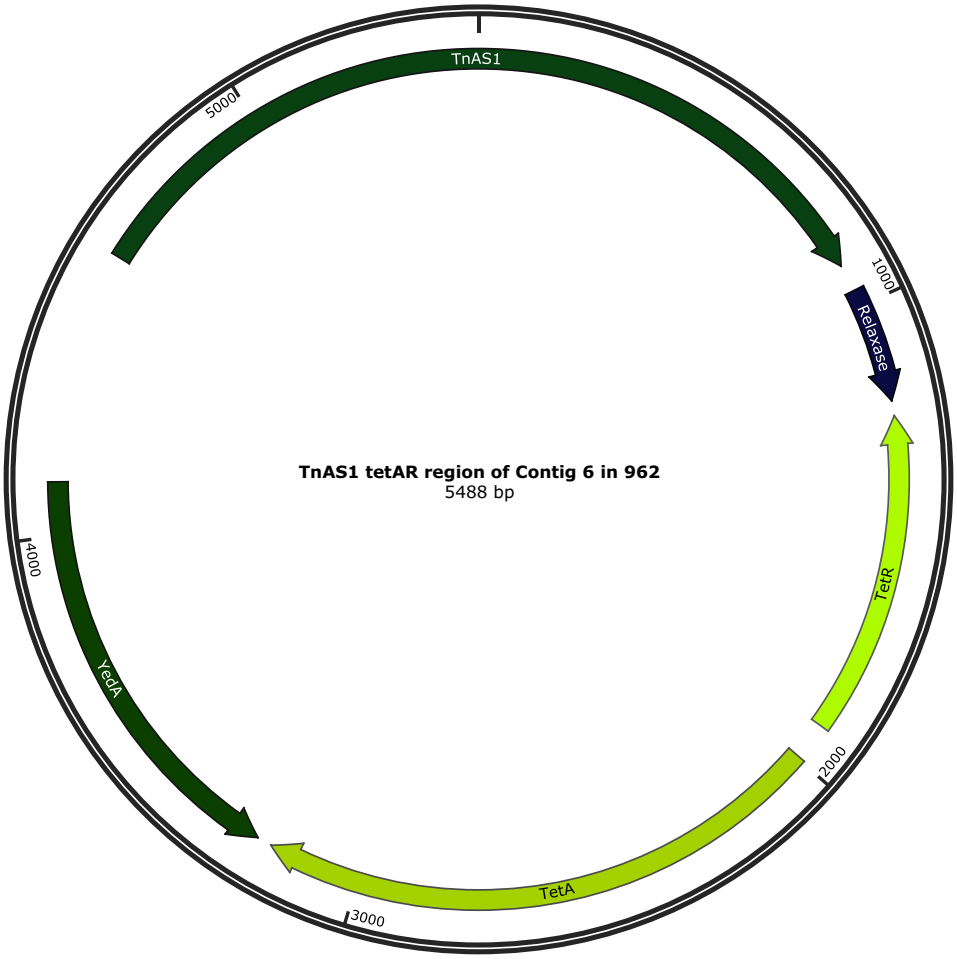


Figure 3.21: The annotated circularised genomic region of 5,488 bp in contig 6 from isolate 962, showing the complete Tn*AS1* transposase, relaxase, *tetAR* and *yedA*

3.7. CONCLUSIONS

From the 47 *E. coli* isolates initially selected for their CTX type phenotype from the main EVAL farms collection of ~1,000 isolates, 39 of these were confirmed as encoding *bla*$_{\text{CTX-M}}$ in association with the mobile element IS*Ecp1*. The primary aim of this chapter was to understand what level of resistance *bla*$_{\text{CTX-M-15}}$ provides and whether the isolates in this group were clonally related, or only related to each other as a result of their carriage of both IS*Ecp1* and *bla*$_{\text{CTX-M-15}}$. In addition, an examination of how the isolates from the *bla*$_{\text{CTX}}$ group explored in this Chapter were related to isolates from the wider database of Enterobase, was also addressed and provided an interesting insight through phylogenetic analysis and SNP distance comparison.

The resistance patterns within the phenotypic disc diffusion assay data provided by EVAL farm had given a good initial indication of the potential genotype of each isolate. Resistance to the broad spectrum aminopenicillin AMP, 3rd generation cephalosporins CTX, CAZ and CPD and the monobactam ATM and susceptibility to the 2nd generation cephalosporin (also known as a cephamycin) FOX and the beta-lactam/beta-lactamase inhibitor combination AMC, was good indication that the causative genotype was due to carriage of *bla*$_{\text{CTX-M}}$ rather than an alternative mechanism such as overexpression of *ampC* (Peter-Getzlaff et al. 2011a; Cantón et al. 2012b). As was outlined in **Section 3.2**, the phenotypic pattern utilised for distinguishing *bla*$_{\text{CTX-M}}$ from overexpression of *ampC* was resistance to the aminopenicillin AMP and the 3rd generation cephalosporin CTX and susceptibility to AMC and FOX.

The selective media utilised during the initial isolation of *E. coli* from the dairy farm by EVAL farms, appeared to have played a key role in the

isolation of *E. coli* encoding *bla*$_{CTX-M}$. CHROMagar ESBL has been noted within the literature by Saito et al. (2010), Lagacé-Wiens et al. (2010) and Khater et al. (2013) as having high specificity and sensitivity for isolating ESBL-producing *E. coli*. The findings within the literature would seem to mirror the results seen with the phenotypic data, as all the *E. coli* in the *bla*$_{CTX}$ group were isolated on CHROMagar ESBL media. Isolating on selective media has both benefits and drawbacks, however. If the isolation of only a subpopulation such as ESBL producers for example is the aim, then CHROMagar ESBL is a good option. However, if a greater amount of the whole population is wanting to be found, then alternative selective media such as TBX or MacConkey can be utilised with the selection of an antibiotic such as CTX, which may isolate other mechanisms of beta-lactamase resistance such as extended spectrum cephalosporin resistant isolates. A good example was the eight isolates that were initially grouped in the *bla*$_{CTX}$ group but were later moved to the *ampC* group which is explored in **Chapter 5**. Those eight that were moved to the *ampC* group, were all isolated on TBX media supplemented with 2 μg ml^{-1} CTX, whereas the rest of the isolates in the *bla*$_{CTX}$ group which were all confirmed as carrying *bla*$_{CTX-M-15}$, were isolated on CHROMagar ESBL. This would suggest from a mixed population of *E. coli*, the specificity and sensitivity of the initial isolation medium is important and CTX selection alone may not be enough to isolate *E. coli* encoding an ESBL such as *bla*$_{CTX-M}$. Duggett et al. (2020) demonstrated the importance and influence of selective media when trying to isolate ESBL type *E. coli* from the same sample and suggested that to allow for the growth and isolation of isolates encoding ESBLs, selective ESBL type media are important. This is because they generally only select for ESBL producers,

whereas MacConkey supplemented with CTX may select for AmpC, ESBL or carbapenemase producers. This was demonstrated within their study within the genotypes of the isolates selected on each medium, reflected from the MacConkey agar supplemented with CTX that showed increased recovery of isolates encoding *bla*$_{\text{CMY-2}}$ and AmpC mutations instead of the ESBL type genotype, whereas the ESBL agars mostly recovered isolates encoding an ESBL genotype. The previous study by Ibrahim et al. (2016) which also looked at *E. coli* isolated from the same dairy farm as this study, also showed the selective isolation medium influenced which subpopulation of resistant isolates was recovered.

As disc assay data had only provided a susceptible/intermediate/resistance (S/I/R) result with no indication of the level of resistance, MICs were conducted within this study to investigate this. High level resistance was seen to AMP, CAZ, CTX, CPD, CFQ and ATM, demonstrating *bla*$_{\text{CTX-M-15}}$ provides an effective mechanism for high level resistance to beta-lactam antibiotics, which was evident with MIC levels above the resistant breakpoint, which were 2 times for CAZ, 3 times for ATM, 6 times for AMP, 8 times for CTX and 9 times for both CPD and CFQ. In addition, as was noted in **Section 3.2.2**, resistance that had been seen in the disc assay data to SXT, STREP and CHLOR in some of the isolates was not present in the MIC data. This finding demonstrated the limitations of the disc diffusion method but also highlighted the importance of repeating experiments, as EVAL farms only conducted the disc assays once. Phenotypic analysis alone using disc diffusion, has its limitations in that you are restricted by the number of discs used within the initial antimicrobial susceptibility testing and you are also restricted by the

upper limit concentration of that particular antibiotic disc. It also does not indicate a resistance mechanism (Anjum 2015). The disc assay data only provided qualitative data in the form of an S/I/R result with no quantitative data with indication of the levels of resistance. Disc assays can also be relatively imprecise, as they are reliant on the measurement of the diameter of the inhibition zone and this cannot be converted to an exact MIC value (Liu et al. 2016a). Disc assays are useful however, as they provide a quick screening method for large numbers of bacterial isolates against a large panel of antibiotics, to give an initial indication of what resistance is likely to be present. MIC assays can then be conducted with either specific antibiotic classes or isolates and with a large number of antibiotic concentrations.

The next stage of the investigation of this Chapter was concerned with the genotypic characterisation of all 39 isolates in the *bla*$_{\text{CTX}}$ group through WGS. Sequencing all isolates via both long read and short read sequencing with the benefit of hybrid assembly, resulted in the ability to confirm that the IS*Ecp1* elements found in all the 39 of the *bla*$_{\text{CTX}}$ isolates were indeed chromosomal, as they were all located in contig 1 and other contigs were found to contain either plasmid DNA or were extremely short linear fragments of DNA (around 4-8 kb in length).

The construction of the genetic environments of IS*Ecp1* for both +*tetAR* that contained the *tetAR* region and Δ*tetAR* that did not contain the *tetAR* region along with the pairwise alignment using Geneious, showed that the majority of the IS*Ecp1* elements were identical with an overall percentage identity of 95.8%, with the lower percentage identity in 950, 953, 955 and 956 shown to be due to the absence of the *tetAR* region. This absence of the

tetracycline resistance genes also matched what was seen within the phenotypic data, as 956 was susceptible to tetracycline. The IS*Ecp1* elements were all found to have all inserted into exactly the same region of the chromosome. As most of the IS*Ecp1* elements were identical, the question of whether the isolates were related clonally or only through the horizontal acquisition of IS*Ecp1* was addressed through WGS, phylogenetics and SNP distance comparison. MLST, whole genome phylogeny and SNP distance comparison showed that all were of ST2325, suggesting a dominant clonal strain, that had spread $bla_{\text{CTX-M-15}}$ across the farm via clonal expansion rather than through the HGT of IS*Ecp1*.

The SNP distance analysis appeared to show that the ST2325 isolates on this particular dairy farm were part of their own separate clade to those in the database with the nearest isolate in the database from a Spanish bovine faecal sample under the isolate name ESC_TA9425AA, which was within 36-45 SNPs of the bla_{CTX} isolates. Other ST2325 isolates in the database also appeared to have formed separate clades of clonality but which were distantly related to any other ST2325 groups from the database. However, it was noted that each of these individual groups appeared to be part of the same individual studies and therefore may have been sampled at a similar time and from a similar location. Also found in the 105 Enterobase ST2325 genomes, were isolates containing IS*Ecp1* also in association with $bla_{\text{CTX-M-15}}$. However, as was seen in the SNP matrix in **Figure 3.9,** the 105 Enterobase isolates were distantly related to the bla_{CTX} isolates from this study and therefore it could be concluded that clonal expansion had indeed very likely played a role in the spread of $bla_{\text{CTX-M-15}}$ on this particular dairy farm and within the other smaller clonal groups in the Enterobase isolates. However, ST2325 strains encoding IS*Ecp1* in association

with *bla*CTX do not appear to have a clonal element to them in the wider community, even if the IS*Ecp1* elements within them are similar. There was also a wide variety of different sampling locations identified from the Enterobase isolates, however 31.4 % of the isolates were of bovine origin. Therefore, it appeared that ST2325 was prevalent among bovine samples in the genomes available from Enterobase, but with such a small sample size it was difficult to truly ascertain whether ST2325 was a bovine-specific ST.

Enterobase contains a total of 224, 390 genomes and searches can be conducted using filters for source niche/type or indeed for specific ST. When other STs were compared to ST2325 by using "bovine" in the search filter in Enterobase for source type, a total of 14, 837 strains were found in the search. The most prominent ST within this search appeared to be ST11. There were found to be a total of 17,175 ST11 *E. coli* genomes in Enterobase with 3,212 of these under the source type of bovine. This could suggest that ST11 could possibly be a bovine-associated ST, although this would need further investigation to prove this hypothesis. It does pose an interesting question of whether specific ST association with *E. coli* from FPA groups, could provide a means to assist AMR surveillance within the food chain.

As noted in **Section 3.6**, it is believed, that the progenitors of *bla*CTX-M were originally mobilised from the chromosome of *Kluyvera* spp. via transposition from elements such as IS*Ecp1* and the studies by Lartigue et al. (2006a) and Nordmann et al. (2008a) showed mobilisation of IS*Ecp1* in association with *bla*CTX-M from a *Kluyvera* spp. chromosome into a plasmid. However, no study was found that demonstrated mobility of IS*Ecp1* in association with *bla*CTX-M from the chromosome of an environmental *E. coli* into

a resident plasmid. Therefore, the question remained, that within a subset of the EVAL farms isolates, could IS*Ecp1* mobilise *bla*$_{\text{CTX-M-15}}$ from the chromosome into one of the resident plasmids? Demonstrating this experimentally, would potentially provide more evidence of an alternative mechanism to clonal expansion, for the dissemination of an ESBL determinant within the environment of a dairy farm. Therefore, the mobility of IS*Ecp1* in association with *bla*$_{\text{CTX-M-15}}$ was explored in more detail in **Chapter 4**.

CHAPTER 4

IS*Ecp1* MOBILITY AND ENHANCED TRANSPOSITION

4.1. INTRODUCTION

The mobile element IS*Ecp1* has been shown not only to have played a key role in the origins of *bla*$_{CTX-M}$ from the chromosome of the progenitor *Kluyvera* spp., (as detailed in **Section 3.6)**, but is also frequently reported upstream of *bla*$_{CTX-M}$ in *E. coli* (Humeniuk et al. 2002a; Rossolini et al. 2008a; Zong et al. 2010b; Bevan et al. 2017b; Afema et al. 2018b). IS*Ecp1* was first identified by Stapleton (1999) within the plasmid pST010 and submitted to GenBank under the accession number AJ242809. As more IS have been discovered, the lines between MGE definitions have become harder to define (Siguier et al. 2014b). IS*Ecp1* could fall between what is classified as an IS or a transposon, in that it has elements of each. IS*Ecp1* is of the IS*1380* family of IS elements and uses a DDE transposase in a 'copy-in' mechanism similar to Tn*3* for mobilisation of the mobile element as seen in **Figure 4.1** (Poirel et al. 2005b; Claeys Bouuaert and Chalmers 2010). Unlike other IS elements, it has the ability to mobilise downstream genes producing what have been termed 'transposition units'. Due to the recognition of an imperfect IR$_R$, these transposition units can be of varying size as a consequence of the one-ended transposition that creates variable end points (Poirel et al. 2005b). The result is downstream genes may be collected or indeed lost as mobilisation takes place (Zong et al. 2010a). This collection and possible loss of downstream genes may have an impact on the evolution of the "pan genome" and, in particular, the so-called variable part of the genome known as the "accessory genome" of a bacterium, by introducing or losing genes associated with resistance, virulence or those genes involved in increased survival and colonisation of niche environments (Medini et al. 2005; Tettelin et al. 2008; Siguier et al. 2014b). Understanding how IS*Ecp1* may

mobilise within the environment of a dairy farm in the presence of sub-lethal levels of beta-lactam antibiotics, should provide an important insight into how antibiotic usage and a single mobile element can produce dissemination of resistance.

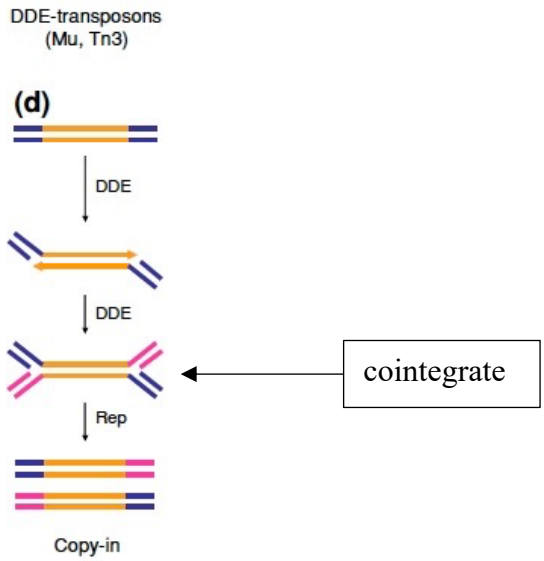


Figure 4.1: 'Copy in' mechanism associated with Tn*3* type DDE transposition, that produces a cointegrate during transposition. Adapted from: (Claeys Bouuaert and Chalmers 2010)

Important questions to answer following the WGS analysis within **Section 3.4** of the 39 IS*Ecp1* positive isolates in the *bla*$_{CTX}$ group were:

i. Could IS*Ecp1* be mobilised out of the chromosome?

ii. Do certain antibiotics at sub-lethal levels enhance IS*Ecp1* mobilisation?

iii. Which plasmids were used as vectors for the IS*Ecp1* element and what genes mobilised alongside it?

iv. Could IS*Ecp1* mobilise back into the chromosome from the plasmid of a transposition transconjugant?

Previous studies have shown successful transposition of a cloned IS*Ecp1* within *Kluyvera ascorbata* from a chromosomal location to an introduced plasmid location, with IS*Ecp1* transposition enhanced in the presence of several antibiotics (Lartigue et al. 2006a; Nordmann et al. 2008a). A further study by Hamamoto et al. (2020b) characterised the transposition of IS*Ecp1*, in association with $bla_{\text{CTX-M-14}}$, from a plasmid construct location to a chromosomal location within an experimental *E. coli* strain. However, no studies were found within the literature that detailed transposition of a naturally-occurring chromosomally-encoded IS*Ecp1* or that addressed the question of whether a resident plasmid could be used as the vector.

This study aimed to address the questions of whether transposition of IS*Ecp1* from the chromosome to a resident plasmid could occur and whether the presence of sub-lethal levels of antibiotics, such as those that might be found in cow faeces, may promote resistance dissemination or selection of resistance determinants such as $bla_{\text{CTX-M}}$ within an environment such as a dairy farm.

4.2. ENHANCED TRANSPOSITION

Transposition of the IS*Ecp1* element using the method detailed in **Section 2.9**, was conducted in the four isolates 687, 876, 956 and 961 that included the plasmid types IncFIC, IncFII, IncI1, IncI2 and IncX4 (detailed in **Table 3.4).** Therefore, within these four chosen isolates, each plasmid type had a representative. The method detailed in **Section 2.9**, utilised the endogenous

plasmids as vectors and, by including all the plasmid replicon types, it was hoped that if there was a preferred plasmid replicon type used in transfer, this could also be identified. The three antibiotics that were chosen to promote transposition included AMP, CAZ and cloxacillin (CLOX) used at sub-lethal levels (**Table 2.8**). The penicillins AMP and CLOX were chosen as these are commonly used within dairy farm environments globally, with cloxacillin often favoured for use in dry cow therapy (González Pereyra et al. 2015; Johnson et al. 2016; Breser et al. 2018; Liu et al. 2018; Whitfield and Laven 2018; Rossi et al. 2019; McDougall et al. 2021). The 3rd generation cephalosporin CAZ was also chosen, to act as a positive control, as previous studies by Lartigue et al. (2006a) and Nordmann et al. (2008b) have shown it may enhance transposition of IS*Ecp1* in association with *bla*$_{\text{CTX-M}}$. It was hoped that utilising the three antibiotics AMP, CAZ and CLOX, might demonstrate whether the presence of antibiotics within a dairy farm environment, even at sub-lethal levels, could enhance IS*Ecp1* transposition under *in vitro* conditions. The transposition experiment was also run with non-selective media to gain a baseline rate of transfer, to allow calculation of any enhanced transfer rate when sub-lethal levels of each antibiotic were included. This baseline rate of transposition was an experimental baseline, rather than an absolute or natural baseline, as it was associated with the experimental conditions under use. This experimental baseline was then challenged by using different antibiotic concentrations to see if an increase from it was detected.

Antibiotic induction of IS*Ecp1* transposition was achieved as described in the method detailed in **Section 2.9.1**. Plate counts were conducted as described in **Section 2.9.2**, from both the donor and recipient cultures and

these are detailed in **Table 4.1**. Following overnight growth of donors in both non-selective and sub-lethal levels of individual antibiotics, a conjugation was undertaken using each of the donors, as described in **Section 2.9.3,** with transconjugants selected on double selective media. The K-12 derivative laboratory recipient *E. coli* strain CV601 which was kanamycin resistant but also labelled with GFP, allowed for the easy identification of successful conjugation of potential IS*Ecp1* encoded plasmids via both antibiotic selection and by secondary confirmation via UV illumination of the GFP at a wavelength of 365 nm (as can be seen in **Figure 4.2**). When selecting transconjugants with only a reliance on antibiotic selection, there is a chance that donors with mutations giving kanamycin resistance can be selected. Therefore, by looking for a positive GFP, this made identifying transconjugants more certain and allowed a visual confirmation without further testing.

The CFU/ml of transconjugants are detailed in **Table 4.1**.

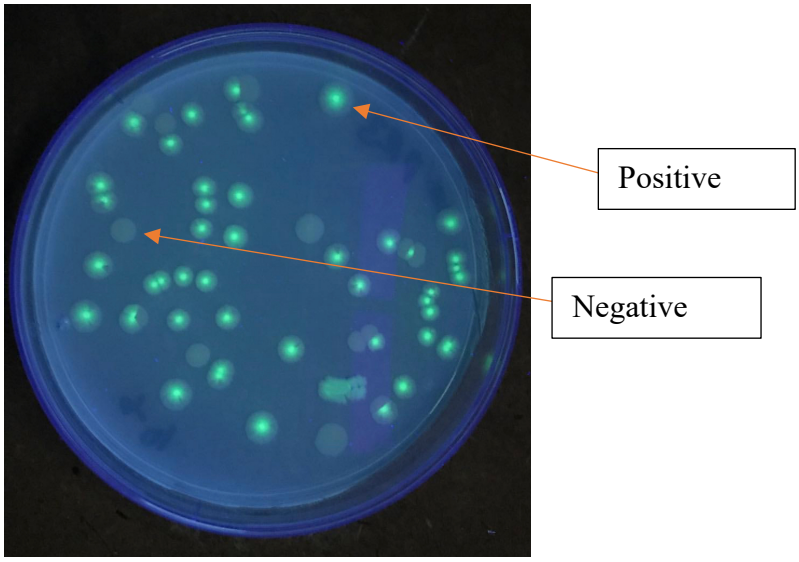


Figure 4.2: Petri dish showing both GFP positive and negative colonies illuminated with UV at 365 nm wavelength to identify isolates positive for GFP and therefore confirmed as true transconjugants

Table 4.1: Plate count and conjugation dilutions plated and CFU/ml for the recipient, the non-selective donor cultures and for each donor culture grown under sub-lethal concentrations of antibiotic

		Plate count Dilutions Plated			**Conjugation Dilution Plated**
Isolate	**Antibiotic Concentration**	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{0}
		CFU/ml			
CV601	KAN 50 μg ml^{-1}	3.4×10^{8}	8.30×10^{8}	3×10^{8}	n/a
687	Non-Selective	4.78×10^{8}	5×10^{8}	8×10^{8}	3×10^{1}
	CAZ 0.5 μg ml^{-1}	TMTC	4.3×10^{8}	4×10^{8}	3×10^{1}
	CAZ 0.25 μg ml^{-1}	5.16×10^{8}	NG	NG	7×10^{2}
	CAZ 0.1 μg ml^{-1}	5.82×10^{8}	5.6×10^{8}	9×10^{8}	1.51×10^{3}
	AMP 16 μg ml^{-1}	NG	6.1×10^{8}	5×10^{8}	3×10^{1}
	AMP 8 μg ml^{-1}	NG	NG	NG	5×10^{1}
	AMP 3.2 μg ml^{-1}	2.99×10^{8}	2.8×10^{8}	4×10^{8}	5×10^{1}
	CLOX 128 μg ml^{-1}	4.38×10^{8}	7.5×10^{8}	1.10×10^{9}	5×10^{1}
	CLOX 64 μg ml^{-1}	7.49×10^{8}	6.9×10^{8}	1×10^{9}	5×10^{1}
	CLOX 25.6 μg ml^{-1}	NG	NG	NG	NG
876	Non-Selective	6.99×10^{8}	9.10×10^{8}	1.2×10^{9}	NG
	CAZ 0.5 μg ml^{-1}	NG	NG	NG	NG
	CAZ 0.25 μg ml^{-1}	5.16×10^{8}	5×10^{8}	4×10^{8}	1×10^{1}
	CAZ 0.1 μg ml^{-1}	NG	NG	NG	NG
	AMP 16 μg ml^{-1}	NG	NG	NG	NG
	AMP 8 μg ml^{-1}	5.52×10^{8}	6×10^{8}	8×10^{8}	2×10^{1}

	AMP 3.2 μg ml^{-1}	NG	NG	NG	NG
	CLOX 128 μg ml^{-1}	7.05 x 10^8	6.6 x 10^8	3 x 10^8	1.6 x 10^2
	CLOX 64 μg ml^{-1}	6.96 x 10^8	8.7 x 10^8	7 x 10^8	1 x 10^1
	CLOX 25.6 μg ml^{-1}	NG	NG	NG	NG
956	Non-Selective	6.84 x 10^8	6.2 x 10^8	1.2 x 10^9	3 x 10^1
	CAZ 0.5 μg ml^{-1}	5.14 x 10^8	5.3 x 10^8	3 x 10^8	3 x 10^1
	CAZ 0.25 μg ml^{-1}	NG	4.6 x 10^8	9 x 10^8	3 x 10^1
	CAZ 0.1 μg ml^{-1}	NG	NG	NG	NG
	AMP 16 μg ml^{-1}	5.3 x 10^8	7.8 x 10^8	6 x 10^8	1 x 10^1
	AMP 8 μg ml^{-1}	NG	5.3 x 10^8	4 x 10^8	1 x 10^1
	AMP 3.2 μg ml^{-1}	NG	NG	NG	NG
	CLOX 128 μg ml^{-1}	4.38 x 10^8	7.5 x 10^8	1.1 x 10^9	5 x 10^1
	CLOX 64 μg ml^{-1}	7.49 x 10^8	6.9 x 10^8	1 x 10^9	5 x 10^1
	CLOX 25.6 μg ml^{-1}	NG	NG	NG	NG
961	Non-Selective	7.79 x 10^8	8.4 x 10^8	9 x 10^8	NG
	CAZ 0.5 μg ml^{-1}	NG	NG	NG	NG
	CAZ 0.25 μg ml^{-1}	NG	NG	NG	NG
	CAZ 0.1 μg ml^{-1}	NG	NG	NG	NG
	AMP 16 μg ml^{-1}	NG	NG	NG	NG
	AMP 8 μg ml^{-1}	NG	NG	NG	NG
	AMP 3.2 μg ml^{-1}	NG	NG	NG	NG
	CLOX 128 μg ml^{-1}	NG	NG	NG	NG
	CLOX 64 μg ml^{-1}	7.99 x 10^8	8 x 10^8	6 x 10^8	1 x 10^1
	CLOX 25.6 μg ml^{-1}	8.21 x 10^8	9.3 x 10^8	7 x 10^8	1 x 10^1

Footnote for Table 4.1*: n/a – not applicable, NG – no growth, TMTC – too many to count*

4.2.1. Transposition Transconjugant (TT) Confirmation

At least 2-3 well isolated single GFP colonies were picked from each double selective conjugation plate and restreaked onto double selective agar to obtain a pure culture (**Section 2.9.3**). A colony from each pure culture restreak plate was utilised in a PCR to confirm they were successful IS*Ecp1* transconjugants, by searching for the donor genes *bla*$_{\text{CTX-M}}$ and IS*Ecp1* and the recipient gene GFP using PCR (*bla*$_{\text{CTX-M}}$, IS*Ecp1* and GFP primers are listed in **Table 2.6** with methods given in **Section 2.6**). This confirmatory PCR was conducted to show both successful transfer of the IS*Ecp1* element in association with *bla*$_{\text{CTX-M-15}}$ and plasmid transfer to the GFP-encoding recipient CV601. The location of where the primers for *bla*$_{\text{CTX-M}}$ and IS*Ecp1* would bind in the forward and reverse locations can be seen in **Figures 2.6** and **2.7**. Bands were present for *bla*$_{\text{CTX-M}}$ (**Figure 4.3**), IS*Ecp1* (**Figure 4.4**) and for GFP (**Figure 4.5**), confirming that all the isolates were the recipient CV601 and contained both *bla*$_{\text{CTX-M-15}}$ and IS*Ecp1,* thus confirming these were all transconjugants. A further control PCR, covering the chromosomal insertion point of IS*Ecp1* at T3SS *prgH/eprH* (partial) as seen in **Figure 3.12**, was conducted to confirm IS*Ecp1* had mobilised away from the chromosome of the parent and into a resident plasmid. The location where the primers denoted as T3SS-ISEcp1-Fwd and T3SS-ISEcp1-Rvs would bind are shown in **Figure 2.8**. This control PCR would identify if the left-hand insertion point at T3SS *prgH*/*eprH* (partial) was still present and should result in no bands seen on the gel if IS*Ecp1* had mobilised out of the chromosome. The results of the T3SS insertion point PCR are shown in **Figure 4.6**. The band covering the T3SS insertion point was absent in all isolates except 687AMP16, suggesting this isolate was a mutated donor rather

than a true transconjugant. However, a very faint band was present for GFP within 687AMP16. This could be a mistake due to contamination within the PCR master mix or alternatively is suggestive of a non-pure culture. However, as there was still GFP expression of the colonies on the sub-culture plate, this would suggest a mixed culture and therefore not all colonies would have been expressing GFP and this could account for the faint band seen on the gel image in **Figure 4.3**. Successful transconjugants confirmed following PCR analysis were designated transposition transconjugants (TTs).

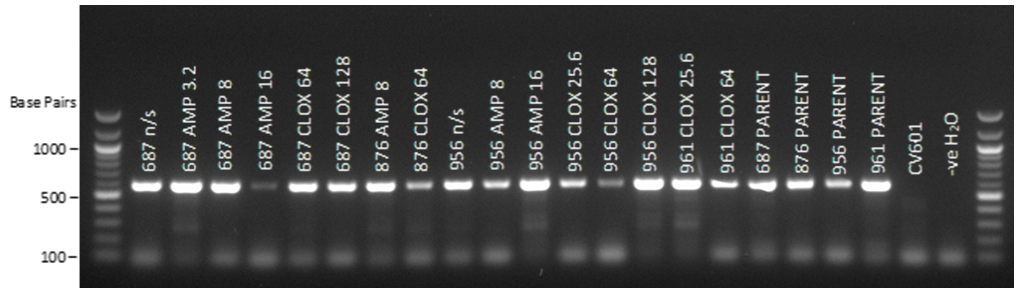


Figure 4.3: Transposition transconjugant CTX PCR confirmation gel detailing 593 bp *bla*$_{CTX-M}$ amplimers. Products were resolved on a 1% TAE agarose gel with a 100 bp NEB quickload ladder (New England Biolabs, (UK) Ltd) at 85 V for 1 hour. The parents 687, 876, 956 and 961 were utilised as positive controls with the recipient CV601 and water utilised as negative controls

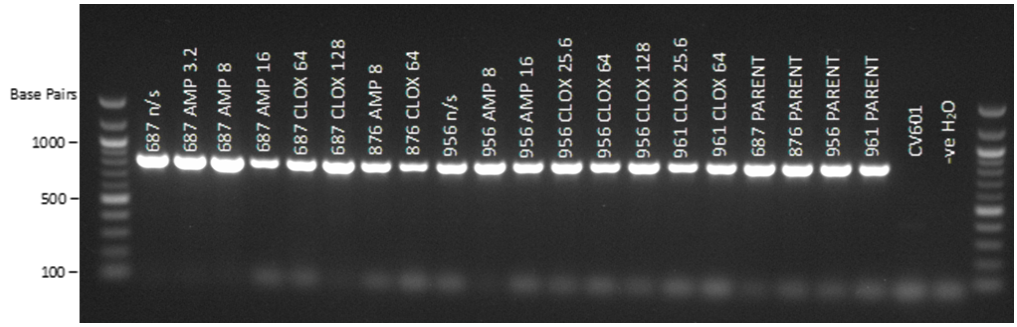


Figure 4.4: Transposition transconjugant IS*Ecp1* PCR confirmation gel detailing 846 bp IS*Ecp1* amplimers. Products were resolved on a 1% TAE agarose gel with a 100 bp NEB quickload ladder (New England Biolabs, (UK) Ltd) at 85 V for 1.5 hours. The parents 687, 876, 956 and 961 were utilised as positive controls with the recipient CV601 and water utilised as negative controls

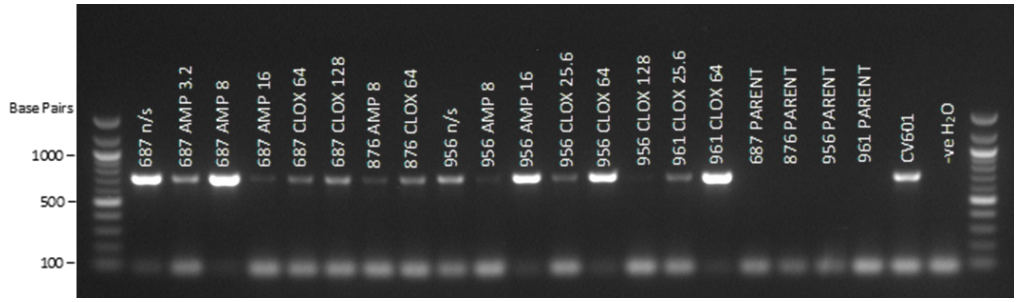


Figure 4.5: Transposition transconjugant GFP PCR confirmation gel detailing 714 bp GFP amplimers. Products were resolved on a 1% TAE agarose gel with a 100 bp NEB quickload ladder (New England Biolabs, (UK) Ltd) at 85 V for 1.5 hours. The parents 687, 876, 956 and 961 and water were utilised as negative controls with the recipient CV601 utilised as a positive control.

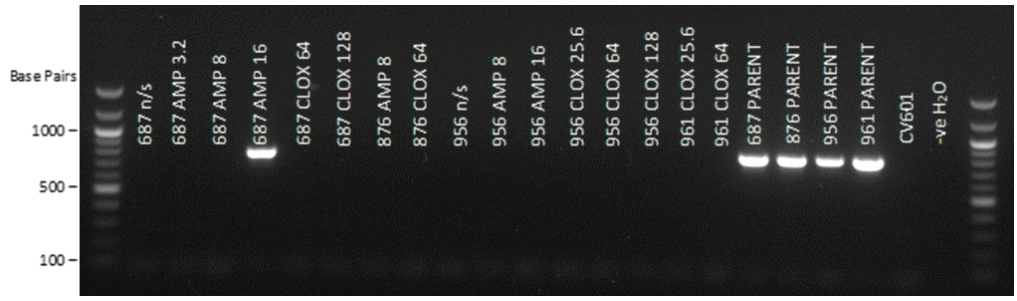


Figure 4.6: Transposition transconjugant T3SS Part Lt PCR confirmation gel detailing 807 bp T3SS Part Lt amplimers. Products were resolved on a 1% TAE agarose gel with a 100 bp ladder NEB quickload ladder (New England Biolabs, (UK) Ltd) at 85 V for 1.5 hours. The parents 687, 876, 956 and 961 were utilised as positive controls with the recipient CV601 and water utilised as negative controls

4.2.2. Transfer Rates of the IS*Ecp1* Element

There were examples of successful transposition of the IS*Ecp1* element, with all antibiotics and concentrations of antibiotic used, however, this varied with each donor strain. Furthermore, enhanced transposition, with an increased rate of transfer compared to the baseline rate of transfer, was successful with only some of the concentrations of each antibiotic depending on the donor strain.

The transfer rate was calculated from a combination of the initial transposition of IS*Ecp1* from the chromosome into a resident plasmid, followed by conjugation of the plasmid into the recipient CV601. The plasmids contained within the donors (which are listed in **Table 3.2**) were an IncFIC in 687, 956 and 961, an IncFII in 876, an IncI1 in 876, 956 and 961, an IncI2 in 687 and an IncX4 in 876 and 961. All plasmids had been found to be cryptic following the WGS analysis conducted in **Section 3.5.3** and therefore they had no usable genetic markers and it was not possible to obtain a conjugation rate for each plasmid

alone. Furthermore, the final transfer rate is a combination of the transposition and conjugation rates and transfer rates were calculated from counting GFP positive colonies, which were confirmed as TTs from UV illumination (**Section 2.9.3**), followed by PCR analysis for the three genes *bla*$_{CTX-M}$, IS*Ecp1* and GFP as detailed in **Sections 2.6.3**, **2.6.4** and **2.6.5**.

The baseline transfer rate of IS*Ecp1* when isolates were grown in non-selective medium with a starting culture of 1 x 10^8 cells was 6 x 10^{-8} with isolate 687 (around 1 in 16 million), 5.03 x 10^{-8} with isolate 876 (1 in 19 million), 4.84 x 10^{-8} with isolate 956 (1 in 20 million) and 3.42 x 10^{-8} with isolate 961 (1 in 29 million). Transfer rates reported in the literature by Lartigue et al. (2006a) and Nordmann et al. (2008a) were around 10^{-7}, however this was with an artificially introduced, chromosomally-encoded IS*Ecp1* and a plasmid construct. The transfer rates with each antibiotic and increase from the baseline rate of transfer can be seen in **Table 4.2**. Transfer of IS*Ecp1* was seen with all antibiotic concentrations (concentrations of antibiotics used are listed in **Table 2.8**), however an enhanced rate of transposition from the baseline rate was only observed in 687, 876 and 956 and with only some of the antibiotics and concentrations tested. Interestingly the majority of enhanced rates were seen with the lower concentrations of antibiotics and this was evident for AMP in 687 which had a slightly higher rate at 1/10 MIC than 1/4 MIC and CLOX in 687 and 956 with slightly higher rates at 1/4 MIC than at 1/2 MIC and at 1/10 MIC than at 1/4 MIC respectively. The biggest increase in enhanced transposition was seen with CAZ in 687, where there was a much higher rate of transposition at 1/10 MIC than at 1/4 MIC. In comparison however in 956, the enhanced rate of transposition with CAZ was greater at 1/2 MIC than at 1/4 MIC. No enhanced

transposition rates were observed for any antibiotics in 961 and there were no enhanced transposition rates for AMP in 876 and 956 or for CAZ in 876.

Table 4.2: Transfer Rates of the IS*Ecp1* element

Isolate	Antibiotic	MIC	Concn	Transfer Rate	Increase from Baseline
EcoSL1010-687	Non-Selective	n/a	n/a	6×10^{-8}	-
EcoHS11212-876	Non-Selective	n/a	n/a	5.03×10^{-8}	-
EcoMHE1801-956	Non-Selective	n/a	n/a	4.84×10^{-8}	-
EcoSS2501-961	Non-Selective	n/a	n/a	3.42×10^{-8}	-
EcoSL1010-687	AMP	1/10	3.2 µg ml^{-1}	1.79×10^{-7}	2.98 Fold
		1/4	8 µg ml^{-1}	1.43×10^{-7}	2.38 Fold
		1/2	16 µg ml^{-1}	4.92×10^{-8}	No Increase
EcoHS11212-876	AMP	1/10	3.2 µg ml^{-1}	No transfer	-
		1/4	8 µg ml^{-1}	3.33×10^{-8}	No Increase
		1/2	16 µg ml^{-1}	No transfer	-
EcoMHE1801-956	AMP	1/10	3.2 µg ml^{-1}	No transfer	-
		1/4	8 µg ml^{-1}	1.89×10^{-8}	No Increase
		1/2	16 µg ml^{-1}	1.28×10^{-8}	No Increase
EcoSS2501-961	AMP	1/10	3.2 µg ml^{-1}	No transfer	-
		1/4	8 µg ml^{-1}	No transfer	-
		1/2	16 µg ml^{-1}	No transfer	-
EcoSL1010-687	CLOX	1/10	25.6 µg ml^{-1}	No transfer	-
		1/4	64 µg ml^{-1}	7.25×10^{-8}	1.2 Fold
		1/2	128 µg ml^{-1}	6.67×10^{-8}	1.1 Fold
EcoHS11212-876	CLOX	1/10	25.6 µg ml^{-1}	No transfer	-
		1/4	64 µg ml^{-1}	1.15×10^{-8}	No Increase

		1/2	128 µg ml^{-1}	2.42×10^{-7}	4.81 Fold
EcoMHE1801-956	CLOX	1/10	25.6 µg ml^{-1}	1.90×10^{-7}	3.93 Fold
		1/4	64 µg ml^{-1}	5.19×10^{-8}	1.07 Fold
		1/2	128 µg ml^{-1}	1.85×10^{-8}	No Increase
EcoSS2501-961	CLOX	1/10	25.6 µg ml^{-1}	1.08×10^{-8}	No Increase
		1/4	64 µg ml^{-1}	1.25×10^{-8}	No Increase
		1/2	128 µg ml^{-1}	No transfer	-
EcoSL1010-687	CAZ	1/10	0.1 µg ml^{-1}	2.70×10^{-6}	45 Fold
		1/4	0.25 µg ml^{-1}	1.25×10^{-6}	21 Fold
		1/2	0.5 µg ml^{-1}	6.98×10^{-8}	1.16 Fold
EcoHS11212-876	CAZ	1/10	0.1 µg ml^{-1}	No transfer	-
		1/4	0.25 µg ml^{-1}	2.00×10^{-8}	No Increase
		1/2	0.5 µg ml^{-1}	No transfer	-
EcoMHE1801-956	CAZ	1/10	0.1 µg ml^{-1}	No transfer	-
		1/4	0.25 µg ml^{-1}	6.52×10^{-8}	1.34 Fold
		1/2	0.5 µg ml^{-1}	5.66×10^{-8}	2.83 Fold
EcoSS2501-961	CAZ	1/10	25.6 µg ml^{-1}	No transfer	-
		1/4	64 µg ml^{-1}	No transfer	-
		1/2	128 µg ml^{-1}	No transfer	-

4.2.3. Transposition Transconjugant (TT) Sequencing

From **Table 4.2**, of the 23 conjugations showing a successful transfer rate (and subsequently confirmed as producing a TT through PCR as per **Section**

2.6), 16 transconjugants were sequenced via Illumina short read sequencing and MinION (ONT) long read sequencing with hybrid assembly (as per **Sections 2.7.3** and **2.8.1**). Isolates that were sequenced along with their new TT names are listed in **Table 4.3**.

WGS was able to confirm through *in silico* MLST analysis using MLST 2.0 (**Section 2.8.5.1**), that all but isolate 687AMP16 of the TTs were of ST10, the same as the recipient CV601. Isolate 687AMP16 was found to be the same ST as the parent ST2325 and was considered to be a mutated donor rather than a true transconjugant. Therefore, 687AMP16 was discounted from any further analysis. The GFP gene was also located successfully within the genome sequence of all of the TTs confirmed as being of ST10.

Table 4.3: Sequenced TTs detailing the new TT name of each isolate and the antibiotic concentration used during enhanced transposition

Parent Donor	Antibiotic	Concentration (μg ml^{-1})	TT Name
EcoSL1010-687	Non-Selective	n/a	687-N
	AMP	3.2	687AMP0.32
	AMP	8	687AMP8
	CLOX	64	687CLOX64
	CLOX	128	687CLOX128
EcoHS11212-876	AMP	8	876AMP8
	CLOX	64	876CLOX64
	CAZ	0.25	876CAZ0.25
EcoMHE1801-956	Non-Selective	n/a	956-N
	AMP	8	956AMP8
	AMP	16	956AMP16
	CLOX	25.6	956CLOX25.6
	CLOX	128	956CLOX128
EcoSS2501-961	CLOX	25.6	961CLOX25.6
	CLOX	64	961CLOX64

4.2.4. Transposition Transconjugant Descriptions and IS*Ecp1* Genetic Environment Graphics

The IS*Ecp1* elements within the TTs listed in **Table 4.3**, were found to have utilised four of the five plasmid replicon types that had been located in the parents (as listed in **Table 3.2**) during conjugative transfer of the IS*Ecp1*

elements from the parents 687, 876, 956 and 961 to the recipient CV601. The parent strains were originally encoding the plasmid replicon types of IncFIC in 687, 956 and 961, IncFII in 876, IncI1 in 687, 876 and 956, IncX4 in 876 and 961 and IncI2 in 687. Plasmid replicon types found in the TT WGS included IncFIC, IncFII, IncI1 and IncX4 but only one plasmid replicon type was found in each TT WGS. The IncI2 plasmid replicon type was not found in any of the TT WGS, which utilised 687 as the parent. As all the plasmids in the parent strains were cryptic, it was unlikely that the antibiotic selection used in the transposition experiments was a factor in which plasmid was utilised by IS*Ecp1*. In addition, it would appear that within each transfer only one plasmid type was used as none of the transconjugants were found with multiple plasmids. Within all but two of the IS*Ecp1* elements, a new imperfect IR_R denoted as $IR_{R(new2)}$ was present. However in two of the isolates, 956-N and 956CLOX128, the $IR_{R(\textit{Kluyvera}\ transposition)}$ was utilised during transfer (detailed in the original IS*Ecp1* element in **Figure 3.12**) and was therefore denoted as $IR_{R(\textit{Kluyvera}\ transposition)}/IR_{R(new2)}$. The 5 bp repeats utilised by each TT during transposition were all AT rich, as has been described in the literature by Poirel et al. (2005c), Smet et al. (2010), Dhanji et al. (2011c) and Yoon et al. (2020). The IS*Ecp1* elements were found to be of varying size, with several a lot smaller than the IS*Ecp1* elements found within the parents 687, 876, 961 (which is detailed in **Figure 3.12)** and 956 (which is detailed in **Figure 3.15**).

Within each of the following sections, the plasmid replicon type utilised by IS*Ecp1*, the IS*Ecp1* size in bp, any truncated genes as a result of IS*Ecp1* insertion and the insertion point in the plasmid along with the IR_R sequences and the bases in the IR_R which were complementary to the IR_L are all

explored. To better describe the TTs, they were sorted into 3 groups according to the size of the IS*Ecp1* element (as shown in **Table 4.4**). Group 1 were all >15 kb in length and are explored in **Section 4.2.4.1**, group 2 were between 10-15 kb in length and are explored in **Section 4.2.4.2** and group 3 were <10 kb in length and are explored in **Section 4.2.4.3**.

The plasmid sequences of each TT were inspected to locate the surrounding plasmid DNA around the IS*Ecp1* elements and any figures produced showed only the immediate surrounding plasmid backbone around the IS*Ecp1* genetic environments and these are detailed in the following sections which describe each group of IS*Ecp1* elements. The plasmid genomic regions immediately either side of the IS*Ecp1* element are shown, as the larger full plasmid maps made identifying individual gene annotations difficult. It was irrelevant in the context of this analysis to show entire plasmid maps, as only the genes immediately either side of the IS*Ecp1* element were relevant to this analysis. The analysis of the WGS of the TTs looked to understand where IS*Ecp1* had inserted in the plasmid, any genes it had interrupted and whether these interrupted genes could possibly have a consequence to plasmid conjugation efficiency or bacterial fitness and importantly if there was any continuity with where IS*Ecp1* had inserted among the TTs. Two remaining TTs 687AMP16 and 876CLOX64 which were not included in groups 1-3 are also described in **Section 4.2.4.4**.

Finally in **Section 4.2.5**, the similarity of the insertion points of each IS*Ecp1* element were analysed and compared to show whether there seemed to be a likely preferred location for IS*Ecp1* insertion into the plasmids.

Table 4.4: The 14 TTs grouped depending on size into Group 1 >15 kb, Group 2 10-15 kb and Group 3 <10 kb and the two remaining TTs not within Groups 1-3

Group	TTs within Group
Group 1 >15 kb	687CLOX128
	961CLOX64
	687AMP0.32
Group 2 10-15 kb	687-N
	956AMP8
	956AMP16
	961CLOX25.6
Group 3 <10 kb	687AMP8
	876AMP8
	956-N
	956CLOX128
	956CLOX25.6
	876CAZ0.25
	687CLOX64
Two remaining TTs	687AMP16
	876CLOX64

4.2.4.1. Group 1 TTs >10 kb in Length

The three TTs placed into group 1 which were all >10 kb in length are listed in **Table 4.5** and details the plasmid replicon type utilised by IS*Ecp1*, the IS*Ecp1* size in bp, any truncated genes as a result of IS*Ecp1* insertion and the plasmid genes near to the insertion point of IS*Ecp1* and the IR_R sequence and

the bases in the IR_R which were complementary to the IR_L. In addition, at the bottom of the table is a graphic detailing the IS*Ecp1* genetic environment. As the IS*Ecp1* genetic environment was identical in all 3 TTs in group 1, only 1 graphic is shown. **Figures 4.7**, **4.8** and **4.9** show a section of the plasmid backbone where the IS*Ecp1* elements had inserted for the TTs 687CLOX128, 961CLOX64 and 687AMP0.32 respectively.

All TTs within group 1 had additional genes from the T3SS included as part of the IS*Ecp1* genetic environment and this was as a result of the recognition of a new imperfect IR_R, which was utilised by IS*Ecp1* during transposition. The additional genes from the T3SS included *prgH*/*eprH* which encodes part of the needle complex, *escF*/*yscF*/*hrpA* which encodes a needle major subunit, *eprJ* which encodes part of the inner membrane ring, *escJ*/*yscJ*/*hrcJ* which encodes part of the inner membrane ring and *orgA*/*mxiK* which encodes part of the sorting platform. Recognition of an imperfect IR_R was noted in **Section 3.6** and has been mentioned several times in the literature as a mechanism utilised by IS*Ecp1* during transposition (Poirel et al. 2005d; Lartigue et al. 2006c; Partridge 2011; Hamamoto et al. 2020c). The consequences of IS*Ecp1* collecting these additional genes from the T3SS could be an increase to virulence in the recipient bacteria, as the T3SS system within *E. coli* is an important factor, critical to virulence in pathogenic *E. coli* strains such as EPEC and EHEC. The T3SS delivers effector proteins to eukaryotic host cells, involved in the subversion of cellular processes, such as signalling pathways within the host and results in attaching and effacing lesion creation (Ideses et al. 2005; Zhou et al. 2014b). A good example of a foodborne pathogen which has a

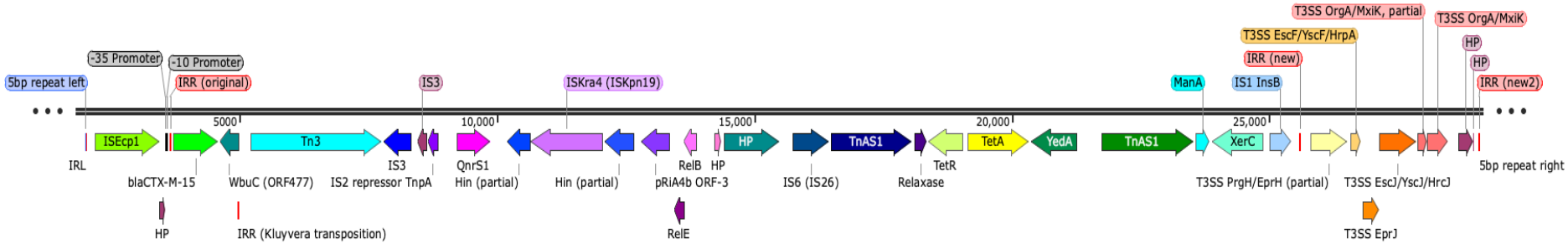
well-defined T3SS, is EHEC O157 and healthy cattle are a known reservoir of EHEC O157 (Lim et al. 2010b).

Interrupted genes as a result of IS*Ecp1* insertion into a plasmid was seen in 687CLOX128 and 687AMP0.32. In 687CLOX128, the insertion of IS*Ecp1* into the IncFIC plasmid, had interrupted the transfer gene *traC*, which is an ATPase involved in F-pilus biogenesis. This interruption could therefore possibly result in a reduced conjugation efficiency and 687CLOX128 did have a slightly lower enhanced transposition rate at 1.1 fold than 687AMP0.32 at 2.98 fold. However, this difference was only small and experimental conditions were different and could have affected rates of transfer, therefore this was merely an observation. In 687AMP0.32, IS*Ecp1* had inserted near to a TIR domain and had interrupted a hypothetical protein (HP), but as the function of the gene was unknown, it could not be assessed on how this interruption may have affected the plasmid. No genes were interrupted in 961CLOX64 by the insertion of IS*Ecp1* into the IncI1 plasmid, however it had inserted between a type 1 toxin anti-toxin (TA) system toxin *hok*/*gef* and a HP.

Table 4.5: Group 1 TTs with IS*Ecp1* elements >15 kb in length

TT Name	**Plasmid Replicon Type Used in Conjugation**	**IS*Ecp1* Size**	**Insertion Point in Plasmid**	**$IR_{R(new2)}$ utilised by IS*Ecp1* with bases complementary to the IR_L shown in red and number of bases complementary to the IRL detailed underneath**
687CLOX128	IncFIC	27,093 bp	Interrupted *traC* and recognition of a new imperfect IR_R took additional genes from T3SS during transfer	 **10 bases complementary to the IR_L**
961CLOX64	IncI1	27,093 bp	Truncated a Hypothetical Protein (HP) and inserted between T1 TA System *hok*/*gef* Toxin and a gene encoding a HP. Recognition of a new imperfect IR_R took additional genes from T3SS during transfer	 **9 bases complementary to the IR_L**

687AMP0.32	IncFIC	27,094 bp	Interrupted a gene encoding a Hypothetical Protein (HP) and recognition of a new imperfect IR_R took additional genes from T3SS during transfer	 **11 bases complementary to the IR_L**



Footnote for Table 4.5*: Detailed is the plasmid replicon type use in the conjugative transfer of ISEcp1, the insertion point of ISEcp1 in the plasmid, the IR_R sequence used with complementary base to IRL shown below and a small graphic of the ISEcp1 element in each TT*

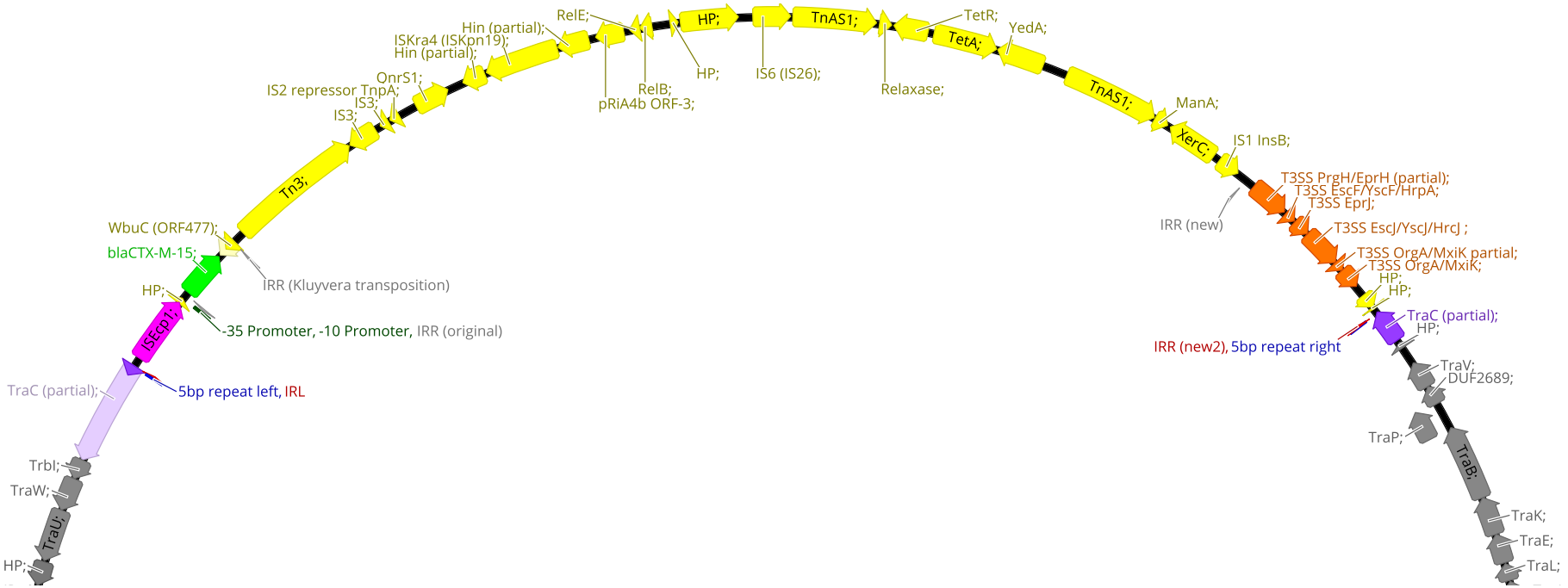


Figure 4.7: The IncFIC partial plasmid backbone of TT 687CLOX128 showing the insertion of the 27,093 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are shown in red, with the 5 bp repeats shown in blue denoting either end of the IS*Ecp1* element. The interrupted gene *traC* at the insertion point at either end of the IS*Ecp1* element is shown in purple, the IS*Ecp1* is shown in pink, $bla_{CTX\text{-}M\text{-}15}$ is shown in light green, the extra genes from the T3SS are shown in orange, the -35 and -10 promoters in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

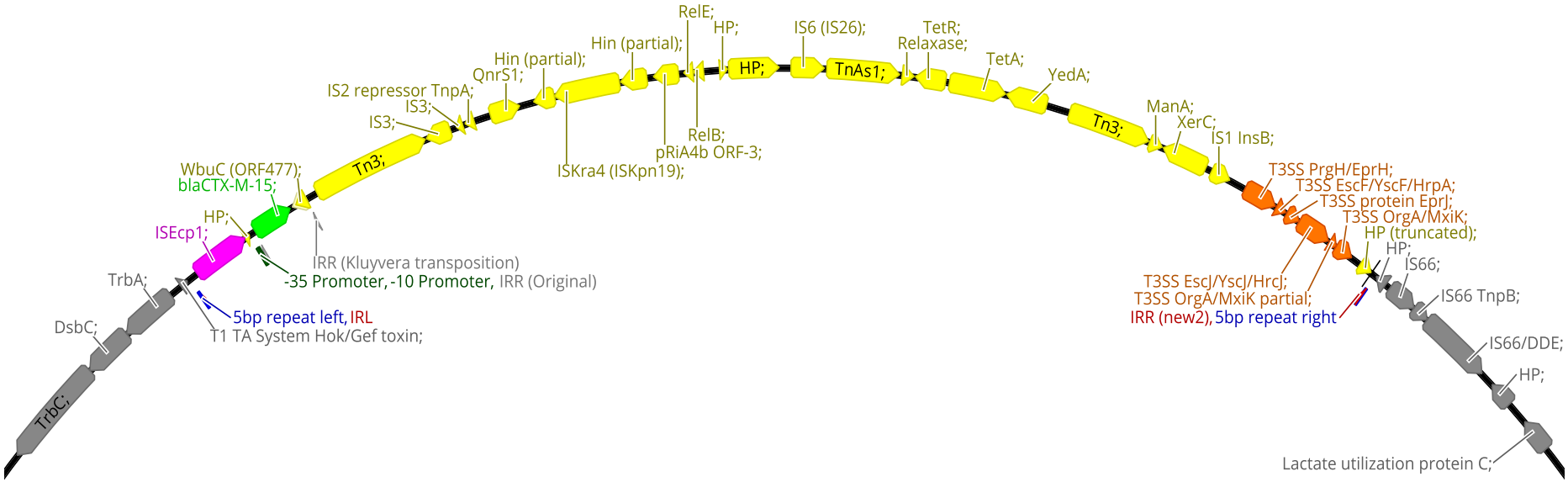


Figure 4.8: The IncI1 partial plasmid backbone of TT 961CLOX64 showing the insertion of the 27,093 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are both shown in red, with the 5 bp repeats shown in blue denoting either end of the IS*Ecp1* element. The IS*Ecp1* is shown in pink, $bla_{CTX\text{-}M\text{-}15}$ is shown in light green, the extra genes from the T3SS are shown in orange, the -35 and -10 promoters in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

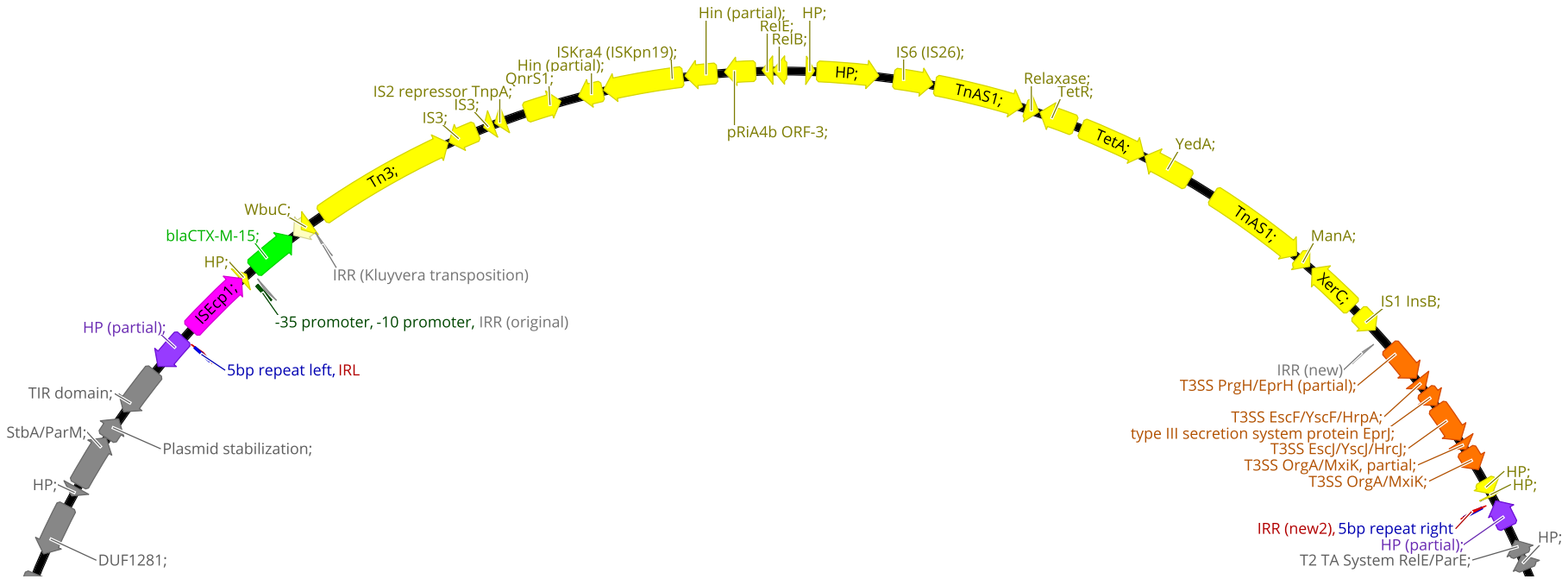


Figure 4.9: The IncFIC partial plasmid backbone of TT 687AMP0.32 showing the insertion of the 27,094 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The interrupted gene encoding a hypothetical protein (HP) at the insertion point at either end of the IS*Ecp1* element is shown in purple, the IS*Ecp1* is shown in pink, *bla*$_{CTX-M-15}$ is shown in light green, the extra genes from the T3SS are shown in orange, the -35 and -10 promoters in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

4.2.4.2. Group 2 TTs 10-15 kb in Length

The four TTs placed into group 2 which were all between 10-15 kb in length are listed in **Table 4.6** which details the plasmid replicon type utilised by IS*Ecp1*, the IS*Ecp1* size in bp, any truncated/interrupted genes as a result of IS*Ecp1* insertion and the plasmid genes near to the insertion point of IS*Ecp1* and the IR_R sequence and the bases in the IR_R which were complementary to the IR_L. In addition, below each TT description in the table is a graphic detailing the IS*Ecp1* genetic environment.

Figures 4.10, **4.11**, **4.12** and **4.13** show a section of the plasmid backbone where the IS*Ecp1* elements had inserted for the TTs 687-N, 956AMP8, 956AMP16 and 961CLOX25.6 respectively.

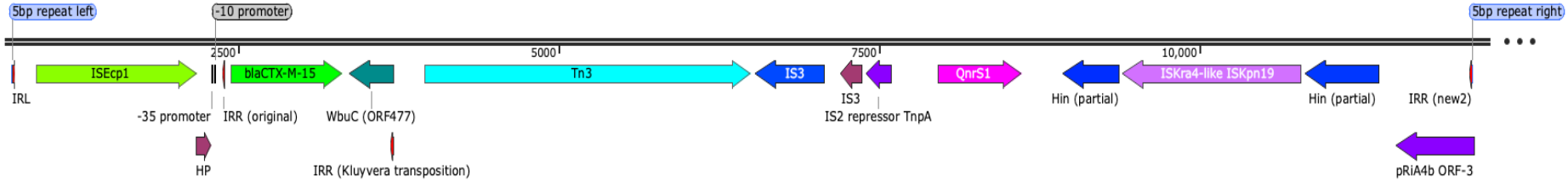
All the TTs within group 2 had lost genes from the IS*Ecp1* and this was the result of the recognition of a new imperfect IR_R and the IS*Ecp1* elements in the TTs were much smaller than in the parent strains. The consequences of the recognition of a new imperfect IR_R, was discussed in **Section 4.2.4.1** and just as this recognition of a new IR_R can gain genes as was seen in group 1, it can also result in the loss of genes as was seen with the TTs in this group. Both 687-N and 961CLOX25.6 had lost the *tetAR* genes as a result of the recognition of the new imperfect IR_R, and the absence of *tetAR*, was a feature of four of the isolates described in **Section 3.5.3** that included 950, 953, 954 and 956. However, this had not occurred in the TTs 956AMP8 and 956AMP16, as the parent 956 was of these strains not encoding *tetAR*.

The TT 961CLOX25.6, was the only TT where the IS*Ecp1* had utilised the IncX4 plasmid during conjugative transfer from the parent 961. In all the remaining TTs in this group the IS*Ecp1* had utilised the IncFIC plasmid.

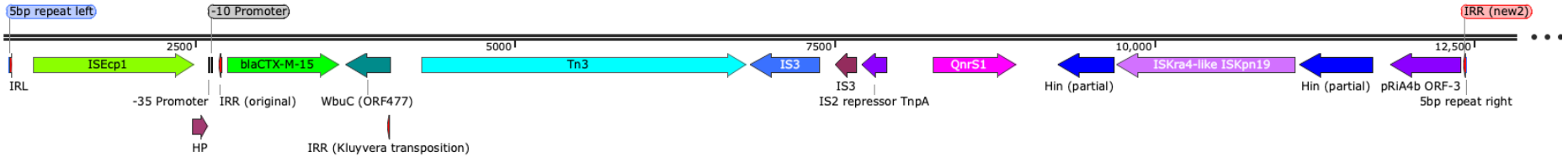
The TTs with 687 and 956 as parents had both used the IncFIC plasmid and IS*Ecp1* had inserted at a different site for each respective TT. In both 687-N and 956AMP8 the IS*Ecp1* element had inserted close to *finO*, with 687-N also in close proximity to *traX* and 956AMP8 in close proximity to DUF2726. In 956AMP16 IS*Ecp1* had inserted between a TIR domain (as was seen in 687AMP0.32) and a plasmid stabilisation protein. Interrupted genes as a result of the insertion of IS*Ecp1* were only seen in 687-N and 961CLOX25.6. In 687-N, insertion of the IS*Ecp1* element had interrupted an alpha/beta hydrolase that was adjacent to *traX* and *finO* and overlapped at the end of pRiA4b ORF-3. The alpha/beta hydrolase superfamily encompasses a functionally diverse family of enzymes involved in a large variety of physiological processes (Zhang et al. 1998). Members of the alpha/beta hydrolase family include proteases which are involved in functions such as proteolysis and therefore the consequences of this interruption by IS*Ecp1* could have an effect on physiological processes. In 961CLOX25.6 insertion of the IS*Ecp1* element had interrupted *virB8*, which forms part of the inner membrane complex of the T4SS (Sgro et al. 2019). The T4SS acts as a DNA translocation system and there are two main families that include those involved in the translocation of DNA during conjugation and those involved in effector translocation to eukaryotic cells in pathogenic bacteria (Christie et al. 2014). The consequences of the interruption of *virB8* could result in a fitness cost to either the ability of this plasmid to conjugate or a reduced pathogenicity fitness.

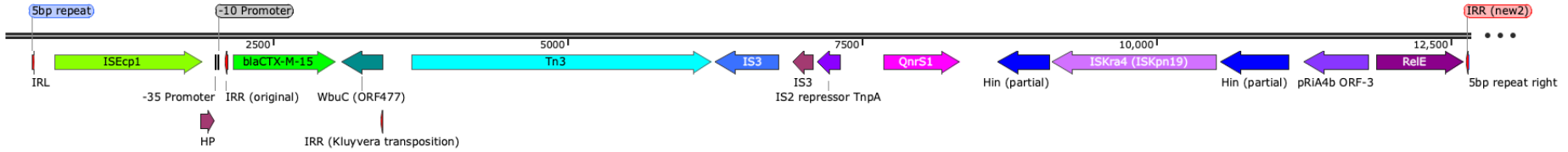
Table 4.6: Group 2 TTs where IS*Ecp1* elements were all between 10-15 kb in length

TT Name	Plasmid Replicon Type Used in Conjugation	IS*Ecp1* Size	Insertion Point in Plasmid	$IR_{R(new2)}$ utilised by IS*Ecp1* with bases complementary to the IR_L shown in red and number of bases complementary to the IR_L detailed underneath
687-N	IncFIC	11,394 bp	Interrupted an Alpha/Beta Hydrolase gene and inserted near *traX* and *finO*. Recognition of a new imperfect IR_R resulted in the loss of Tn*AS1* region encoding *tetAR*.	 **10 bases complementary to the IR_L**

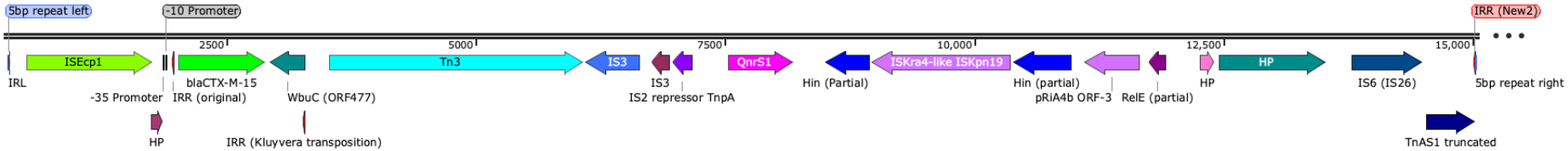


956AMP8	IncFIC	11,394 bp	No interrupted genes but inserted between a gene encoding a DUF2726 and *finO*	 **10 bases complementary to the IR_L**
956AMP16	IncFIC	12,196 bp	No interrupted genes but inserted between genes encoding a TIR Domain and a Plasmid Stabilization Protein	 **6 bases complementary to the IR_L**





961CLOX25.6	IncX4	14,722 bp	Truncated Tn*AS1* and Interrupted *virB8*. Recognition of a new imperfect IR_R resulted in the loss of *tetAR*.	 **7 bases complementary to the IR_L**



***Footnote for Table 4.6**: Detailed is the plasmid replicon type use in the conjugative transfer of ISEcp1, the insertion point of ISEcp1 in the plasmid, the IR_R sequence used with complementary base to IR_L shown below and a small graphic of the ISEcp1 element in each TT*

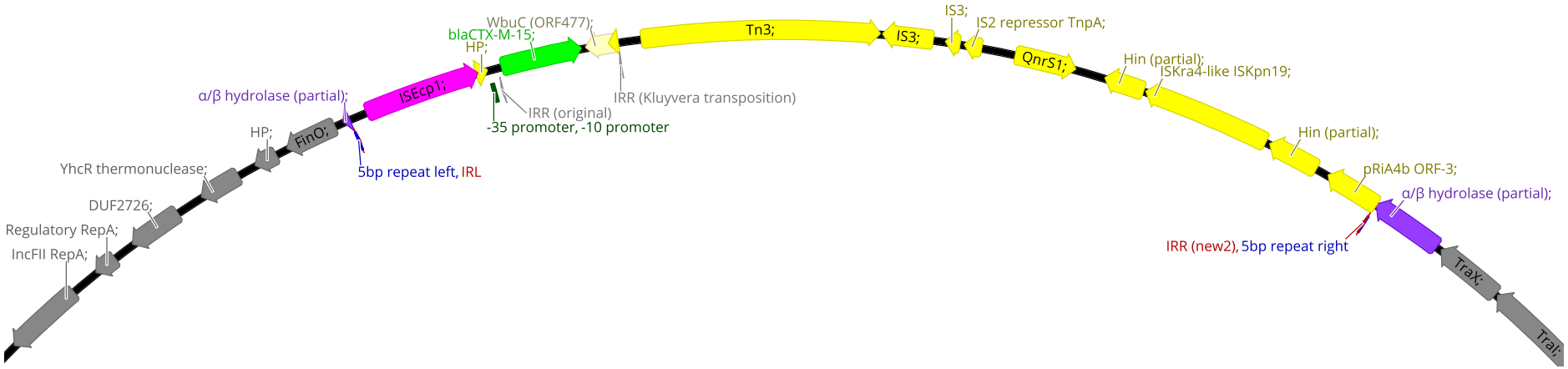


Figure 4.10: **The IncFIC partial plasmid backbone of TT 687-N showing the insertion of the 11,394 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The interrupted gene encoding an alpha/beta hydrolase at the insertion point at either end of the IS*Ecp1* element is shown in purple, the IS*Ecp1* is shown in pink, *bla*$_{CTX-M-15}$ is shown in light green, the -35 and -10 promoters in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element**

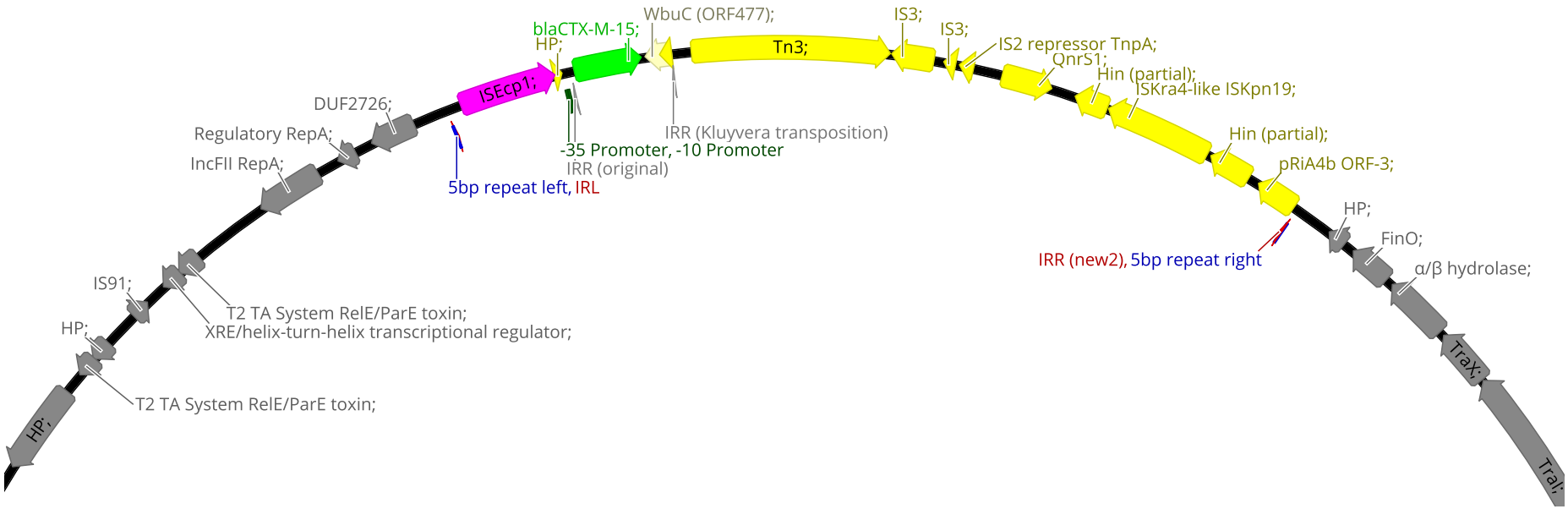


Figure 4.11: The IncFIC partial plasmid backbone of TT 956AMP8 showing the insertion of the 11,394 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The IS*Ecp1* is shown in pink, $bla_{CTX\text{-}M\text{-}15}$ is shown in light green, the -35 and -10 promoters in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

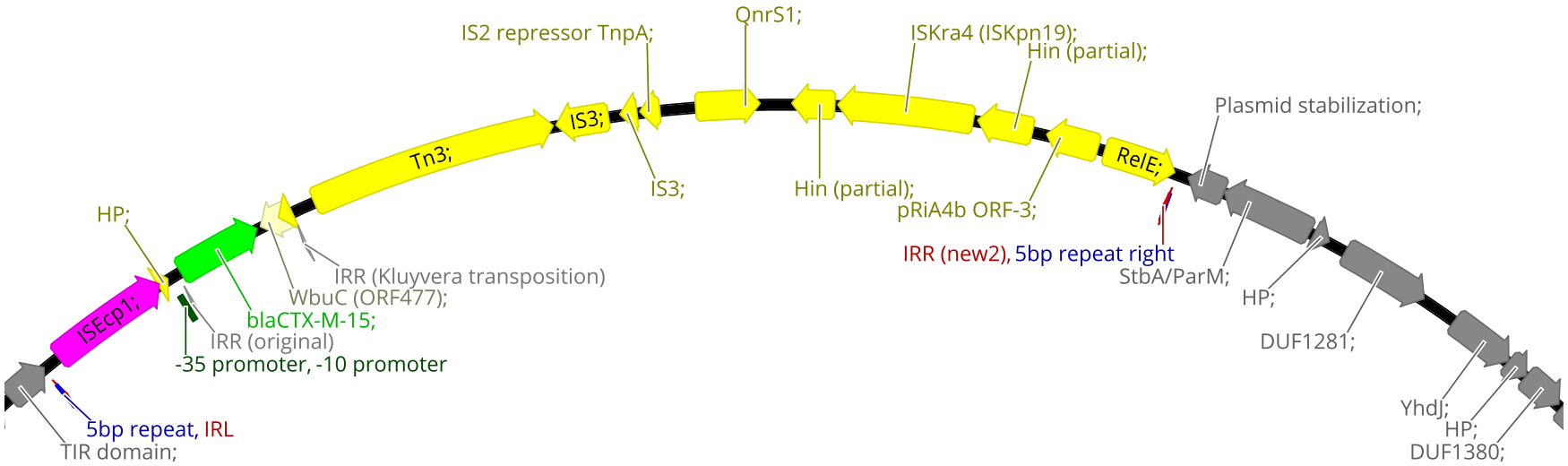


Figure 4.12: The IncFIC partial plasmid backbone of TT 956AMP16 showing the insertion of the 12,196 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The IS*Ecp1* is shown in pink, *bla*$_{CTX-M-15}$ is shown in light green, the -35 and -10 promoters in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

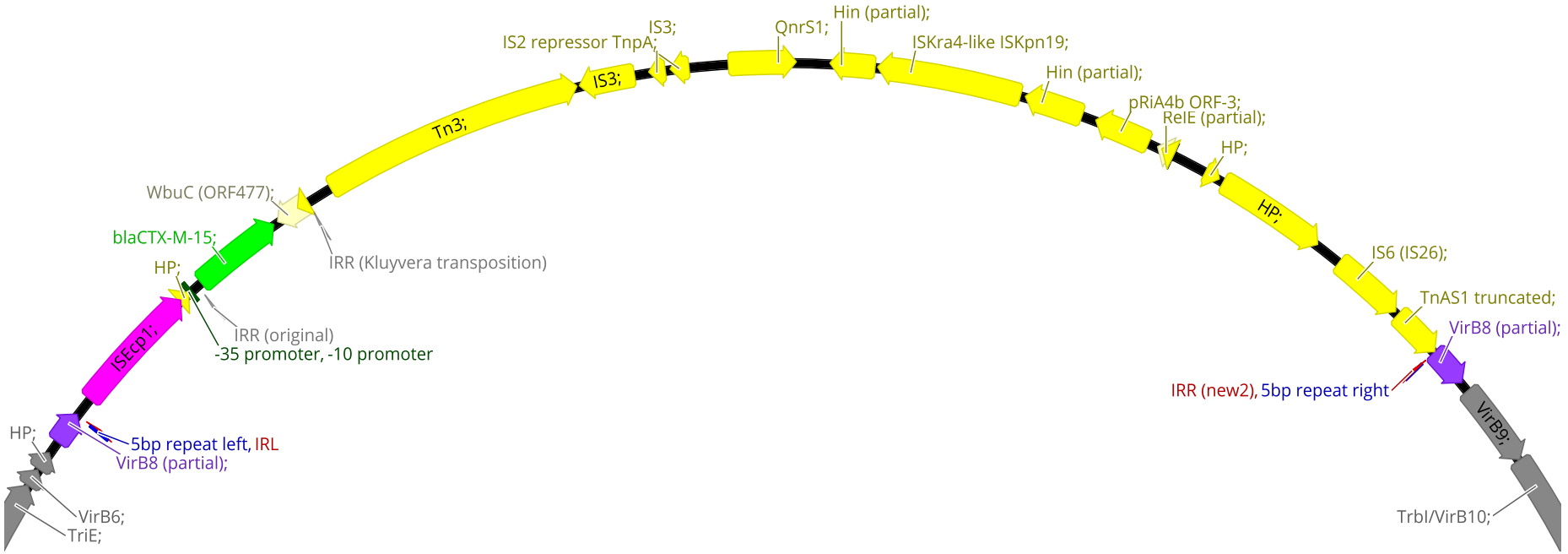


Figure 4.13: The IncX4 partial plasmid backbone of TT 961CLOX25.6 showing the insertion of the 14,722 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The interrupted gene encoding *virB8* at the insertion point at either end of the IS*Ecp1* element is shown in purple, the IS*Ecp1* is shown in pink, $bla_{CTX\text{-}M\text{-}15}$ is shown in light green, the -35 and -10 promoters are shown in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

4.2.4.3. Group 3 TTs <10 kb in Length

The seven TTs placed into group 3 which were all <10 kb in length are listed in **Table 4.7** which details the plasmid replicon type utilised by IS*Ecp1*, the IS*Ecp1* size in bp, any truncated/interrupted genes as a result of IS*Ecp1* insertion and the plasmid genes near to the insertion point of IS*Ecp1* and the IR_R sequence and the bases in the IR_R which were complementary to the IR_L. In addition, below each TT description in the table is a graphic detailing the IS*Ecp1* genetic environment. **Figures 4.14**, **4.15** and **4.16** show a section of the plasmid backbone where the IS*Ecp1* elements had inserted, with **Figure 4.14** showing 687AMP8, 876AMP8 and 956-N, **Figure 4.15** showing 876CAZ0.25, 956CLOX25.6 and 956CLOX128 and **Figure 4.16** showing 687CLOX64.

The IS*Ecp1* had utilised the IncFIC plasmid during conjugative transfer from the parents to the recipient in all but 876CAZ0.25, where it had utilised the IncFII plasmid.

The TTs placed in group 3 included 687AMP8, 876AMP8, 956-N, 956CLOX128, 956CLOX25.6, 876CAZ0.25 and 687CLOX64. The TTs within this group had lost almost all of the genes that had been present in the IS*Ecp1* genetic environment of the parent strains. Again, this loss of genes was due to the recognition by IS*Ecp1* of an imperfect IR_R, as had been seen in the TTs in group 2. Most of the TTs had no further genes downstream of *wbuC* and in 687AMP8, 876AMP8 and 956-N the recognition of the $IR_{R(new2)}$ had truncated *wbuC*. Only 687CLOX64 retained genes of the parent IS*Ecp1* element that were downstream of *wbuC*, which included Tn*3*, two IS3s, an IS*2* repressor TnpA and *qnrS1*. However, 687CLOX64 lost *tetAR* and all the remaining TTs had all lost both *qnrS1* and *tetAR*. However, the loss of *tetAR* had not occurred in 956-N,

956CLOX128 and 956CLOX25.6, due to the absence of *tetAR* in the parent 956 as was stated in **Section 4.2.4.2**.

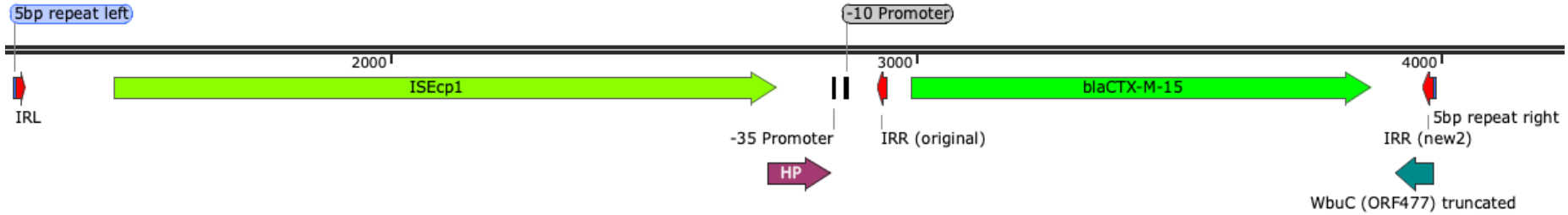
Genes that were interrupted as a result of the insertion of IS*Ecp1* into the plasmid backbone were seen in 876AMP8 where *traX* had been interrupted, 956CLOX25.6 where an alpha/beta hydrolase had been interrupted (which had also been seen in 687-N as described in **Section 4.2.4.1**) and 876CAZ0.25 where *traD* had been interrupted resulting in the stop codon being separated from the coding sequence. The *traX* gene encodes for TraX that is involved in acetylation of pilin subunits. Maneewannakul et al. (1995) observed that a *traX* mutant plasmid, pOX38-*traX* construct, was able to transfer under standard mating conditions with normal efficiency. It was observed however that *traX* mutants within liquid culture clumped together, suggesting high adhesiveness within non-acetylated cells. Therefore, *traX* was deemed to be non-essential to F plasmid conjugation. The gene *traD* encodes for the coupling protein TraD involved in F-plasmid mediated conjugation. When F conjugation is initiated, the F-pilus brings the two cells into close contact and a cytoplasmic pore called the transferosome links the two cells, which consists of a T4SS which spans the cell membrane connected to the coupling protein, TraD (Lawley et al. 2003; Lu et al. 2008). The consequences of this interruption of *traD* and the subsequent loss of the stop codon TGA could be readthrough of the gene and a resultant loss of function, which could possibly have a fitness cost to conjugation ability.

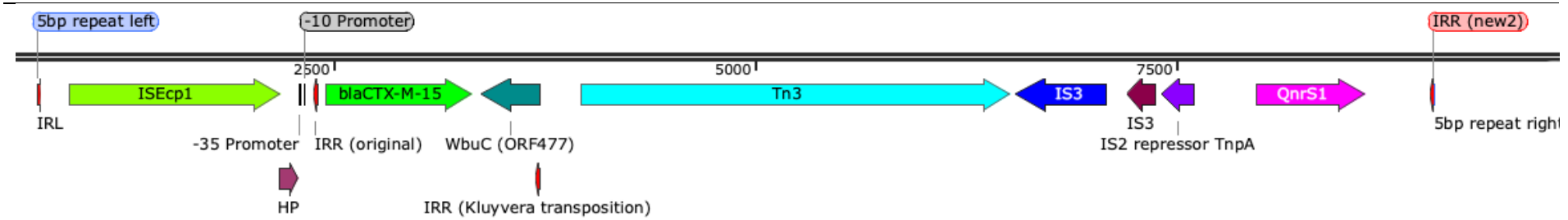
In 687AMP8 the IS*Ecp1* had inserted between the IncFIC RepA and DUF2776, which was a similar location to 956AMP8 described in **Section 4.2.4.2**, 956-N had inserted between *parB* and *repB* and 687CLOX64 had

inserted between *traX* and *traI* which was a similar location to 876AMP8 and also 687-N described in **Section 4.2.4.2**.

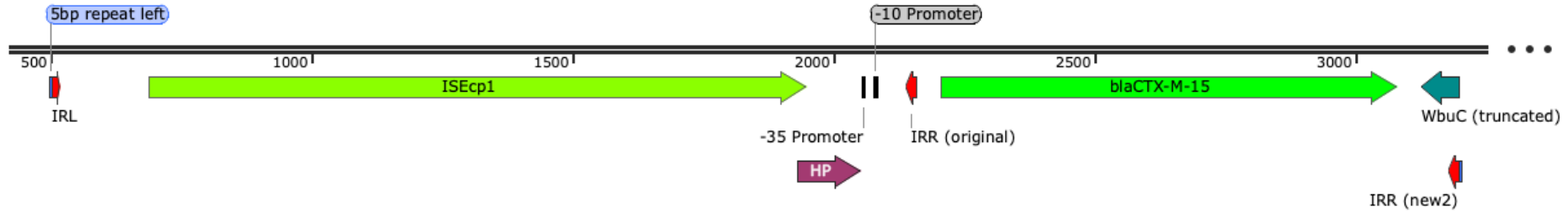
Table 4.7: Group 3 TTs where IS*Ecp1* elements were all between <10 kb in length

TT Name	Plasmid Used by IS*Ecp1*	IS*Ecp1* Size	Truncated genes and insertion point in plasmid	$IR_{R(new2)}$ utilised by IS*Ecp1* with bases complementary to the IR_L shown in red and detailed underneath
687AMP8	IncFIC	2,706 bp	Truncated *wbuC* and inserted between IncFII *repA* and a gene encoding a DUF2726. Recognition of a new imperfect IR_R resulted in the loss of all genes beyond *wbuC*, that included the Tn*3*, IS*3*, *qnrS1*, the two partial halves of *hin*, IS*Kra4*, *relEB*, IS*6* and the Tn*AS1* region encoding *tetAR*, *manA* and *xerC*	5′ AGCTCCGTGGTTCCTGGT 3′ 18 3′ TCGAGGCACCAAGGACCA 5′ IRR (new2) **8 bases complementary to the IR_L**
687CLOX64	IncFIC	8,284 bp	No interrupted genes but insertion was between *traI* and *traX*. Recognition of a new imperfect IR_R resulted in the loss of all genes beyond *qnrS1* that included the two partial halves of h*in*, IS*Kra4*, *relEB*, IS*6* and the Tn*AS1* region encoding *tetAR*, *manA* and *xerC*	5′ CTGTACAAAGTTTGCCGG 3′ 18 3′ GACATGTTTCAAACGGCC 5′ IRR (new2) **6 bases complementary to the IR_L**

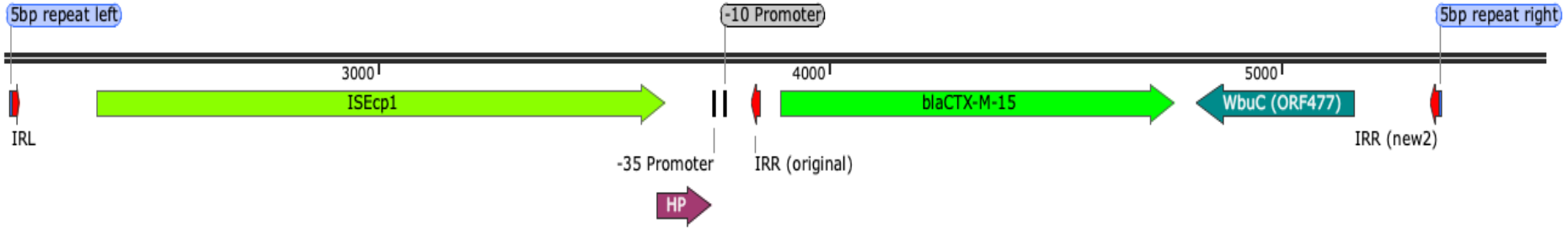
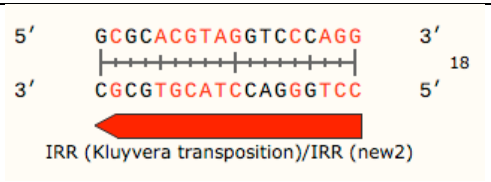


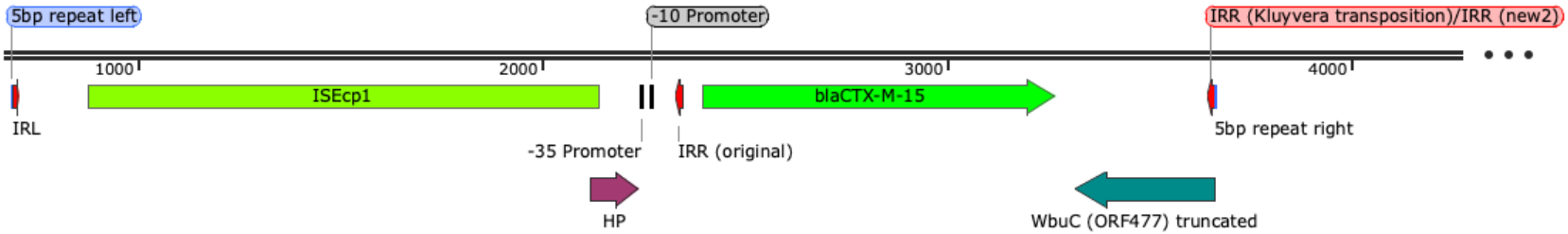


876AMP8	IncI1	2,706 bp	Truncated *wbuC* and interrupted *traX*. Recognition of a new imperfect IR_R resulted in the loss of all genes beyond *wbuC*, that included the Tn*3*, IS*3*, *qnrS1*, the two partial halves of *hin*, IS*Kra4*, *relEB*, IS*6* and the Tn*AS1* region encoding *tetAR*, *manA* and *xerC*	5′ AGCTCCGTGGTTCCTGGT 3′ 18 3′ TCGAGGCACCAAGGACCA 5′ IRR (new2) **8 bases complementary to the IR_L**

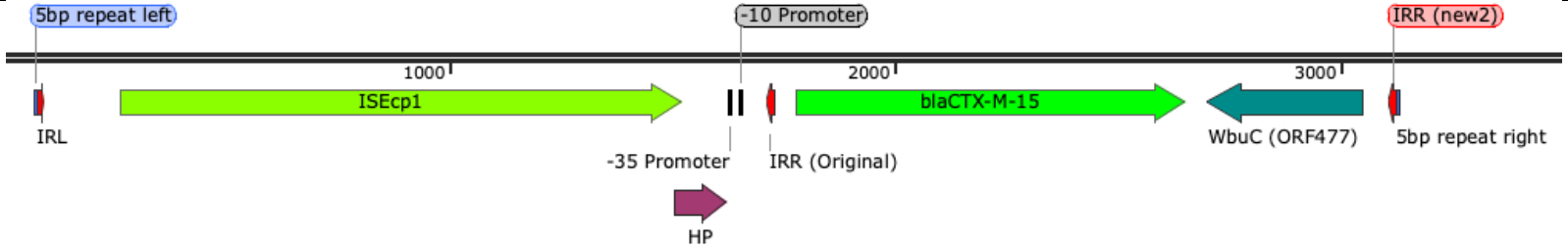


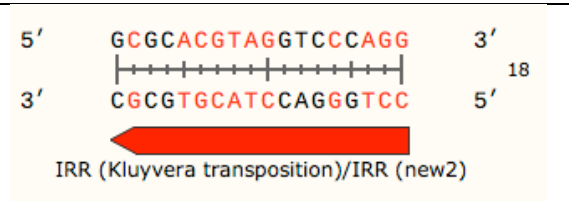
876CAZ0.25	IncFII	3,172 bp	Truncated *traD* by inserting adjacent to the stop codon TGA, resulting in readthrough of *traD*. Recognition of a new imperfect IR_R resulted in the loss of all genes beyond *wbuC*,	5′ GTCTTTTATATACCCGGG 3′ 18 3′ CAGAAAATATATGGGCCC 5′ IRR (new2)

			that included the Tn*3*, IS*3*, *qnrS1*, the two partial halves of *hin*, IS*Kra4*, *relEB*, IS*6* and the Tn*AS1* region encoding *tetAR*, *manA* and *xerC*	**4 bases complementary to the IR_L**
				
956-N	IncFIC	2,981 bp	Truncated *wbuC* and used $IR_{R(Kluyvera\ transposition)}$ during transfer. Recognition of a new imperfect IR_R resulted in the loss of all genes beyond *wbuC*, that included the Tn*3*, IS*3*, *qnrS1*, the two partial halves of *hin*, IS*Kra4*, *relEB*, IS*6* and the Tn*AS1* region encoding *tetAR*	 **11 bases complementary to the IR_L**

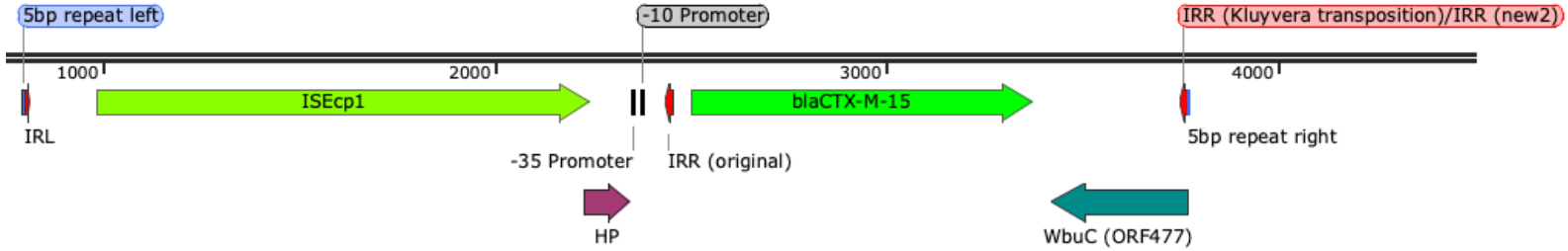


956CLOX25.6	IncFIC	3,060 bp	Interrupted an Alpha/Beta Hydrolase and inserted near *traX* and *finO*. Recognition of a new imperfect IR_R resulted in the loss of all genes beyond *wbuC*, that included the Tn*3*, IS*3*, *qnrS1*, the two partial halves of *hin*, IS*Kra4*, *relEB*, IS*6* and the Tn*AS1* region encoding *tetAR*	 **8 bases complementary to the IR_L**

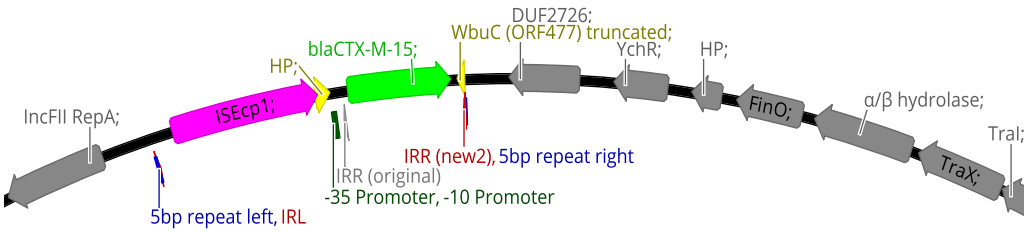


956CLOX128	IncFIC	2,981 bp	No interrupted genes but inserted between an Alpha/Beta hydrolase gene and *finO*. Used $IR_{R(Kluyvera\ transposition)}$ during transfer. Recognition of a new imperfect IR_R resulted in the loss of all genes beyond *wbuC*, that included the Tn*3*, IS*3*,	

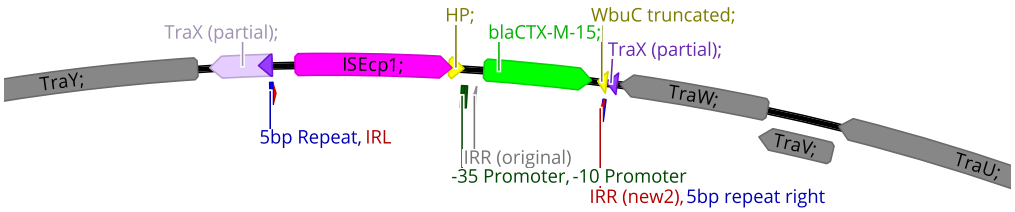
			qnrS1, the two partial halves of *hin*, IS*Kra4*, *relEB*, IS*6* and the Tn*AS1* region encoding *tetAR*	**11 bases complementary to the IR_L**



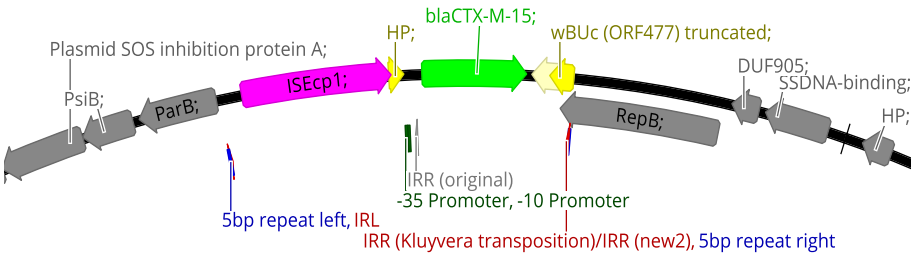
***Footnote for Table 4.7**: Detailed is the plasmid replicon type use in the conjugative transfer of ISEcp1, the insertion point of ISEcp1 in the plasmid, the IR_R sequence used with complementary base to IR_L shown below and a small graphic of the ISEcp1 element in each TT*



687AMP8 (IncFIC, 2,706 bp)

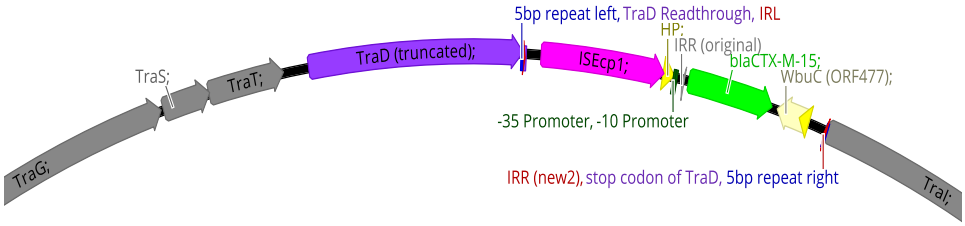


876AMP8 (IncI1, 2,706 bp)

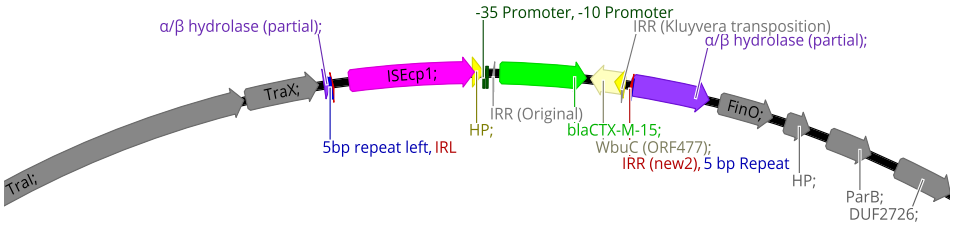


956-N (IncFIC, 2,981 bp)

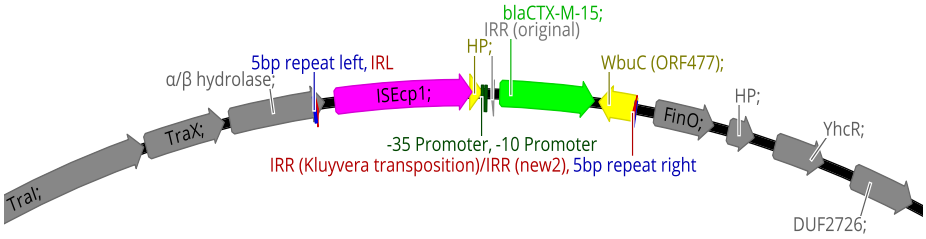
Figure 4.14: The partial plasmid backbone of TTs which all had a truncated *wbuC* including 687AMP8, 876AMP8 and 956-N with plasmid types used by IS*Ecp1* and IS*Ecp1* length (bp) both in brackets. The IR_L used in combination with the $IR_{R(new2)}$ are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The only interrupted gene was *traX* in 876AMP8 which is shown in purple at the insertion point at either end of the IS*Ecp1* element in the 876AMP8 plasmid backbone. In all plasmid backbones, the IS*Ecp1* is shown in pink, $bla_{CTX-M-15}$ is shown in light green, the -35 and -10 promoters are shown in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element



876CAZ0.25 (IncFII, 3,172 bp)



956CLOX25.6 (IncFIC, 3,060 bp)



956CLOX128 (IncFIC, 2,981 bp)

Figure 4.15: The partial plasmid backbone of TTs which all had similar IS*Ecp1* genetic environments with no further genes downstream of *wbuC* which included 876CAZ0.25, 956CLOX25.6 and 956CLOX128 with plasmid types used by IS*Ecp1* and IS*Ecp1* length (bp) both in brackets. The IR_L used in combination with the $IR_{R(new2)}$ are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The only interrupted genes were a truncated *traD* in 876CAZ0.25 and an alpha/beta hydrolase in 956CLOX25.6 which are shown in purple at the insertion point either end of the IS*Ecp1* element in 876CAZ0.25 and 956CLOX25.6 plasmid backbones. In all plasmid backbones, the IS*Ecp1* is shown in pink, $bla_{CTX\text{-}M\text{-}15}$ is shown in light green, the -35 and -10 promoters are shown in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

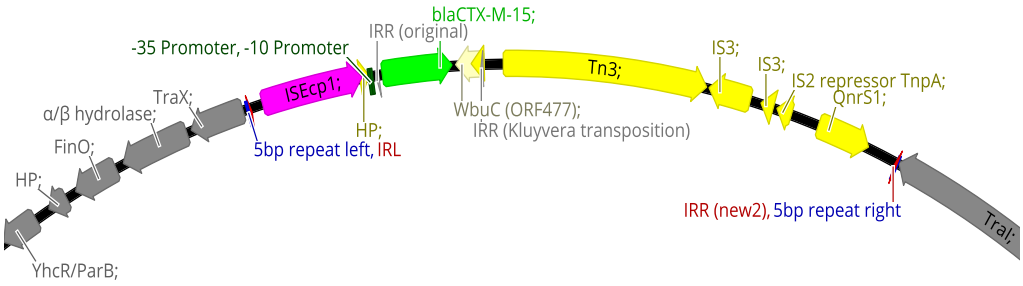


Figure 4.16: The IncFIC partial plasmid backbone of TT 687CLOX64 showing the insertion of the 8,284 bp IS*Ecp1* element which used the IR_L in combination with the $IR_{R(new2)}$ which are both shown in red, with the 5 bp repeats shown in blue which denote either end of the IS*Ecp1* element. The IS*Ecp1* is shown in pink, $bla_{CTX\text{-}M\text{-}15}$ is shown in light green, the -35 and -10 promoters are shown in dark green and any other genes within the IS*Ecp1* element are yellow. Any annotation in grey denotes plasmid DNA outside the boundaries of the IS*Ecp1* element

4.2.4.4. The Remaining Two TTs 687AMP16 and 876CLOX64

Two remaining isolates were initially included in the main group of 16 TTs, however these were not sorted into groups 1-3 as 687AMP16 was found to be a mutated donor and 876CLOX64 appeared to have a *bla*$_{\text{CTX-M-15}}$ that was chromosomally encoded in CV601. It was confirmed that 687AMP16 was a mutated donor from MLST (**Section 2.8.5.1**) on the WGS which showed it to be ST2325, which was the same as the parent and through the absence of the GFP gene of CV601.

In 876CLOX64 only *bla*$_{\text{CTX-M-15}}$ was found along with *wbuC* (ORF477) that had been truncated further from 348 bp to 207 bp. **Figure 4.17** details the *bla*$_{\text{CTX-M-15}}$ and truncated *wbuC* found within the genome sequence of 876CLOX64. Manual investigation of the entire WGS, found that IS*Ecp1* appeared to be missing, which was unexpected, as the PCR conducted on 876CLOX64 in **Section 4.2.1** and shown in **Figure 4.4** was positive for IS*Ecp1* and therefore this suggests the loss happened subsequently after transposition and possibly upon recovery from frozen culture. In addition to the loss of IS*Ecp1*, other genes associated with the IS*Ecp1* element were also missing, including the IRs and 5 bp repeats as detailed in **Figure 3.12**. This could suggest that a second transposition had occurred from the plasmid and *bla*$_{\text{CTX-M-15}}$ was transferred to the chromosome of CV601 but, after this event, IS*Ecp1* was lost. From the WGS of CV601, the *bla*$_{\text{CTX-M-15}}$ and *wbuC* were found alone in a short contig and separate from the chromosomal assembly, therefore it was not possible to determine exactly where in the chromosome *bla*$_{\text{CTX-M-15}}$ had inserted.

Figure 4.17: The two genes *bla*$_{CTX-M-15}$ and the truncated 207 bp *wbuC* found within the chromosome of 876CLOX64, appearing to show that transposition of IS*Ecp1* had occurred, followed by insertion into the chromosome of the recipient CV601

4.2.5. Similarity of Insertion Sites of IS*Ecp1*

There were some similarities noted between each of the TTs in relation to where IS*Ecp1* had inserted into the plasmid backbone. This was only really evident with IncFIC plasmid, as the majority of IS*Ecp1* elements were found to have utilised the IncFIC plasmid during conjugative transfer into the recipient CV601. The overall structure and size of the IncFIC plasmids of the parents were identical at 61,878 bp in all except 956 where the IncFIC plasmid was 11 bp smaller at 61,867 bp. All but two of the TTs had inserted into the same general region of the plasmid which included the genes *traI*, *traX*, an alpha/beta hydrolase, *finO*, a HP, *yhcR*, DUF2726, *repA*, IncFIC RepA, *relE*/*parE*, XRE transcriptional regulator, IS91, a HP, *relE*/*parE*, a HP, TIR domain and a gene encoding for a plasmid stabilisation protein. However, the insertion point found for the only IS*Ecp1* element to utilise the IncFII plasmid, TT 876CAZ0.25 was also in a similar location, as it had interrupted *traD* which was found to be immediately adjacent to *traI*. There didn't appear to be any continuity in the insertion locations into the IncI1 plasmid, however with only two IS*Ecp1* elements having utilised the IncI1 plasmid in TTs 961CLOX64 and 876AMP8, this was difficult to assess. In addition, only one IS*Ecp1* element had utilised the IncX4 plasmid which was TT 961CLOX25.6, so only the information from that

one TT in relation to preferred insertion site could be utilised for the IncX4 plasmid.

To compare and visualise the insertion site location in greater detail for each of the TT's that were found within an IncFIC plasmid, a plasmid map was constructed using Geneious Prime (**Section 2.8.2.1**) from the WGS of the parent strain 687. As all the IncFIC plasmids within the parent strains 687, 956 and 961 were extremely similar with only around 11 bp difference between them and the regions of interest for the analysis in this section identical, only one plasmid map was constructed. **Figure 4.18** shows a plasmid map of the IncFIC plasmid and identifies on it each of the three regions that were utilised by the TTs as insertion sites, with the green region encoding *repB* indicating where the IS*Ecp1* in TT 956-N had inserted, the pink region encoding *traC* indicating where the IS*Ecp1* in TT 687CLOX128 had inserted and blue indicating the large genomic region used by the remaining IS*Ecp1* elements found inserted at various points into an IncFIC plasmid that included 687AMP0.32, 687-N, 956AMP8, 956AMP16, 687AMP8, 956-N, 956CLOX25.6, 956CLOX128 and 687CLOX64. In addition, the *traD* gene is highlighted in orange on **Figure 4.18**, to show where 876CAZ0.25 had inserted into the IncFII plasmid, which had a very similar plasmid backbone to the IncFIC plasmid, with the region of interest for the analysis in this section identical. Each of the TTs are labelled on the plasmid map in **Figure 4.18** as red annotations of the TT name, that identifies on the map, the 5 bp repeat from either end of the IS*Ecp1* element, signifying where they each had inserted into the plasmid.

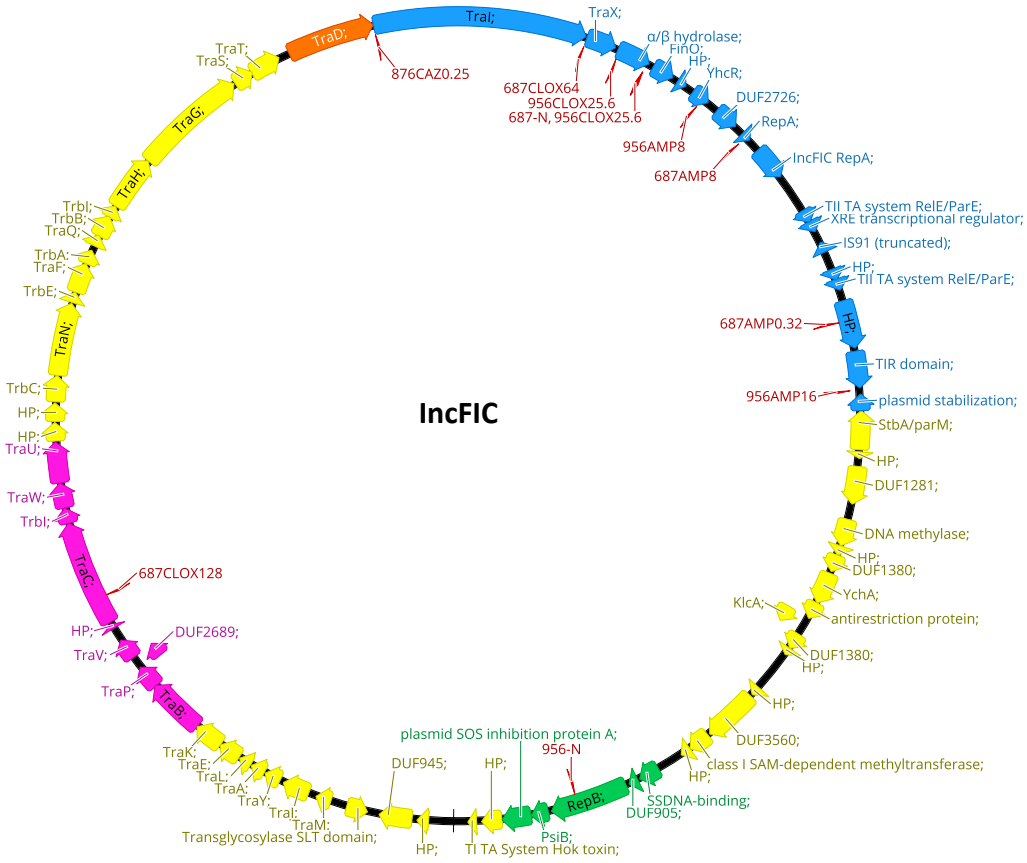


Figure 4.18: The plasmid map of the IncFIC plasmid from the parent strains, which was 61, 878 bp in 687 and 961 and 61, 867 bp in 956. The plasmid map details the insertion locations seen within the TTs as red annotations of the TT names and the main regions of IS*Ecp1* insertion. The main regions of insertion of IS*Ecp1* identified were the green region indicating TT 956-N, the pink region indicating TT 687CLOX128 and the blue region indicating the insertion region used by the remaining IS*Ecp1* elements found in an IncFIC plasmid. In addition, the *traD* gene is highlighted in orange on this plasmid map to show where 876CAZ0.25 had inserted into the similarly structured IncFII plasmid

4.3. MIC DETERMINATION OF TRANSPOSITION TRANSCONJUGANTS

To determine the effects on phenotypic resistance from the transposition of IS*Ecp1* into the recipient CV601, MICs were performed on the transposon transconjugants as per the method detailed in **Section 2.4.1**. This MIC aimed to assess any changes in the level of resistance that was seen in the MICs performed on the parent strains in **Chapter 3** (**Section 3.3.1** with results in **Tables 3.2** and **3.3**) and also assess whether the concentrations of AMP, CLOX and CAZ utilised for enhanced transposition were truly sub-lethal, which could be assessed by analysing the result for both ATCC25922 and CV601, which were both susceptible to all of these antibiotics. A panel of 15 antibiotics was selected from the original MIC panel of 25 antibiotics (listed in **Table 2.8**) that included AMP, CAZ, CTX, CPD, CFQ, ATM, AMC, FOX, ERT, NEO, TET, NAL, CIP and ENR and in addition CLOX was also included and a literature stated breakpoint was utilised (Hertz et al. 2014). The rationale for choosing these 15 antibiotics was firstly that the selection of AMP, CAZ, CTX, CPD, CFQ and ATM would assess the level of resistance provided by $bla_{\text{CTX-M-15}}$. CLOX was selected as this was one of the antibiotics utilised in the enhanced transposition experiments of **Section 4.2**. AMC and FOX were chosen to assess if any mutations to *ampC* had occurred and ERT would assess any porin loss or mutations. NEO was included as the recipient was encoding aminoglycoside resistance through the aminoglycoside 3'-phosphotransferase *aph3*' and this addition would act as an additional confirmation that this activity in CV601 was still present and unaffected by the transposition. TET was included to assess the level of resistance provided by those TTs which still contained *tetAR* and acted

as secondary confirmation that tetracycline resistance was no longer present in those that had lost *tetAR* and finally NAL, CIP and ENR were included to assess resistance provided by *qnrS1* and, as with *tetAR,* this would assess the level of resistance in those that had lost *qnrS1* and those that still contained *qnrS1*. **Table 4.8** details the MIC results for all 16 TTs, which included both the mutated donor as an additional confirmation to show it was indeed a mutated donor and the TT 876CLOX64 where the $bla_{\text{CTX-M-15}}$ had transferred into the chromosome of CV601.

The resistance profile of CV601 was assessed first, as this was the baseline background resistance profile, which changed following the introduction of the IS*Ecp1* element, which in turn resulted in a gain of resistance. CV601 was only resistant to NEO with an MIC of 64 mg L^{-1} and had susceptible MIC results for all other antibiotics. The results for AMP, CLOX and CAZ in CV601 and ATCC25922 of $\leq$4 mg L^{-1}, 256 mg L^{-1} and $\leq$0.5 mg L^{-1} respectively suggested the concentrations chosen for CLOX and CAZ were all at sub-lethal levels but only concentration 3.2 mg L^{-1} for AMP was at a sublethal level. The calculations of the 1/2, 1/4 and 1/10 MIC enhanced transposition concentrations for AMP and CAZ were taken from breakpoints in CLSI (CLSI 2022) rather than EUCAST (EUCAST 2022) and the resistant breakpoint for AMP in CLSI is 32 mg L^{-1}. Therefore, 16 mg L^{-1} for example would be 1/2 the MIC according to CLSI, but according to these results and the EUCAST breakpoint, 1/2 MIC would be only 4 mg L^{-1}. This demonstrates there can often be discrepancy between the two different S/I/R breakpoints stated by CLSI and EUCAST. The results here would suggest that the EUCAST breakpoint is closer to the susceptibility breakpoint for the isolates used within this study and to the pan

susceptible control ATCC25922. However, breakpoints listed by EUCAST and CLSI are "clinical breakpoints" and dictate the likelihood of therapeutic success when antibiotics are utilised at a specific concentration dictated by the dosing regimen (as was described in **Section 2.4**). Therefore, the comparison in an *in vitro* environment may differ to an *in vivo* environment.

All TTs except 876CLOX64 were resistant to AMP, CLOX, CAZ, CTX, CPD, CFQ and ATM with identical MICs of >512 mg L^{-1}, >512 mg L^{-1}, 16 mg L^{-1}, 512 mg L^{-1}, 128 mg L^{-1} and 32 mg L^{-1} respectively and these results were also identical to the IS*Ecp1* donor parent MICs detailed in **Chapter 3**, **Table 3.2**. The MIC results for 876CLOX64 were identical to the results seen for CV601. The MIC results for AMC, FOX, ERT and NAL were also all identical within the TTs and identical to the parent MIC results shown in **Chapter 3**, **Table 3.3**. As expected, the mutated donor 687AMP16 returned a susceptible result for NEO at ≤4 mg L^{-1} with the remaining TTs all returning the same resistant MIC result as CV601. Only 687CLOX128, 961CLOX64 and 687AMP0.32 returned a resistant MIC result for TET of 64 mg L^{-1}, which was the same MIC result as the parents in **Chapter 3**, **Table 3.3** all other TTs tested returned a susceptible result for TET.

Some changes to MIC results between the parent MIC results in **Chapter 3**, **Table 3.3** and the TT MIC results were noted for CIP and ENR. In 8 of the TT's which included 687-N, 687AMP0.32, 687CLOX64, 687CLOX128, 956AMP8, 956AMP16, 961CLOX25.6 and 961CLOX64 the MIC results for CIP and ENR were 0.25 mg L^{-1} and 1 mg L^{-1} respectively, which was the same as the parent MIC results in **Chapter 3**, **Table 3.3**. In the remaining

TTs which had lost *qnrS1* during IS*Ecp1* transposition, the MIC for CIP and ENR was the same as CV601.

The MIC results demonstrated, that in all but the mutated donor 687AMP16 and TT 876CLOX64, high level resistance to the beta-lactams that was seen in the IS*Ecp1* donor parent MICs results seen in **Chapter 3**, **Section 3.3.1,** had not changed following IS*Ecp1* transposition. It would appear in 876CLOX64, the transfer of $bla_{\text{CTX-M-15}}$ to the chromosome had resulted in a loss of gene function, as this TT had lost all beta-lactam resistance. It was noted in **Section 4.2.4.4**, that $bla_{\text{CTX-M-15}}$ was found in the chromosome without IS*Ecp1* and it was noted in **Chapter 3**, **Section 3.7** that IS*Ecp1* brings the strong promoter sequences -35 and -10 for the upregulation of $bla_{\text{CTX-M-15}}$ expression and no promoter sequences were found upstream of $bla_{\text{CTX-M-15}}$ in 876CLOX64. Most of the published literature on chromosomally-encoded $bla_{\text{CTX-M-15}}$ describes $bla_{\text{CTX-M-15}}$ in association with IS*Ecp1* (Fabre et al. 2009; Coelho et al. 2010; Mahrouki et al. 2012; Hirai et al. 2013b; Rodríguez et al. 2014a; Mshana et al. 2015; van Aartsen et al. 2019; Shawa et al. 2021). Therefore, the loss of IS*Ecp1* and its promoters, could have resulted in the loss of gene function of $bla_{\text{CTX-M-15}}$ in 876CLOX64. However, this was not investigated further experimentally, so the loss of function can only be theorised through the published data on the frequent association of $bla_{\text{CTX-M-15}}$ with IS*Ecp1*.

The results for NEO were all resistant and identical to the CV601 result in all but the mutated donor 687AMP16, further confirming this was a mutated donor.

For the TET MIC results, as only the parents 687, 876 and 961 were encoding *tetAR*, the TET results for any TTs that had 956 as the parent were

susceptible as expected. All TTs in group 2 (**Section 4.2.4.2**) and group 3 (**Section 4.2.4.3**) with 687, 876 and 961 as parents, had lost *tetAR* following IS*Ecp1* transposition and they had a susceptible result as expected. The only TTs not to lose *tetAR* following IS*Ecp1* transposition were all in group 1 (**Sections 4.2.4.1**), and as expected they all had the same level of resistance as the IS*Ecp1* donor parents 687 and 961.

The 8 TTs returning the same result for CIP and ENR as the donor parents, had not lost the *qnrS1* gene following IS*Ecp1* transposition. For the remaining TTs, the lower result for CIP and ENR MIC was the same as CV601 and appeared to be a consequence of all of these TTs having lost the *qnrS1* gene following IS*Ecp1* transposition. Despite the differences in the MIC results for CIP and ENR between the TT isolates, all were still susceptible, but it did appear to show that *qnrS1* provides a very low-level mechanism against fluoroquinolones, however, was unable to provide resistance or even intermediate resistance.

Table 4.8: MIC results for the TTs and the recipient CV601 along with the control isolate ATCC25922

Antibiotics	AMP	CLOX	AMC	FOX	CAZ	CTX	CPD	CFQ	ATM	ERT	NEO	TET	NAL	CIP	ENR
Breakpoints ↓	**Sensitive ≤ / Resistant > (mg/L) →**														
EUCAST	8	-	8	8/32	1/4	1/2	1	-	1/4	0.5	8/16	4/16	-	0.25/0.5	-
Literature Stated	-	256	-	-	-	-	-	0.25	-	-	-	-	32	-	2
ECOFF	8	-	-	≥32	0.5	0.25	1	0.125	0.25	0.032	-	-	8	-	0.125
Isolates ↓	**MIC →**														
687-N	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	0.25	1
687AMP0.32	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	64	32	0.25	1
687AMP8	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	≤0.064	≤0.032
687AMP16	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	≤4	64	32	0.25	1
687CLOX64	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	0.25	1
687CLOX128	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	64	32	0.25	1
876AMP8	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	≤0.064	≤0.032
876CLOX64	≤4	256	≤4	2	≤0.5	≤0.25	0.5	≤0.125	≤0.25	≤0.032	64	≤2	32	≤0.064	≤0.032
876CAZ0.25	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	≤0.064	≤0.032
956-N	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	≤0.064	≤0.032

956AMP8	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	0.25	1
956AMP16	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	0.25	1
956CLOX25.6	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	≤0.064	≤0.032
956CLOX128	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	≤0.064	≤0.032
961CLOX25.6	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	≤2	32	0.25	1
961CLOX64	>512	>512	≤4	2	16	>512	512	128	32	≤0.032	64	64	32	0.25	1
ATCC25922	≤4	256	≤4	2	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	≤0.032	≤4	≤2	32	≤0.064	≤0.032
CV601	≤4	256	≤4	2	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	≤0.032	64	≤2	32	≤0.064	≤0.032

Footnote for Table 4.8: *Any resistant results are highlighted in yellow*

4.4. TRANSPOSITION TRANSCONJUGANTS CONJUGATED INTO TG2

Within several TT strains, including 687CLOX128, 876CAZ0.25, 961CLOX25.6 and 876AMP8, the insertion of IS*Ecp1* had interrupted genes that could possibly be important to conjugation including *traC, traD*, *virB8* and *traX*. Therefore, it was decided to conduct a second conjugation, with a selected few of the TTs detailed in **Table 4.4**. The new recipient TG2, encoding the selective marker for tetracycline resistance, was utilised for this second conjugation and therefore any TTs selected as donor strains would need to be lacking the *tetAR* genes. Four TTs were chosen for the second conjugation and included two that had interrupted *tra* genes (876AMP8, 876CAZ0.25), one with an interrupted gene not thought to be important to conjugation (956CLOX25.6) and one that had no interrupted genes (956AMP16). Details of the interruptions in genes within 876AMP8, 876CAZ0.25 and 956CLOX25.6 can be seen within **Figures 4.19**, **4.20** and **4.21**, which detail the original gene from the parent donor and how the interruptions were presented (detailed as partial LT and partial RT) within the TT. No figure was constructed for 956AMP16 as no gene interruptions occurred during transposition of IS*Ecp1*.

The TTs 687CLOX128, 876CAZ0.25, 961CLOX25.6 and 876AMP8 used as donors in this second conjugation, all successfully conjugated their plasmids into the new recipient TG2. The success of the conjugation in the two TTs with interrupted conjugation genes, which was *traX* in 876AMP8 and *traD* in 876CAZ0.25, suggested that the insertion of the IS*Ecp1* into these transfer genes had not reduced the efficiency of conjugation in these plasmids to such an extent, that conjugation could not take place. However, it could be argued that if

the interruption of the *tra* genes had prevented the plasmid from conjugating, this would not have occurred into the recipient CV601 in the first instance. In addition, 956CLOX25.6 also conjugated successfully confirming that the interruption to the alpha/beta hydrolase had not affected the plasmid conjugation ability. As expected 956AMP16 with no gene interruptions also conjugated successfully.

Table 4.9: TTs that successfully conjugated with the recipient TG2

TT isolate	**Plasmid**	**Conjugation Rate**	**Insertion point Mutagenesis**
876AMP8	IncI1	1 x 10^{-5}	IS*Ecp1* insertion truncated *traX*
876CAZ0.25	IncFII	1 x 10^{-6}	IS*Ecp1* insertion adjacent to the TGA stop codon of *traD*
956AMP16	IncFIC	1 x 10^{-3}	IS*Ecp1* insertion without interruption of any genes
956CLOX25.6	IncFIC	1 x 10^{-2}	IS*Ecp1* insertion split an alpha/beta hydrolase gene

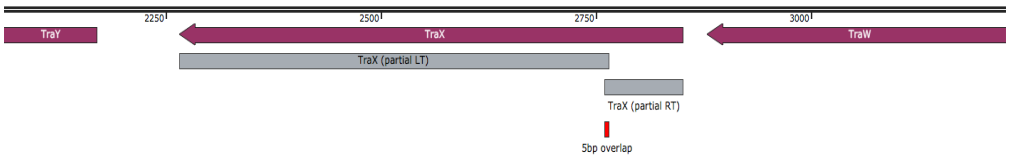


Figure 4.19: TT 876AMP8 showing the split within *traX* following insertion of IS*Ecp1*. *traX* as it appears within the parent is represented at the maroon coding region, with the two halves of the gene represented by the grey boxes underneath. There was also a 5 bp overlap (shown in red) between the end of TraX (partial LT) and TraX (partial RT)

Figure 4.20: TT 876CAZ0.25 showing the insertion of IS*Ecp1* adjacent to the TGA stop codon of *traD*. *traD* as it appeared within the parent 876 is shown as a maroon coding region with the TraD (partial LT) and TraD (partial RT stop codon) shown as a grey box below

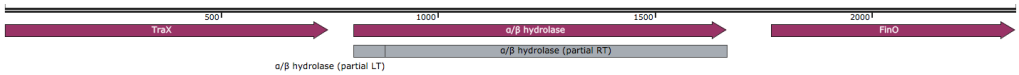


Figure 4.21: TT 956CLOX25.6 showing the insertion of IS*Ecp1* which split an alpha/beta hydrolase in half. The alpha beta hydrolase (denoted as α/β hydrolase) as it appeared within the parent 956 is shown as a maroon coding region with the two halves of the alpha beta hydrolase shown as two grey boxes below denoted as α/β hydrolase (partial LT) and α/β hydrolase (partial RT)

4.5. CONCLUSIONS

The ESBL *bla*$_{\text{CTX-M-15}}$ in association with the mobile element IS*Ecp1*, was found to be present within a number of multi-drug resistant isolates sampled from differing locations within a dairy farm. In all but isolates 950, 953, 955 and 956, which had lost the *tetAR* region (**Section 3.7.1** and **Figure 3.15**) and 962 where the *tetAR* region was not located within the IS*Ecp1* genetic environment (**Section 3.7.1.2** and **Figure 3.21**), the IS*Ecp1* elements were clonal (as detailed in **Section 3.7.1.2**). The IS*Ecp1* was shown to mobilise *bla*$_{\text{CTX-M-15}}$ both in a non-selective and selective (sub-lethal levels of antibiotics) environment. Mobilisation of IS*Ecp1* readily in the non-selective environment suggested the response to the experimental conditions such as the ideal physiochemical conditions, high nutrient availability and a stable temperature, were sufficient for mobilisation of IS*Ecp1* to occur. However, the inclusion of the antibiotics CAZ, AMP and CLOX at sub-lethal levels enhanced transposition of the IS*Ecp1* element in association with *bla*$_{\text{CTX-M-15}}$ and was likely the result of the sub-lethal levels of antibiotics acting upon the IS*Ecp1* element as opposed to *bla*$_{\text{CTX-M-15}}$. This result was anticipated, as it has been mentioned several times within the literature by Miller et al. (2004), Aminov et al. (2011b) and Beceiro et al. (2013), that sub-lethal levels of beta-lactam antibiotics can induce an SOS response, which may lead to increased mutagenetic activity and genetic variability with resultant increased MGE mobilisation (Kuan et al. 1991; Capy et al. 2000b; Foster 2007). It was also noted by Hastings et al. (2004) that other antibiotics may induce the SOS response, including ciprofloxacin, trimethoprim and some other quinolones, demonstrating that exposure to one antibiotic could result in the dissemination of resistance to an unrelated antibiotic. Lartigue et al. (2006a)

utilised nalidixic acid at 1/10, 1/4 and 1/2 the MIC in enhanced transposition of IS*Ecp1* experiments but saw no increase in transposition of IS*Ecp1* in the presence of this antibiotic. However, no studies were found that utilised the potent inducer of the SOS response fluoroquinolones (Blázquez et al. 2012; Baharoglu and Mazel 2014; Qin et al. 2015; Recacha et al. 2019).

Previous studies by Lartigue et al. (2006a) and Nordmann et al. (2008b) have shown the presence of CAZ can enhance transposition of an IS*Ecp1* element. This study was also able to demonstrate that sub-lethal levels of CAZ enhanced transposition, however the other 2 antibiotics utilised as part of this study, including AMP and CLOX, further showed some level of influence to enhanced transposition. When these results are related to the dairy farm environment, this demonstrates that even when the use of 3rd and 4th generation cephalosporins is avoided, the selection of an ESBL determinant such as *bla*$_{\text{CTX-M-15}}$ may still occur. This is due to the association of *bla*$_{\text{CTX-M-15}}$ with IS*Ecp1* and the resultant enhanced transposition and mobilisation and dissemination of *bla*$_{\text{CTX-M-15}}$, that confers multi drug resistance to beta-lactam antibiotics including the penicillins, 3rd and 4th generation cephalosporins and the monobactams. Thus, even if farmers resort to using the older generation antibiotics such as AMP and CLOX to treat mastitis, as opposed to 3rd and 4th generation cephalosporins, then this would not prevent cephalosporin resistance being selected for and spreading. In addition, even at sub-lethal levels antibiotics may still have an effect on resistance transmission, through the effects associated with increased mutagenetic activity and genetic variability from the SOS response induction.

The experimental baseline utilised in this study, used nutritious media, a steady temperature and no bacterial competition. Alternative baselines could have been set within this study, for example by using minimal media or different temperatures but that was not attempted in this study. Obviously environmental conditions could be different, making the rate of transposition in the natural environment of for example the cow gut different, where the atmosphere may not be aerobic and the temperature, nutrient availability and bacterial competition may all play a role in changing the rate of transposition. In addition, in the environment of the farm, other factors may play a role in stressing the bacteria such as low nutrient availability, UV damage, competition etc, which can induce the SOS response which in turn can affect the transposition of IS*Ecp1*. Therefore, it is questionable whether an absolute or natural baseline can even exist in nature, as this would be influenced by the constantly changing environmental conditions of the bacteria.

One experiment that was not investigated was to check the binding affinity of LexA to the region of IS*Ecp1*. However, a search of the sequence was conducted using the LexA consensus sequence TACTG$(TA)_5$CAGTA (Fernández De Henestrosa et al. 2000; Wade et al. 2005) and two other search patterns that included CTGNNTNNNNNNNCAG and CTGDNTDNNHNNHCAG that were listed in the study by Fernández De Henestrosa et al. (2000). A few possible LexA binding sites were located, with one in the vicinity of the IR_R as detailed in **Figure 4.22**. This putative LexA binding site in the vicinity of the IS*Ecp1* IR_R, could indicated that IS*Ecp1* may be under the repression of LexA and the SOS response. In addition, this finding is also consistent with other similar transposons under LexA repression, such as

Tn*5* which is a composite transposon bracketed by two IS*50,* with a LexA binding site on the right IS50 (Ross et al. 2014). In addition, deviations from the LexA binding site consensus sequence have been noted in the literature by De Henestrosa et al. (2000), so the alternative sequence found within the vicinity of the IR_R IS*Ecp1*, could be a potential LexA binding site. However, this would need to be challenged more thoroughly experimentally.

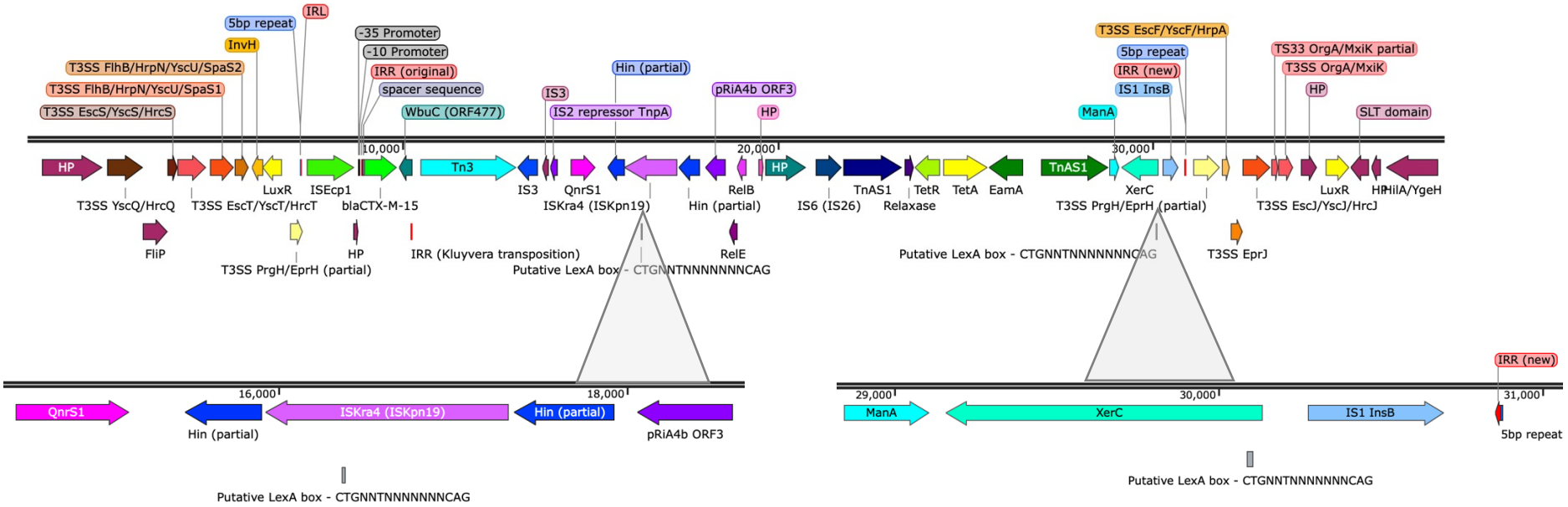


Figure 4.22: IS*Ecp1* genetic environment showing the location of two possible LexA binding sites. Areas with putative LexA binding sites are highlighted underneath the main IS*Ecp1* genetic environment as zoomed figures.

It has been reported in the literature by Zhang and Li (2011), Kulkarni et al. (2017) and Rodriguez-Mozaz et al. (2020) that levels of beta-lactams are usually at the limit of detection (LOD) within wastewater/effluent or very low levels, as they are often degraded quickly due to either beta-lactamase activity or through their susceptibility to hydrolysis. Concentrations reported within effluent have been in the region of LOD – 99.4 ng L^{-1} for AMP (Lin et al. 2008; Rodriguez-Mozaz et al. 2020), 15 ng L^{-1} for CLOX (Watkinson et al. 2007) and LOD, 34 ng L^{-1} and <12 ng L^{-1} for CTX (Gulkowska et al. 2008). However as was shown within the enhanced transposition experimental results, increased rates of IS*Ecp1* transposition were happening at sub-lethal levels, which could demonstrate that concentrations of antibiotics previously thought to be unimportant for causing resistance through the dilution of waste streams, may be significant to the spread of resistance determinants.

Many of the TTs were showing an enhanced level of transfer from the baseline rate of transfer and this demonstrated that IS*Ecp1* transposition does not appear to be a rare event. Only a small number of strains were utilised in the enhanced transposition experiments and a large number of TTs were generated, with some transconjugant plates containing 1 colony from an initial culture plating of only 100 µl. In addition, within the TT 876CLOX64 it was found that the IS*Ecp1* element had transposed from the chromosome of the parent 876, into a plasmid and back into the chromosome of the recipient CV601, in turn losing the IS*Ecp1* element and the plasmid it had utilised for transfer, leaving behind only the $bla_{\text{CTX-M-15}}$ gene and *wbuC* that was truncated to 207 bp. This demonstrated that no curing mechanism was necessary for the plasmid to be lost and transposition back into the chromosome to occur and shows IS*Ecp1* transfers

readily within isolates both in a non-selective and selective environment. However, as this plasmid curing and IS*Ecp1* transfer into the chromosome of CV601 was only seen in 876CLOX64, it cannot be assessed for certain if this was a rare event or not, as only 16 TTs were analysed as part of this study, which is quite a small sample size. On the other hand it could be argued, that with such a small sample size and this having occurred in only 1 isolate, this could suggest this is not a rare event.

The genetic environments of the TTs also varied a great deal and several of the IS*Ecp1* elements had lost both the *tetAR* region and *qnrS1*. The loss of the *tetAR* region and *qnrS1*, demonstrated that IS*Ecp1* transposition does not always have beneficial results in relation to resistance, as transposition can sometimes reduce the resistance conferred by genes encoded by the bacteria through the recognition of a new imperfect $IR_{R,}$ and loss of resistance genes. However, in a non-selective environment, it may indeed be beneficial for the bacteria to lose surplus genes such as resistance genes and mobile elements such as transposons and plasmids, as there is a fitness cost required to maintain these large pieces of external DNA and this is not always favourable when the selective pressure of antibiotics are absent. In an environment like a farm or a hospital, where antibiotic selective pressure is present, it may indeed be more favourable to maintain a large IS*Ecp1* element that encodes multiple resistance genes and plasmids that enable HGT of this IS*Ecp1* element.

It is well documented in the literature and was first described in **Chapter 3**, **Section 3.7** and was noted in this chapter in **Section 4.2.4.1**, that the commonly seen signature of IS*Ecp1* mobilisation is the recognition of an imperfect IR_R (Poirel et al. 2003; Poirel et al. 2005a; Lartigue et al. 2006c;

Dhanji et al. 2011c; Zowawi et al. 2015; Sun et al. 2016; Hamamoto et al. 2020a; Widyatama et al. 2021b) and through this the collection of new genes may occur which have been termed transposition units (Zong et al. 2010a; Widyatama et al. 2021c; Yagi et al. 2021). However, it does not appear to be documented in the literature that the IS*Ecp1* mobilisation mechanism may result in gene loss and therefore this appears to be a novel finding. The recognition of the new imperfect IR_R is not completely random as there is commonly some homology to the IR_L, but it does appear however to be somewhat random how far downstream from the IR_L this recognition happens (Poirel et al. 2008). Therefore, gene gain or indeed loss as a consequence of this may be equally possible and the TTs analysed in this study would appear to support this. Another transposon that mobilises in a similar way, by the recognition of variable different IR_R sequences is T*n2* and the insertion sequence IS91 also mobilises in this way. The transposable elements' right extremity is therefore defined in both T*n2* and IS91 through this mechanism (Poirel et al. 2008).

Three of the TTs 687AMP0.32, 687CLOX128 and 961CLOX64, had actually gained the additional genes from the T3SS: *prgH*/*eprH*, *escF*/*yscF*/*hrpA*, *eprJ*, escJ/*yscJ*/*hrcJ* and *orgA*/*mxiK* by recognising a new imperfect IR_R further along the genome during transfer which was first described in **Section 3.7** and is discussed in detail in **Section 4.2.4.1**. This demonstrated that through IS*Ecp1* transposition, there is the potential for movement of important virulence genes, which might generate new variants of pathogens, which is a key fundamental biological process.

CHAPTER 5

FURTHER MECHANISMS OF BETA-LACTAMASE RESISTANCE

5.1. INTRODUCTION

As was discussed within **Chapter 3**, ESBLs such as *bla*$_{\text{CTX-M-15}}$ are of great concern to the efficacy of antibiotics, such as the 3rd and 4th generation cephalosporins, monobactams and broad-spectrum penicillins. Within the environment of the dairy farm, it was shown in **Chapter 3**, that *bla*$_{\text{CTX-M-15}}$ had the ability to cause resistance to multiple antibiotics and the potential to mobilise resistance around the dairy farm environment in association with the mobile element ISE*cp1* as detailed in **Chapter 4**. To gain a greater understanding of the beta-lactamase community within the dairy farm, other mechanisms of beta-lactamase type resistance were investigated. These other potential resistance mechanisms included overexpression of chromosomal *ampC* (often termed extended spectrum cephalosporinases (ESCs)) and the two beta-lactamases *bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA}}$. ESCs and ESBLs are noted in the literature as being of concern to both the clinical and agricultural/veterinary environments (Feria 2002; Pérez-Pérez and Hanson 2002; Peter-Getzlaff et al. 2011b; Haenni et al. 2014c). Within this chapter, these other mechanisms responsible for beta-lactamase type resistance will be explored through both phenotypic and genotypic analyses, that included the initial disc assay data provided by EVAL farms, MIC assays, PCR analysis of the promoter region of *ampC* and WGS. In addition, the sequencing of all isolates via both short and long read with hybrid assembly, allowed for a more in depth look at the isolates in this group.

The designated *ampC* group initially consisted of only 40 isolates and therefore preliminary PCR analysis of the promoter regions of the *ampC* gene, that was conducted prior to MICs and WGS, was only conducted on those 40. Within that group of 40 isolates was isolate 348, which with subsequent WGS

was found to be a *Citrobacter* spp., therefore this isolate was present in initial PCR analysis conducted prior to WGS but was deleted from all other figures and tables and discounted from any further analysis. As was detailed in **Chapter 3**, the 8 isolates 854, 855, 856, 870, 871, 872, 968 and 969 originally placed in the *bla*$_{CTX}$ group, were thought to be more likely to be encoding an ESC such as overexpression of *ampC*, rather than the ESBL *bla*$_{CTX-M}$ as the initial phenotypic picture suggested overexpression of *ampC* and the subsequent PCR confirmed absence of *bla*$_{CTX-M}$. These additional 8 were not subject to PCR analysis of the promoter regions of *ampC*, as this was deemed unnecessary because WGS was later conducted. Therefore, the final number of isolates in the *ampC* group was 47.

5.2. EXPLORING *ampC* IN THE DAIRY FARM *E. coli* ISOLATES

Within the EVAL farms isolates, overexpression of *ampC* was first found and analysed in isolate EcoUR2111-869, which had been sequenced via PacBio as part of the EVAL farms study (**Table 2.3**). Following searches of the WGS of EcoUR2111-869 (referred to simply as 869 in the proceeding text), it was found to be encoding the mutation C→T at position -42 and G→A at position -18, which created two alternative new stronger -35 and -10 promoter sequences (Caroff et al. 2000; Mulvey et al. 2005a; Peter-Getzlaff et al. 2011a; Haenni et al. 2014a; Mandal et al. 2016), which was detailed in **Section 1.5.3.2** and is shown in **Figure 5.1**. Isolate 869 acted as a positive control for all

subsequent assays regarding *ampC* analysis. The PacBio sequenced isolates BCC2 (Ibrahim et al. 2016) (**Table 2.4**) and EcoSL3110-774 (**Table 2.2**) were also used within the following assays, as they encoded $bla_{\text{CTX-M-14}}$ (Ibrahim et al. 2016) and $bla_{\text{CTX-M-15}}$ respectively. The pan susceptible control strain ATCC25922, encoding a WT *ampC* gene expressed at a constitutively low level, acted as a control throughout. Previous studies including those by Tracz et al. (2005; 2007) have also utilised ATCC25922 as a control when assessing *ampC* overexpression. **Figure 5.1** shows the promoter and attenuator regions upstream of the *ampC* and details where the -35 and -10 sit upstream of the attenuator for both the WT promoters (annotated as Wild Type below) and the new -35 and -10 promoters (annotated as Alternative above) created from mutations at positions -42 and -18.

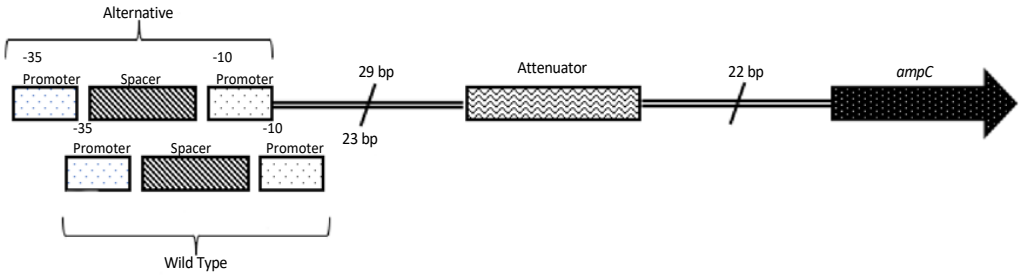


Figure 5.1 The *ampC* promoter region. This shows the area encoding the two promoter boxes -35 and -10, the spacer between them, the attenuator and *ampC*. The CDS regions along the bottom, show the wild type, with the alternative CDS regions above, resulting from the introduction of mutations which shift the position of the promoters and spacer along the genome, increasing the distance between *ampC* and the promoters. Original figure produced for this study from WGS of 869 and based on data published by (Mulvey et al. 2005; Peter-Getzlaff et al. 2011)

5.2.1. Susceptibility Patterns Associated with Overexpression of *ampC*

Overexpression of *ampC* in the EVAL farms isolates, was first explored by utilising phenotypic data. The usual phenotypic picture associated with overexpression of *ampC* is reduced susceptibility to cephamycins including FOX and cefotetan, broad spectrum penicillins such as AMP, the beta-lactam/beta-lactamase inhibitor combination AMC, 3rd generation cephalosporins including CTX, EFT and CAZ and the monobactam ATM (Caroff et al. 1999c; Haenni et al. 2014d). When *ampC* overexpression is coupled to porin loss, reduced susceptibility to carbapenems can also occur (Mammeri et al. 2008a). In contrast *bla*$_{\text{CTX-M}}$ phenotypic resistance profiles usually show resistance to CFQ, but susceptibility to FOX and AMC (Drawz and Bonomo 2010b). Discerning resistance as a result of overexpression of *ampC* from ESBL types such as *bla*$_{\text{CTX}}$, can be challenging within the laboratory environment (Gupta et al. 2014). However, by utilising susceptibility patterns, it is possible to distinguish overexpression of *ampC* from *bla*$_{\text{CTX-M}}$ by using the discernible markers as discriminatory parameters (Peter-Getzlaff et al. 2011b). Cephamycins are generally a reliable susceptibility marker when discerning resistance as a result of overexpression of *ampC* activity against ESBLs, as bacteria overexpressing *ampC*, are reliably resistant to them. The picture may become complicated however, when in addition to overexpression of *ampC*, there are also other beta-lactamases such as ESBLs or carbapenemases present in the same organism (Reuland et al. 2014; Tamma et al. 2019).

5.2.2. Phenotypic Disc Assay Data of 47 *ampC* Isolates

E. coli isolates were firstly selected from the EVAL farms collection based upon the presenting phenotypic resistance profile from disc diffusion assay data supplied by EVAL farms (with full details described in **Section 2.2.1**). Isolates presenting with reduced susceptibility to CTX, CAZ, CPD, FOX, AMC, AMP and ATM, were suspected of exhibiting a likely overexpression of *ampC* genotype but without the production of an ESBL. All isolates are detailed in **Table 2.3** of **Chapter 2 (**which includes full isolate codes and isolation date, sampling location and media isolated on). The heatmap shown in **Figure 5.2** details the resistance profiles from disc assay data of the 47 *ampC* isolates, which was created using Microsoft Excel conditional formatting (**Section 2.4.1**). Detailed at the base of the heatmap are also the number of resistant results (RES), intermediate results (INT) and susceptible results (SUS), along with the representative percentages. Within the succeeding text, figures and tables, full isolate codes are shortened to only the number for example EcoSL2906-99 is shortened to 99 and EcoBHSSC2111-867 is shortened to 867.

From the disc diffusion assay data provided by EVAL farms for the 47 *E. coli* isolates, resistance to FOX, CPD and CTX among isolates was high at 89.4%, 85.1% and 85.1% of isolates respectively, with moderate resistance towards AMP, AMC, CAZ, STREP and SXT at 63.8%, 55.3%, 44.7%, 31.9% and 31.9% respectively. Only a low frequency of isolates were showing resistance to NAL, CIP, NIT, ATM, AZM, TET, CHLOR, and IMP at 23.4%, 19.1%, 19.1%, 17%, 14.9%, 10.6%, 8.5% and 4.3% respectively. Only a low frequency of intermediate resistance was seen among isolates with CAZ at

25.5%, ATM at 17%, STREP at 14.9%, CTX at 10.6%, CIP at 6.4%, FOX at 4.3% and CPD, SXT and NIT all at 2.1%.

Disc assay data allowed for the quick identification of multi-drug resistance, isolates showing similar or identical resistance profiles and also allowed for the potential prediction of genotype. However, predicting genotype from phenotype can be more difficult when multiple mechanisms of resistance are present within the same isolate, for example when *bla*$_{\text{OXA}}$ and overexpression of *ampC* are both present.

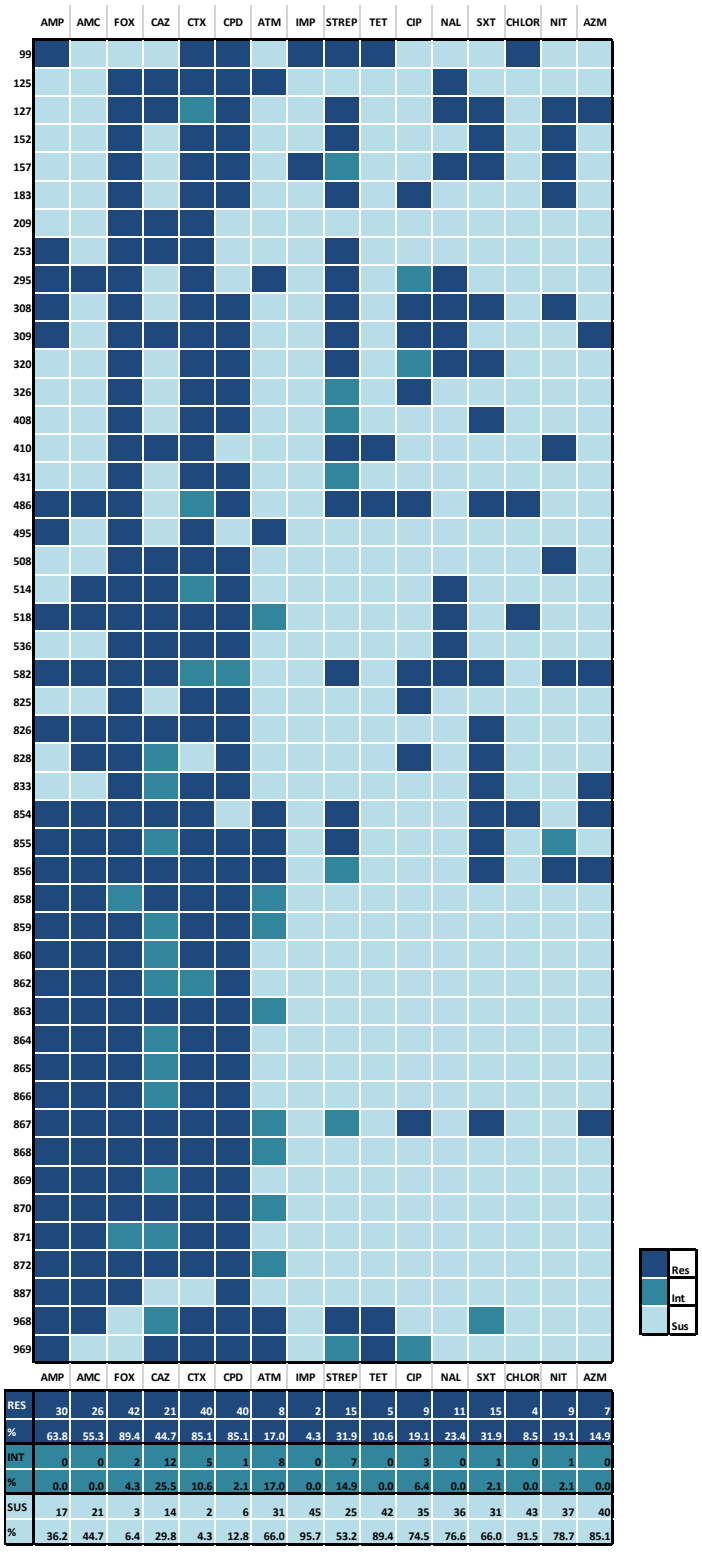


Figure 5.2: Heatmap of resistance profiles of 47 isolates in the *ampC* group, produced from disc diffusion assay data supplied by EVAL farms. The number of Resistant (RES), Intermediate Resistant (INT) and Susceptible (SUS) results along with the representative percentages are detailed along the bottom of the heatmap

5.2.3. MIC Determination of the 47 *E. coli ampC* Isolates

For further clarification of likely genotype and the level of resistance of the 47 isolates in the *ampC* group, MIC assays were conducted using the agar dilution method as detailed in **Section 2.4.1**. The MIC assays utilised the same extended panel of antibiotics used for the isolates in the *bla*$_{CTX}$ group, which are detailed in **Table 2.3** of **Chapter 2**. Of most interest concerning overexpression of *ampC* identification, was the level of resistance to the penicillin AMP, the beta-lactam/beta-lactamase inhibitor combination AMC, the cephamycin FOX, the 3rd generation cephalosporins CTX, CAZ and CPD and the monobactam AZM. With the addition of CFQ in the extended panel, the discriminatory parameters were all present for identifying overexpression of *ampC* from *bla*$_{CTX}$ from phenotypic patterns of susceptibility.

The MIC results for each isolate are listed in **Table 5.1** that includes EUCAST and literature stated breakpoints (shown in red text) and ECOFF values for each antibiotic (where available). The definition stated by EUCAST for the categories of susceptible/intermediate/resistant (S/I/R) is detailed in **Section 2.4**, however it should be noted that intermediate is now termed 'susceptible, increased exposure', but for the purposes of this study the term intermediate was used. An example of how an MIC result was determined as intermediate would be a result of 2 mg L^{-1} for CTX which has clinical breakpoints of $\leq$1 mg L^{-1} / >2 mg L^{-1}. As the result for resistant must be >2 the result of 2 mg L^{-1} would be classed as an intermediate result. Equally any result between two S/R breakpoints (with STREP an example that has clinical breakpoints of $\leq$8 mg L^{-1} / >64 mg L^{-1}) would be classed as an intermediate result.

Of the 24 antibiotics tested, only 11 returned a susceptibility result in line with what either EUCAST or the literature stated as a resistance breakpoint. Therefore, the results listed in **Table 5.1** only include the 11 antibiotics that had resistant results (with resistant results highlighted in yellow, intermediate results highlighted in blue and susceptible results as plain black text). Of the remaining 15 antibiotics where no resistance was found in any of the 47 isolates, the majority of the MICs were identical. Only three exceptions were noted where there was a slight increase of the MIC, and these included, CFQ where the MIC increased from ≤0.125 to 0.25 in isolates 968 and 969, ERT where the MIC increased from ≤0.032 to 0.064 in isolates 865, 869, 870, 872 and 969 and CIP where the MIC increased from ≤0.064 to 0.25 in 865 and 866. However, none of these slight increases in MICs in CFQ, ERT and CIP changed the end result and all isolates were still susceptible to these antibiotics. The study by Guillon et al. (2011) noted that ESCs may contribute to ertapenem resistance, but this is usually in combination with porin loss (Mammeri et al. 2008a; Guillon et al. 2011). Therefore, this slight increase in the MIC concentration seen with ERT, could be a result of overexpression of *ampC*. However, as the increase did not result in reduced susceptibility, this was merely an observation. **Table 5.2** details the results of the remaining 15 antibiotics not listed in **Table 5.1**.

Table 5.1: MIC results of the 47 *ampC* isolates, detailing the resistant results only which were found for only 11 of the 26 antibiotics tested.

Also detailed are the EUCAST and literature stated breakpoints (where applicable) and ECOFF values

Antibiotics →	**AMP**	**AMC**	**FOX**	**CAZ**	**CTX**	**CPD**	**CFQ**	**ATM**	**STREP**	**TET**	**SXT**
Breakpoints ↓	**Sensitive ≤ / Resistant > (mg L^{-1})**										
EUCAST	8	8	8/32	¼	½	1	-	¼	8/64	4/16	2/4
Literature Stated	-	-	-	-	-	-	0.25	-	-	-	-
ECOFF	8	-	≥32	0.5	0.25	1	0.125	0.25	16	-	0.5
Isolates ↓	**MIC →**										
99	512	8	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	256	64	2
125	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	4	≤2	1
127	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	8	4	≤2	1
152	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	4	≤2	≤0.5
157	4	8	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	4	≤2	1
183	4	≤4	0.5	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	4	≤2	1
209	4	≤4	0.5	≤0.5	≤0.25	0.5	≤0.125	≤0.25	4	≤2	1
253	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	4	≤2	0.5

295	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	4	≤2	1
308	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	4	≤2	1
309	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	≤4	≤2	≤0.5
320	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	4	≤2	≤0.5
326	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	4	≤2	1
408	4	≤4	0.5	≤0.5	≤0.25	1	≤0.125	≤0.25	4	≤2	1
410	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	≤4	≤2	1
431	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	4	≤2	1
486	512	64	2	≤0.5	≤0.25	0.5	2	≤0.25	128	128	32
495	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	≤4	≤2	≤0.5
508	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	4	≤2	1
514	4	≤4	1	≤0.5	≤0.25	≤0.25	≤0.125	≤0.25	≤4	≤2	1
518	128	64	32	16	2	64	≤0.125	4	≤4	≤2	1
536	4	≤4	2	≤0.5	≤0.25	0.25	≤0.125	≤0.25	≤4	≤2	1
582	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	≤4	≤2	1
825	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	4	2	1

826	128	64	32	8	2	64	≤0.125	1	4	≤2	1
828	4	8	2	1	≤0.25	0.5	≤0.125	≤0.25	≤4	≤2	≤0.5
833	4	≤4	1	≤0.5	≤0.25	0.5	≤0.125	≤0.25	4	≤2	≤0.5
854	512	128	64	32	4	64	≤0.125	8	8	≤2	≤0.5
855	256	64	32	16	2	64	≤0.125	2	4	≤2	≤0.5
856	512	128	64	32	4	64	≤0.125	8	4	≤2	≤0.5
858	256	64	32	8	2	64	≤0.125	2	4	≤2	1
859	256	64	32	8	2	64	≤0.125	2	≤4	≤2	1
860	256	64	32	8	2	64	≤0.125	2	≤4	≤2	1
862	256	64	32	8	2	64	≤0.125	2	≤4	≤2	≤0.5
863	256	64	32	8	2	64	≤0.125	2	4	≤2	≤0.5
864	16	64	32	8	2	64	≤0.125	2	≤4	≤2	≤0.5
865	256	64	32	8	2	64	≤0.125	2	≤4	64	≤0.5
866	256	64	32	8	2	64	≤0.125	2	≤4	64	≤0.5
867	256	64	32	8	2	64	≤0.125	2	≤4	≤2	≤0.5
868	256	64	32	8	2	64	≤0.125	2	≤4	≤2	≤0.5

869	256	64	32	8	2	64	≤0.125	2	≤4	≤2	1
870	256	64	32	16	2	64	≤0.125	4	4	≤2	≤0.5
871	256	64	32	16	2	64	≤0.125	2	4	≤2	≤0.5
872	512	128	64	32	4	64	≤0.125	4	8	≤2	≤0.5
887	256	64	32	8	2	64	≤0.125	2	≤4	≤2	≤0.5
968	512	128	64	32	4	64	0.25	2	64	128	2
969	512	128	64	16	4	64	0.25	4	64	128	2
ATCC25922	≤4	≤4		≤0.5	≤0.25	0.5	≤0.125	≤0.25	≤4	≤2	1

Footnote for Table 5.1: *Any resistant results are highlighted in yellow with intermediate results highlighted in blue. Any literature stated breakpoints that were utilised are shown in red text*

Table 5.2: The 15 antibiotics that had no resistance results in the MIC assays

Antibiotic	EUCAST Breakpoints	Majority Result	Notes on Result
IMP	2/4	≤0.5	All identical to majority result
ERT	0.5	≤0.032	Isolates 865, 869, 870, 872 and 969 all had a result of 0.064. The remaining isolates were all ≤0.032.
MER	2/8	≤0.064	All identical to majority result
GENT	2	≤1	All identical to majority result
NEO	8/16	≤4	All identical to majority result
APR	8/64	≤4	All identical to majority result
TOB	2	≤1	Isolates 295, 308, 431, 833, 863 all had results of 1 and 157 had a result of 2. The remaining isolates were all ≤1.

TIG	0.5	≤0.25	All identical to majority result
CIP	0.25/0.5	≤0.064	Isolates 865 and 866 both had a result of 0.25. The remaining isolates were all ≤0.064
ENR	2	≤0.032	Isolates 865 and 866 both had a result of 1, isolates 127, 157, 209, 308, 431, 582, 825 and 828 all had a result of 0.064, isolates 309, 410, 508, 514, 536 and 833 all had a result of 0.032 and the remaining isolates all had a result of ≤0.032
NAL	32	4	All identical to majority result
CHLOR	8	≤4	All identical to majority result
AZM	32	<8	All identical to majority result
COL	2	≤1	All identical to majority result
NIT	64	≤32	All identical to majority result

Footnote for Table 5.2*: Any literature stated breakpoints that were utilised are noted in this table in red text. The table details the antibiotic as an abbreviation, the EUCAST breakpoint as Sensitive ≤ / Resistant > (mg L^{-1}), the majority result and any notes on the result.*

5.2.3.1. The Levels of Beta-Lactam Resistance in the Block of 22 Isolates Presumptively Overexpressing *ampC*

From looking at the susceptibility pattern of beta-lactam resistance in all 47 of the isolates, the block of 22 isolates that included 518, 826, 854, 855, 856, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 887, 968 and 969, were considered as showing likely overexpression of *ampC*, due to their level of resistance. **Table 5.3** lists the antibiotics with the susceptible/resistant (S/R) breakpoint in brackets, followed by the relevant isolates from the block of 22, their MIC values and either how many concentrations above the S/R breakpoint these results were or if the result was susceptible or intermediate (S/I).

Resistance to AMP, AMC, FOX, CAZ and CPD was present in all 22 isolates. The majority of AMP MICs were the same at 256 mg L^{-1}, with five isolates with an MIC of 512 mg L^{-1} and a single isolate that had an MIC of 16 mg L^{-1}. The MICs for AMC were 64 mg L^{-1} within all but five isolates which had an MIC of 128 mg L^{-1}. The MICs of FOX were also very similar in most isolates, with MICs of 32 mg L^{-1} within all but five isolates which had an MIC of 64 mg L^{-1}. MICs for CAZ were more varied among the 22 isolates, with 13 with an MIC of 8 mg L^{-1}, five with an MIC of 16 mg L^{-1} and four with an MIC of 32 mg L^{-1}. The MICs for CPD were all identical at 64 mg L^{-1}.

The majority of the 22 isolates were either susceptible or had intermediate resistance to CTX and ATM. Only five isolates were resistant to CTX with an MIC of 4 mg L^{-1} and in the remaining isolates all results were intermediate and identical with MICs of 2 mg L^{-1}. The MICs of ATM however were more varied with only three isolates resistant to ATM with an MIC of 8 mg

L^{-1}, with the remaining isolates consisting of one susceptible isolate with an MIC of 1 mg L^{-1}, four intermediate isolates with an MIC of 4 mg L^{-1} with the remaining intermediate isolates all with an MIC of 2 mg L^{-1}.

All 22 isolates were susceptible to CFQ and all with an MIC of $\leq$0.125 mg L^{-1} except 968 and 969 with an MIC of 0.25 mg L^{-1}, which were two and one concentration(s) below the S/R breakpoint of $\leq$/>0.5 mg L^{-1} respectively.

The resistance in this group to the penicillin AMP and the 3rd generation cephalosporins CAZ and CPD, the reduced susceptibility to CTX and the monobactam ATM and significantly the high-level resistance to the cephamycin FOX and the beta-lactam/beta-lactamase inhibitor combination AMC along with the susceptibility to the 4th generation cephalosporin CFQ, all gave a good indication that this resistance mechanism was indeed likely to be overexpression of *ampC* rather than $bla_{\text{CTX-M}}$.

Two other isolates also had beta-lactam resistance, which included isolate 99 and 486. Isolates 99 and 486 both had high AMP MICs of 512 mg L^{-1} and isolate 486 also had an AMC MIC of 64 mg L^{-1}. The resistances in 99 and 486, were thought to be possibly the result of other beta-lactamase mechanisms such as bla_{TEM} or bla_{OXA}, as no 3rd generation cephalosporin resistance was present in either of these isolates, making $bla_{\text{CTX-M}}$ or an ESC unlikely. However, 486 was resistant to the 4th generation cephalosporin CFQ.

Table 5.3: Table detailing the number of times above the resistance breakpoint the MIC results were for the beta-lactam resistance in the block of 22 isolates. Beta-lactam antibiotics are listed above each result with the S/R breakpoint in brackets

Isolate(s)	MIC	Number of concentrations above resistance breakpoint or a S/I Result
Ampicillin (≤/>8 mg L^{-1})		
864	16 mg L^{-1}	One
518, 826, 855, 858, 859, 860, 862, 863, 865, 866, 867, 868, 869, 870, 871 and 887	256 mg L^{-1}	Five
854, 856, 872, 968 and 969	512 mg L^{-1}	Six
Amoxicillin/Clavulanic Acid (≤/>8 mg L^{-1})		
518, 826, 855, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871 and 887	64	Three
854, 856, 872, 968 and 969	128	Four

Ceftazidime (≤1/>4 mg L^{-1})		
826, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 869 and 887	8	One
518, 855, 870, 871 and 969	16	Two
854, 856, 872 and 968	32	Three
Cefotaxime (≤1/>2 mg L^{-1})		
855, 858, 859, 860, 862, 864, 867, 869 and 887	1	Susceptible result
518, 826, 863, 865, 866, 868, 870 and 871	2	Intermediate result
854, 856, 872, 968 and 969	4	One
Cefpodoxime (</>1 mg L^{-1})		
518, 826, 854, 855, 856, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 887, 968 and 969	8	Three

Aztreonam (≤1/>4 mg L^{-1})		
826	1	Susceptible
855, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 869, 871, 887 and 968	2	Intermediate result
518, 870, 872 and 969	4	Intermediate result
854 and 856	8	One

5.2.3.2. Changes to Phenotypic Susceptibility from Disc Assay Data Following MIC Assays

The resistance results following MIC assays revealed significant changes in phenotypic susceptibility when compared to the disc assay data supplied by EVAL farms. Therefore, a second heatmap was constructed to better show how the resistance had changed following MIC assays. The following text outlines where there were losses, decreases, gains and increases in resistance to specific antibiotics in each of the EVAL farms isolates and this is shown in the heatmap of **Figure 5.3.** **Figure 5.3** details the MIC results for all 47 isolates in the *ampC* group, which was constructed in the same way as the heatmap of disc assay data in **Figure 5.2**. This additional heatmap allowed for easy comparison between the two assay methods by identification of changes to resistances in the two heatmaps in **Figures 5.2** and **5.3**. What the heatmap in **Figure 5.3** also revealed was a clear indication of the isolates that were thought likely to be overexpressing *ampC*, as these were mostly clustered together in the bottom half of the group of isolates. **Figure 5.3** details the percentages of susceptible/intermediate/resistant isolates for both the disc assay data and the MIC data with the percentage decrease (displayed as a negative number) or gain at the bottom. Increases in resistance percentages from disc assay to MIC were seen for CAZ and TET at 4.3% and 2.1% respectively. There were far more decreases in resistance percentages however (with percentage decreases in brackets) for AMP (12.8%), AMC (6.4%), FOX (40.4%), CTX (74.5%), CPD (38.3%), ATM (10.6%), IMP (4.3%), STREP (23.4%), CIP (19.2%), NAL (23.4%), SXT (29.8%), CHLOR (8.5%), NIT (19.2%) and AZM (14.9%). The huge decrease in resistance percentage for CTX of 74.5% was in some part due

to an increase in intermediate resistance of 25.5%, however there was also a large increase in susceptibility of 48.9% and ATM had an increase in intermediate resistance of 23.4%. There were also decreases in intermediate resistance (with percentage decreases in brackets) for FOX (4.3%), CAZ (25.5%), CPD (2.1%), STREP (14.9%), CIP (6.4%), SXT (2.1) and NIT (2.1%).

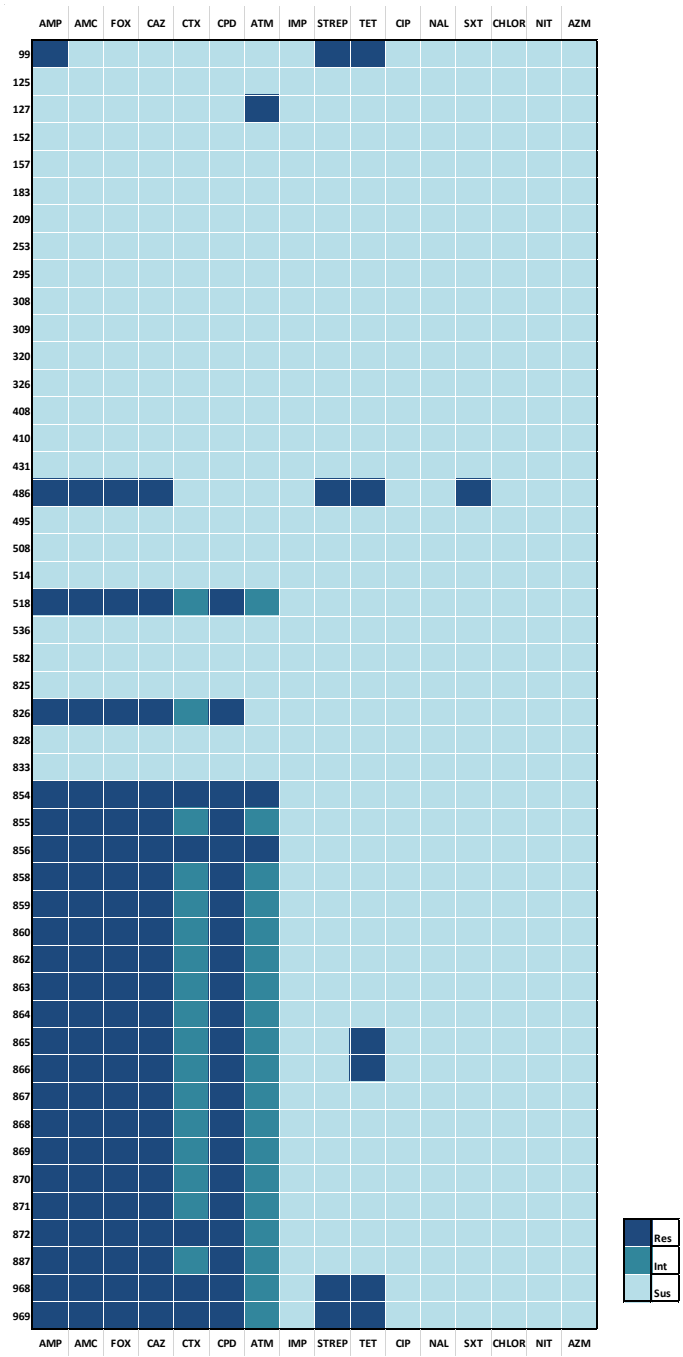
From this analysis it is clear that a large number of isolates that had originally shown resistance results following disc assays, as shown in the heatmap of **Figure 5.2** and detailed in **Section 5.2.2**, were now pan-susceptible to all the antibiotics tested including isolates 125, 152, 183, 209, 253, 295, 308, 309, 320, 326, 408, 410, 431, 495, 508, 514, 536, 582, 825, 828 and 833, which is detailed in **Figure 5.3**.

A large block of isolates also had a small decrease or increase in the number of resistances when the two heatmaps of **Figures 5.2** and **5.3** were compared, including 99, 127, 157 and 486. Isolate 99 lost resistance to CTX, CPD, IMP and CHLOR and was now only resistant to AMP, STREP and TET. Isolates 127 and 157 lost all the resistances noted in the disc assay but following MICs, isolate 127 had gained resistance to ATM. Isolate 486 also lost most of the resistance noted in the disc assay and was now only resistant to AMP, AMC, STREP, TET and SXT.

There was a block of 22 isolates including 518, 826, 854, 855, 856, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 887, 968 and 969, showing resistance with some intermediate resistance following MICs to the same group of beta-lactams including AMP, AMC, CAZ, CTX, CPD and ATM, which was very similar to the disc assay data. However, there were some small changes of note to the susceptibility between MICs and disc assay data in

this block of 22 isolates. Additions to resistance following MIC assays were seen in 854 which gained resistance to CPD, isolates 865 and 866 which gained resistance to TET, isolate 870 which gained intermediate resistance to ATM, isolate 887 which gained intermediate resistance to CTX and ATM and resistance to CAZ and finally isolate 969 which gained resistance to AMC. Finally, there was an increase from intermediate to resistant for CAZ in isolates 855, 859, 860, 862, 864, 865, 866, 869, 871, 887 and 968.

In this block of 22 isolates, several had also completely lost resistances and were now susceptible when compared to the disc assay data. Losses to resistance included STREP in 854, 855, 856, 867, 968 and 969, CIP in 867, SXT in 826, 854, 855 856 867 and 968, NIT in 856 and AZM in 854, 856 and 867. Losses to intermediate resistance included SXT in 969, CHLOR in 854, NIT in 855 and CIP in 969. There were also reductions amongst these 22 isolates where resistance had reduced to intermediate, including CTX in isolates 518, 826, 855, 858, 859, 860, 863, 864, 865, 866, 867, 868, 869, 870 and 871 and ATM in isolates 855, 968 and 969. This block of 22 isolates were thought likely to all be overexpressing *ampC* due to the resistance to both FOX and AMC in addition to 3rd generation cephalosporin and penicillin resistance and reduced susceptibility to ATM. The second heatmap in **Figure 5.3** gave a clearer indication of the 22 isolates likely to be encoding an overexpression of *ampC*, rather than the heatmap in **Figure 5.2**. The exact mechanism of the resistances in all of the 22 isolates with beta-lactam resistance was further investigated with genotypic data from WGS, along with the mechanisms for any other resistances seen in the MIC results including TET, STREP and SXT resistance.



	AMP	AMC	FOX	CAZ	CTX	CPD	ATM	IMP	STREP	TET	CIP	NAL	SXT	CHLOR	NIT	AZM
RES	24	23	23	23	5	22	3	0	4	6	0	0	1	0	0	0
%	51.1	48.9	48.9	48.9	10.6	46.8	6.4	0.0	8.5	12.8	0.0	0.0	2.1	0.0	0.0	0.0
INT	0	0	0	0	17	0	19	0	0	0	0	0	0	0	0	0
%	0.0	0.0	0.0	0.0	36.2	0.0	40.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SUS	23	24	24	24	25	25	25	47	43	41	47	47	46	47	47	47
%	48.9	51.1	51.1	51.1	53.2	53.2	53.2	100.0	91.5	87.2	100.0	100.0	97.9	100.0	100.0	100.0

Figure 5.3: Heatmap of resistance profiles of 47 isolates in the *ampC* group, produced from MIC assay data. The number of Resistant (RES), Intermediate Resistant (INT) and Susceptible (SUS) results along with the representative percentages are detailed along the bottom of the heatmap

Disc assay resistant/intermediate/susceptible numbers and percentages

	AMP	AMC	FOX	CAZ	CTX	CPD	ATM	IMP	STREP	TET	CIP	NAL	SXT	CHLOR	NIT	AZM
RES	30	26	42	21	40	40	8	2	15	5	9	11	15	4	9	7
%	63.8	55.3	89.4	44.7	85.1	85.1	17.0	4.3	31.9	10.6	19.2	23.4	31.9	8.5	19.2	14.9
INT	0	0	2	12	5	1	8	0	7	0	3	0	1	0	1	0
%	0	0	4.3	25.5	10.6	2.1	17.0	0	14.9	0	6.4	0	2.1	0	2.1	0
SUS	17	21	3	14	2	6	31	45	25	42	35	36	31	43	37	40
%	36.2	44.7	6.4	29.8	4.3	12.8	66.0	95.8	53.2	89.4	74.5	76.6	66.0	91.5	78.7	85.1

MIC resistant/intermediate/susceptible numbers and percentages

	AMP	AMC	FOX	CAZ	CTX	CPD	ATM	IMP	STREP	TET	CIP	NAL	SXT	CHLOR	NIT	AZM
RES	24	23	23	23	5	22	3	0	4	6	0	0	1	0	0	0
%	51.1	48.9	48.9	48.9	10.6	46.8	6.4	0.0	8.5	12.8	0.0	0.0	2.1	0.0	0.0	0.0
INT	0	0	0	0	17	0	19	0	0	0	0	0	0	0	0	0
%	0	0	0	0	36.2	0	40.4	0	0	0	0	0	0	0	0	0
SUS	23	24	24	24	25	25	25	47	43	41	47	47	46	47	47	47
%	48.9	51.1	51.1	51.1	53.2	53.2	53.2	100.0	91.5	87.2	100.0	100.0	97.9	100.0	100.0	100.0

Percentage loss or gain of resistant/intermediate/susceptible between disc assay and MIC assay

	AMP	AMC	FOX	CAZ	CTX	CPD	ATM	IMP	STREP	TET	CIP	NAL	SXT	CHLOR	NIT	AZM
RES	-12.8	-6.4	-40.4	4.3	-74.5	-38.3	-10.6	-4.3	-23.4	2.1	-19.2	-23.4	-29.8	-8.5	-19.2	-14.9
INT	0	0	-4.3	-25.5	25.5	-2.1	23.4	0	-14.9	0.0	-6.4	0	-2.1	0	-2.1	0
SUS	12.8	6.4	44.7	21.3	48.9	40.4	-12.8	4.3	38.3	-2.1	25.5	23.4	31.9	8.5	21.3	14.9

Figure 5.4: The resistant/intermediate/susceptible results and percentages from both the disc assays and MIC assays. Along the bottom are listed the percentage differences between the two assays, which was calculated by subtracting the disc assay percentage from the equivalent MIC percentage, with any decreases displayed as a negative figure

5.3. *ampC* GENOTYPIC DATA

All isolates within the *ampC* group were sequenced via both MinION (ONT) long and Illumina short read platforms (as per **Section 2.7.3)** with hybrid assembly as detailed in **Section 2.8.1**. In the subsequent sections, isolates within the group that were overexpressing *ampC* were identified and additional resistance genes, virulence genes and plasmids were characterised along with the identification of ST through MLST. A whole genome phylogeny was also conducted as per **Section 2.8.7** and a small subset of isolates with the same ST were subjected to SNP analysis using Snippy as per **Section 2.8.8**.

It was decided for the purposes of this study that the *ampC* encoded by *E. coli* K12 MG1655 (Accession Number: U00096) would be utilised as the WT sequence control, when identifying any *ampC* promoter mutations within the 47 *E. coli* isolates.

5.3.1. Mutations Associated with Overexpression of Chromosomal *ampC*

Mutations at positions -42, -32, -18 and -11 along with bp insertions between -13 and -14 at positions -13.1 and -13.2, have been frequently described to produce the strong *ampC* promoter and are often associated with various polymorphisms at positions -88, -82, -1, +58, +70 and +81: and mutations within the attenuator region between +17 and +37 have also been described (Caroff et al. 1999b; Forward et al. 2001; Siu et al. 2003a; Mulvey et al. 2005a; Tracz et al. 2005; Corvec et al. 2007; Yu et al. 2009b; Peter-Getzlaff et al. 2011a; Haenni et al. 2014e). **Figure 5.5** details the WT *ampC* region of MG1655 and

demonstrates exactly where the mutation positions may occur in relation to the promoters, spacer, attenuator and start of the coding region of *ampC*, with the variation positions of potential base changes denoted in red with the position number above.

Depending on the position of the mutation, promoter mutations can either mutate the existing WT promoter to be closer in resemblance to the consensus promoter or displace the WT promoter to create a new promoter box identical to the consensus promoter. Mutations to the existing WT promoter include a **T→A** at position -32, changing the -35 promoter from TTG**T**CA to TTG**A**CA and a **C→T** at position -11, changing the -10 promoter from TA**C**AAT to TA**T**AAT (base changes are highlighted in bold). Mutations that displace the WT promoter to create a new promoter include **C→T** at position -42 creating the new promoter **T**TGACA and **G→A** at position -18 creating the new promoter T**A**TAAT (base changes are highlighted in bold). In addition to promoter changes, the insertion of 1-2 bases at positions -13.1 and -13.2, results in the spacer sequence increasing from 16 bp to 17 or 18 bp. (Siu et al. 2003a; Mulvey et al. 2005a; Haenni et al. 2014e). Mutations have also been reported in the attenuator region which is responsible for regulating the *amp* operon expression levels. The attenuator is a gene regulatory region, which acts as a terminator of transcription due to the formation of an mRNA-stem loop (Turnbough 2019). Mutations in the attenuator region of *ampC* are believed to allow greater read-through via the destabilisation of the stem loop structure (shown in **Figure 5.6**) (Siu et al. 2003b; Mulvey et al. 2005a). Forward et al. (2001) found that mutations that shortened the RNA hairpin, resulted in a reduction in thermodynamic stability. Unfortunately, site directed mutagenesis

in this area is difficult, as a result of the stem-loop structure, and therefore this has been a challenging hypothesis to investigate (Tracz et al. 2005).

Figure 5.5: The 145 bp promoter region of the WT sequence from *E. coli* K12 MG1655 detailing the promoters, spacers and attenuator and the first 33 bp (11 amino acid residues) of the coding region of *ampC*. Mutation location numbers are above with bp substitution locations denoted in red

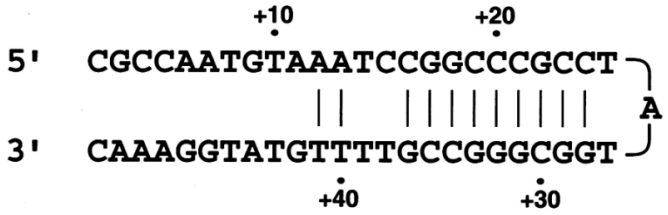


Figure 5.6 The stem-loop structure (also known as a hairpin) formed by the attenuator in *ampC*. Adapted from (Siu et al. 2003)

5.3.2. PCR of Promoter and Attenuator Regions of *ampC*

Initial screenings for mutations in the promoter and attenuator regions of *ampC* were conducted on 40 of the isolates within the *ampC* group and the controls BCC2 and ATCC25922 using PCR (**Section 2.6.7**), followed by Sanger sequencing (**Section 2.9.1**) of the PCR products to identify the locations of the mutations. As outlined in the introduction of this Chapter however, isolate 348 was discounted from further analysis as it was found to be a *Citrobacter* spp. and the additional 8 isolates had not been added to the group prior to PCR analysis, therefore 348 is present but the additional 8 are absent on the gel image in **Figure 5.7**. The primers used within this study are listed in **Table 2.6** and were taken from the study by Peter-Getzlaff et al. (2011c). Crude DNA preps were used as the template DNA (**Section 2.5.1**) and PCR conditions are detailed in **Section 2.6.7**. As detailed in **Figure 5.7**, there was a PCR product of the expected size of 271 bp for all isolates. However, both isolate 183 and ATCC25922 had non-specific bands at around 380 bp and 700 bp. Isolate 183 also had what appeared to be two non-specific bands combined at around 400-450 bp. Even though 183 gave multiple bands the PCR product was taken forward for further analysis as detailed in the next section. The additional bands in 183 and ATCC25922, could have been due to incomplete binding of the primer or mismatched sequences within the genome sequence. As all isolates were taken forward for WGS, PCRs for isolates with multiple bands were not repeated.

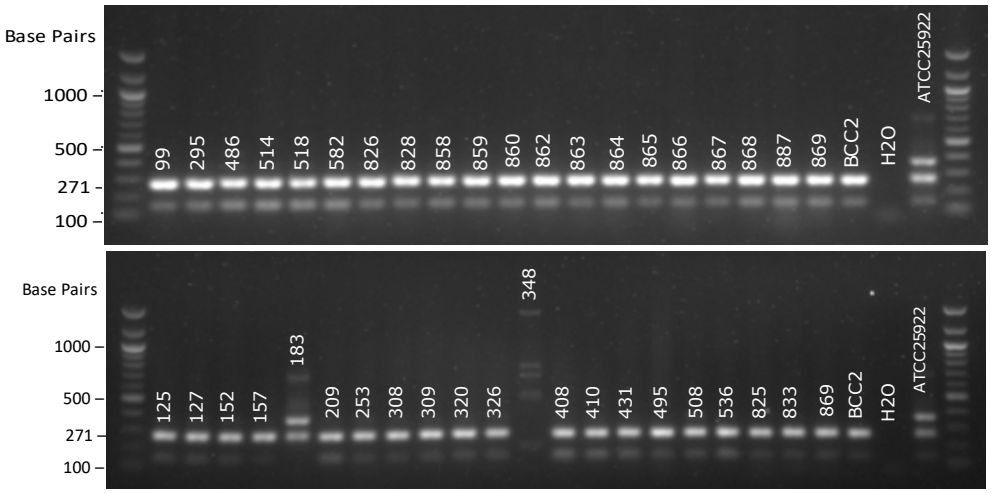


Figure 5.7: *ampC* PCR products from selected isolates with *ampC* overexpression phenotype. Gel electrophoresis images showing the 271 bp *ampC* amplimers. PCR products were resolved on a 1% TAE agarose gel with a 100 bp NEB quickload ladder (New England Biolabs, (UK) Ltd) at 85 V for 45 minutes. Isolates BCC2, 869 and ATCC25922 were utilised as positive controls with water utilised as a negative control

5.3.3. *ampC* Mutation Types

PCR products purified using a NEB T1030 Monarch® PCR & DNA Cleanup Kit (NEB, UK) (**Section 2.5.2**) from the 39 isolates along with BCC2 (Ibrahim et al. 2016) and ATCC25922, were sent for Sanger sequencing (Eurofins, Germany) (**Section 2.8.1**) to determine mutation types of overexpression of *ampC*. All *ampC* PCR products that were sequenced, returned successful sequence data that was then aligned against the WT *ampC* promoter coding region from the sequence of *E. coli* K12 MGl655 (Accession Number: U00096) and the mutation types were then compared to what has been noted in the literature such as the studies by Mulvey et al. (2005b) and Peter-Getzlaff et al. (2011b). Isolate 774 was also included in this analysis as an additional

control, by utilising the *ampC* promoter region sequence taken from the whole genome PacBio sequence.

From the group of 39 isolates, 36 had the G→A at position -18, with 14 of these having the additional mutation C→T at position -42 and 3 isolates had no *ampC* mutations. All but the 3 isolates with no *ampC* mutations, had an additional mutation of C→T at position -1 but this has not been reported to have an effect on the overexpression of *ampC*. As detailed in **Section 5.3.1**, the -42 and -18 mutations are associated with the displacement of the WT promoter sequences and the creation of two new -35 and -10 promoter boxes as shown in **Figure 5.1**. However, when comparing the MIC results in **Section 5.2.3** to the isolates with only the -18 mutation, it revealed that the -18 mutation alone was not sufficient to produce an overexpression of *ampC* phenotype which would show reduced susceptibility to AMP, AMC, CTX, CAZ, CPD and ATM. However, the 22 isolates with the mutations at -42 and -18, which according to previous studies by Mulvey et al. (2005b) and Peter-Getzlaff et al. (2011b) should be overexpressing *ampC*, would appear to match the reduced susceptibility phenotype, as the MIC results in **Section 5.2.3** reflect this resistance mechanism.

5.4. WHOLE GENOME SEQUENCING OF *ampC* ISOLATES

WGS of all 47 isolates placed into the *ampC* group was conducted via MinION (ONT) long read and Illumina short read sequencing with hybrid assembly as described in **Section 2.7.3**. The remaining 8 isolates that had been placed into the *ampC* group following PCR testing for bla_{CTX} in **Chapter 3** (**Section 3.2.2**) were also analysed for *ampC* mutation genotyping via the WGS. In addition to confirming the *ampC* mutation type, obtaining WGS of all isolates within the *ampC* group allowed for further investigation to be conducted, including a whole genome phylogeny of all the 47 isolates within the *ampC* group followed by MLST analysis. The whole genome phylogeny and MLST was conducted to investigate whether there was the potential for the spread of clonal groups of ESCs on this particular farm and whether they were associated with the same ST or multiple STs. This did not assume that overexpression of *ampC* was associated with a specific ST but rather used the latter as a marker to investigate how much of a part clonality was playing in the dissemination of overexpression of *ampC* on this specific farm. In addition, WGS also allowed for MLST to be conducted using MLST 2.0 (**Section 2.8.6.1**), the identification of the resistance genes using ResFinder 4.1 (**Section 2.8.6.2**), virulence factors using VirulenceFinder 2.0 (**Section 2.8.6.3**) and plasmid replicon typing using PlasmidFinder 2.1 (**Section 2.8.6.4**).

5.4.1. *ampC* Mutation Type in Remaining Eight Isolates Added to the *ampC* Group

The full *ampC* region including the promoters, spacer sequence, attenuator and start of the *ampC* coding region (as shown in **Figure 5.5**), was analysed from the WGS in the additional 8 isolates added to the *ampC* group from the bla_{CTX} group, which included 854, 855, 856, 870, 871, 872, 968 and 969. It was found that all were encoding identical mutations to the 14 isolates found to be encoding an overexpression of *ampC* genotype detailed in **Section 5.3.3**, which included the G→A at position -18, C→T at position -42 and C→T at position -1. As was described in **Section 5.3.3**, the mutations at -42 and -18 are associated with the creation of two new stronger -35 and -10 promoters, which results in overexpression of *ampC*. This additional analysis using WGS, resulted in a total of 22 isolates being confirmed as overexpressing *ampC* and would account for the phenotypic results seen following MIC assays as per **Section 5.2.3**.

Figure 5.8 shows the *ampC* promoter region of the 22 isolates encoding an overexpression of *ampC* genotype with the new -35 and -10 promoter and new spacer detailed. The mutations at positions -42 and -18 and the additional mutation at -1 are all denoted in blue.

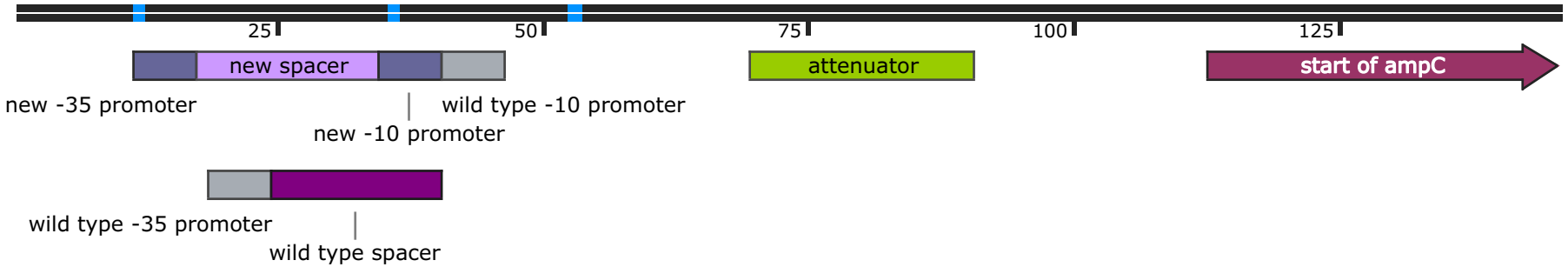


Figure 5.8: The *ampC* promoter region of the overexpression of *ampC* mutation type found in the 21 isolates 518, 826, 854, 855, 856, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 870, 871, 872, 887, 968 and 969 and the control 869. Mutations at -42 and -18 which created the new -35 and -10 promoter boxes and a new spacer and an additional mutation at -1 are all denoted in blue

5.4.2. Assembly Statistics and Contigs with Plasmids from *ampC* Isolates

The assembly statistics and open reading frame numbers were calculated for all the sequenced isolates in the *ampC* group as per **Section 2.8.1**, using the free software Bandage (available at: https://rrwick.github.io/Bandage) and the online ORFinder software (available at: https://www.ncbi.nlm.nih.gov/orffinder/). PlasmidFinder 2.1 was utilised to locate contigs containing plasmids as per **Section 2.8.6.4**. All the assembly statistics, open reading frame numbers are detailed in **Table 5.4** which also includes the contig number, contig numbers containing plasmids, whether the plasmids were complete, overall genome size, overall %GC content, N50 number and whether the chromosome was complete. The definition of the N50 number was described in **Section 3.4.1** of **Chapter 3**.

As can be seen in **Table 5.4** many of the hybrid assemblies resulted in only 1-6 contigs and there were many complete chromosomes and plasmids, making resistance and virulence gene locating much easier. In addition, plasmids were often found complete within a single contig. Therefore, genes found located in contig 1 were thought very likely to be chromosomally-encoded and this was confirmed with manual investigation of the WGS. Genes located in any other contig but contig 1, were cross referenced to the PlasmidFinder results to confirm whether that contig was plasmid sequence and again this was confirmed with manual investigation of the WGS.

Table 5.4: Assembly statistics for the 47 sequenced isolates in the *ampC* group sequenced via Illumina short read and MinION (ONT) long read sequencing with hybrid assembly

Isolate	Number of ORFs	Total Number of Contigs	Contig Number Containing a Plasmid (replicon and plasmid size (bp))	Complete Plasmid	Overall Genome Size (bp)	Overall %GC Content	N50 number	Complete Chromosome
99	4,771	3	Contig 2 (IncI1 109,925 bp)	Yes	5,087,489 bp	50.8%	4,974,203 bp	Yes
125	4,601	3	Contig 2 (IncI2 58, 676 bp) Contig 3 (IncX4 32, 451 bp)	Yes Yes	4,913,426 bp	50.5%	4,822,299 bp	Yes
127	4,788	26	Contig 6 (IncFIA/IB/IC 87,298 bp)	Yes	4,900,569 bp	50.8%	953,457 bp	No
152	4,646	10	Contig 2 (IncFIA/IB/IC 98,442 bp)	Yes	4,997,026 bp	50.7%	4,881,799 bp	Yes
157	4,449	5	Contig 2 (IncFIA/IB/IC 72,141 bp)	Yes	4,781,825 bp	50.5%	4,703,482 bp	Yes
183	4,447	2	None	n/a	4,896,262 bp	50.5%	4,894,711 bp	Yes
209	4,645	18	None	n/a	4,835,703 bp	50.8%	2,482,222 bp	No
253	4,420	99	None	n/a	4,798,034 bp	50.7%	252,041 bp	No

295	4,615	4	Contig 2 (IncI2 58,667 bp) Contig 3 (IncFIA/IB/IC 57,294 bp)	Yes Yes	4,942,218 bp	50.5%	4,822,747 bp	Yes
308	4,448	2	Contig 2 (IncFIA/IB/IC 65,454 bp)	Yes	4,801,829 bp	50.6%	4,736,375 bp	Yes
309	4,508	8	None	n/a	4,874,422 bp	50.6%	4,861,701 bp	Yes
320	5,147	5	Contig 2 (IncFII/IA 115,030 bp) Contig 3 (IncFIB 111,486 bp) Contig 4 (IncFII/IB 95,969 bp)	Yes Yes Yes	5,414,437 bp	50.5%	5,090,484 bp	Yes
326	4,733	6	Contig 2 (IncFIA/IB/IC 102,044 bp)	Yes	5,062,331 bp	50.8%	4,948,049 bp	Yes
408	4,614	4	Contig 2 (IncFIA/IB/IC 129,980 bp)	Yes	4,868,027 bp	50.7%	4,733,216 bp	Yes
410	4,614	4	Contig 2 (IncFIB/IC 84,085 bp)	Yes	4,939,845 bp	50.6%	4,850,060 bp	Yes
431	4,653	24	Contig 5 (IncFIB 111,420 bp) Contig 6 (IncFII/Y 69,141 bp)	Yes No	4,968,782 bp	50.6%	3,157,702 bp	No
486	4,735	2	None	n/a	5,117,830 bp	50.6%	5,116,568 bp	Yes

495	4,988	6	Contig 2 (IncI1 94,354 bp) Contig 3 (IncFIC 71,727 bp) Contig 4 (IncFIB/IC 70,202 bp)	Yes Yes Yes	5,264,353 bp	50.6%	5,024,553 bp	Yes
508	4,709	12	Contig 2 (IncI1 93,771 bp)	Yes	5,024,182 bp	50.7%	4,811,402 bp	Yes
514	4,810	1	None	n/a	5,223,907 bp	50.7%	5,223,907 bp	Yes
518	4,592	3	Contig 2 (IncFIA/IB/IC 53,248 bp)	Yes	4,895,323 bp	50.7%	4,840,683 bp	Yes
536	4,747	2	Contig 2 (IncFIA/IB/IC 101,914 bp)	Yes	5,086,424 bp	50.8%	4,984,510 bp	Yes
582	4,534	3	Contig 2 (IncFIA/IB/IC 106,577 bp)	Yes	4,909,379 bp	50.8%	4,801,442 bp	Yes
825	4,729	2	Contig 2 (IncFIA/IB/IC 192,985 bp)	Yes	5,048,981 bp	50.6%	4,822,996 bp	Yes
826	4,658	3	Contig 2 (IncFIA/IB/IC 53,138 bp)	Yes	4,947,988 bp	50.6%	4,892,547 bp	Yes
828	4,733	6	Contig 2 (IncFIA/IB/IC 203,832 bp)	Yes	5,050,353 bp	50.6%	4,836,285 bp	Yes
833	4,514	6	Contig 2 (IncFIB/IC/R 89,318 bp)	Yes	4,858,095 bp	50.6%	4,754,738 bp	Yes
854	4,634	6	Contig 2 (IncFIA/IB/IC 53,247 bp)	Yes	4,931,836 bp	50.7%	4,865,382 bp	Yes
855	4,646	3	Contig 2 (IncFIA/IB/IC 53,246 bp)	Yes	4,940,827 bp	50.7%	4,883,783 bp	Yes
856	4,636	3	Contig 2 (IncFIA/IB/IC 53,243 bp)	Yes	4,929,540 bp	50.7%	4,874,913 bp	Yes

858	4,661	4	Contig 2 (IncFIA/IB/IC 53,465 bp)	Yes	4,949,964 bp	50.6%	4,892,426 bp	Yes
859	4,673	12	Contig 2 (IncFIA/IB/IC 52,919 bp)	Yes	4,954,942 bp	50.6%	4,845,664 bp	Yes
860	4,632	10	Contig 2 (IncFIA/IB/IC 51,701 bp	No	4,933,056 bp	50.7%	4, 865, 489 bp	Yes
862	4,632	3	Contig 2 (IncFIA/IB/IC 53,247 bp)	Yes	4,929,734 bp	50.7%	4,874,944 bp	Yes
863	4,659	2	Contig 2 (IncFIA/IB/IC 53,264 bp)	Yes	4,945,979 bp	50.6%	4,892,715 bp	Yes
864	4,657	8	Contig 2 (IncFIA/IB/IC 53,241 bp)	Yes	4,943,619 bp	50.7%	4,882,904 bp	Yes
865	4,645	2	Contig 2 (IncFIA/IB/IC 52,814 bp)	Yes	4,936,354 bp	50.7%	4,883,540 bp	Yes
866	4,644	2	Contig 2 (IncFIA/IB/IC 53,247 bp)	Yes	4,936,921 bp	50.7%	4,883,674 bp	Yes
867	4,661	3	Contig 2 (IncFIA/IB/IC 51,897 bp)	Yes	4,948,919 bp	50.7%	4,895,454 bp	Yes
868	4,648	13	Contig 4 (IncFIA/IB/IC 53,239 bp)	Yes	4,934,320 bp	50.7%	2,912,719 bp	No
869	4,643	4	Contig 3 (IncFIA/IB/IC 53,139 bp)	Yes	4,939,109 bp	50.7%	4,544,496 bp	Yes
870	4,658	4	Contig 3 (IncFIA/IB/IC 53,247 bp)	Yes	4,945,704 bp	50.6%	4,620,359 bp	Yes
871	4,650	3	Contig 2 (IncFIA/IB/IC 53,247 bp)	Yes	4,943,192 bp	50.6%	4,883,592 bp	Yes
872	4,663	4	Contig 3 (IncFIA/IB/IC 53,237 bp)	Yes	4,949,119 bp	50.6%	4,623,396 bp	Yes

887	4,649	5	Contig 2 (IncFIA/IB/IC 53,251 bp)	Yes	4,942,647 bp	50.6%	4,847,438 bp	Yes
968	4,869	3	None	n/a	5,224,556 bp	50.7%	5,222,334 bp	Yes
969	4,869	1	None	n/a	5,235,760 bp	50.6%	5,235,760 bp	Yes

Footnote for Table 5.4: *Assembly statistic included the number of ORFs, total number of contigs, contig number containing a plasmid with plasmid replicon and size (bp), whether the plasmid was complete, overall genome size (bp), %GC content, N50 number and whether chromosome was complete*

5.4.3. Additional Resistance Genes of the 47 *ampC* Isolates

ResFinder 4.1 (**Section 2.8.6.2**) was utilised to identify the resistance genes located in the 47 isolates in the *ampC* group. In addition, the contig where the gene was encoded was located and confirmed as either chromosomally or plasmid- located. The phenotypic resistance to AMP, AMC, FOX, CAZ, CTX, CPD and ATM in the 22 isolates described in **Sections 5.3.3** and **5.4.1**, was thought extremely likely to be a consequence of the association of those 22 strains with overexpression of *ampC*. The resistance mechanism of any other isolates displaying phenotypic resistance on the heatmap in **Figure 5.3** were also investigated, including resistance to AMP, STREP and TET in isolate 99, resistance to ATM in isolate 127, resistance to AMP, AMC, FOX, CAZ, CPD, STREP, TET and SXT and intermediate resistance to CTX and ATM in isolate 486, TET resistance in isolates 865 and 866 and resistance to STREP and TET in isolates 968 and 969. **Table 5.5** details the resistance genes, resistance gene full name, description, which isolates were encoding them and whether they were encoded on the chromosome or on plasmids. The majority of the resistance genes were all located on contig 1 and therefore were assumed to be chromosomally-encoded, which was then confirmed with manual searches of the WGS. Four chromosomally-encoded aminoglycoside type resistance genes were found that included *aac2'* in 127, a 2′-N-acetyltransferase which confers resistance to kasugamycin, *ant3''* in 486, a 3"-nucleotidyltransferase which confers resistance to spectinomycin and streptomycin and in isolates 486, 968 and 969, an aminoglycoside phosphotransferase *aph3''* and an aminoglycoside O-phosphotransferase *aph6* which both confer resistance to streptomycin (with a full description of the mechanism of these gene groups described in **Chapter**

3, **Section 3.2.1**). In isolate 486 only, the narrow spectrum beta-lactamase *bla*$_{\text{OXA-1}}$, the dihydrofolate reductase *dfrA36* conferring trimethoprim resistance, the florfenicol efflux pump gene *floR* conferring resistance to florfenicol, the efflux pump gene *qacE* conferring resistance to quaternary ammonium compounds, the dihydropteroate synthase *sul1* conferring resistance to sulphonamides and the tetracycline efflux gene *tetA* conferring tetracycline resistance were located and were all chromosomally-encoded. Finally, *fosA7* a fosfomycin thiol transferase was found chromosomally-located in 508. Only isolate 99 had plasmid-encoded resistance genes which were located on contig 2 which was found to contain an IncI1 plasmid. The resistance genes encoded by the IncI1 plasmid of isolate 99 included *aph3'*, *aph6*, the beta-lactamase *bla*$_{\text{TEM-1}}$ conferring penicillin resistance, *floR*, *sul2* a dihydropteroate synthase conferring resistance to sulphonamides and *tetA* and *tetC* (also known as *tetR*) a tetracycline/H+ antiporter conferring tetracycline resistance.

When comparing the resistance genes to the phenotypic results, these results appear to reflect what was found in the MIC assays. Isolate 99 had resistance to AMP, STREP and TET following MICs and the presence of *bla*$_{\text{TEM}}$ would likely account for the AMP resistance with the aminoglycoside genes *aph3'* and *aph6* accounting for the STREP and the *tetAC* genes accounting for the TET resistance. However even though there was a *floR* gene found, there was no resistance to CHLOR in the MIC assays. The MIC assays also did not test a sulphonamide alone and instead the combination SXT was utilised and so it was not possible to assess whether the presence of *sul2* in isolate 99 was producing phenotypic sulphonamide resistance. In isolate 127 the resistance gene *aac2'*did not appear to confer resistance to any of the aminoglycosides in

the panel of antibiotics tested in the MIC assays and no mechanism could be found for the resistance to ATM. In isolate 486 there was phenotypic resistance to AMP, AMC, CPD, STREP, TET and SXT following MIC assays. The AMP and CPD resistances were most likely a consequence of the $bla_{\text{OXA-1}}$ which could also possibly account for the AMC resistance. The presence of the aminoglycoside resistance genes *ant3''*, *aph3''* and *aph6* most likely resulted in the STREP resistance seen in 486 and the tetracycline resistance was likely due to the *tetA* with the SXT resistance most probably due to the *drfA36* and *sul1* resistance genes. As with isolate 99, *floR* did not confer resistance to CHLOR in 486 and this would suggest *floR* alone is not sufficient for CHLOR resistance. As no quaternary ammonium compounds were tested in the phenotypic assays, the resistance produced by *qacE* in 486 was not assessed. In addition, fosfomycin was not included in the MIC antibiotic panel and therefore the phenotypic resistance produced by *fosA7* in isolate 508, was also not assessed.

Table 5.5: Resistance genes located in the WGS of the 47 *ampC* isolates with results obtained from ResFinder

Resistance Genes	Full Name	Description	Isolates	Location Encoded
aac2'	2′-N-Acetyltransferase	Kasugamycin resistance	127	Chromosome
ant3"	3"-nucleotidyltransferase	Spectinomycin and streptomycin resistance	486	Chromosome
aph3"	Aminoglycoside phosphotransferase	Streptomycin resistance	99, 486, 968 and 969	99 – IncI1 Plasmid 486, 968 and 969 – Chromosome
aph6	Aminoglycoside O-phosphotransferase	Streptomycin resistance	99, 486, 968 and 969	99 – IncI1 Plasmid 486, 968 and 969 – Chromosome
$bla_{\text{OXA-1}}$	Beta-lactamase OXA-1	Narrow spectrum oxacillinase type beta-lactamase	486	Chromosome
$bla_{\text{TEM-1}}$	Beta-lactamase TEM-1	Penicillin resistance	99	IncI1 Plasmid
dfrA36	Dihydrofolate reductase	Trimethoprim resistance	486	Chromosome

floR	Florfenicol R	Efflux pump of florfenicol and chloramphenicol	99, 486	99 – IncI1 Plasmid 486 – Chromosome
fosA7	Fosfomycin thiol transferase	Fosfomycin resistance	508	Chromosome
qacE	Quaternary ammonium compound efflux pump	Quaternary ammonium compound resistance	486	Chromosome
sul1	Dihydropteroate synthase	Sulphonamide resistance	486	Chromosome
sul2	Dihydropteroate synthase	Sulphonamide resistance	99, 486, 968 and 969	99 – IncI1 Plasmid 486, 968 and 969 – Chromosome
tetA	Tetracycline resistance protein, class A	Tetracycline efflux	99	IncI1 Plasmid
tetB	Tetracycline resistance protein, class B	Metal-tetracycline/H+ antiporter	486, 968, 969	All on Chromosome
tetC (also known as *tetR*)	Tetracycline resistance protein, class C	Metal-tetracycline/H+ antiporter	99	IncI1 Plasmid

Footnote for Table 5.5*: Details resistance gene, full name of resistance gene, description, isolates and location encoded*

5.4.4. Whole Genome Phylogeny Tree for *ampC* Isolates

A whole genome phylogeny maximum likelihood tree shown in **Figure 5.9** was produced for all 47 of the isolates in the *ampC* group using IQtree v2.0, with annotation achieved using the iTOL v.5.7 (**Section 2.8.7**). The MLST (MLST 2.0 as per **Section 2.8.6.1**), plasmid carriage (PlasmidFinder 2.1 as per **Section 2.8.6.4**) and virulence genes (VirulenceFinder 2.0 as per **Section 2.8.6.3**) along with those isolates confirmed as overexpressing *ampC*, were all annotated on the tree shown in **Figure 5.9**. The whole genome phylogeny demonstrated there was a lot of diversity amongst the isolates placed into the *ampC* group with only a small number appearing to be either closely or clonally related. The group consisting of ST1308 was made up of the 21 isolates 320, 518, 866, 872, 859, 855, 856, 826, 860, 854, 868, 867, 871, 862, 887, 858, 865, 863, 864, 870 and 869, and mostly appeared to be either very closely related or clonal and this was further established through SNP analysis of this small group of 21 detailed in **Section 5.4.1.2**. The group of ST75 made up of the two isolates 968 and 969 also appeared to be closely related, but without SNP data this was merely an observation. These two ST groups ST1308 and ST75 appeared to be unrelated to each other but within both groups all but isolate 320 was confirmed as overexpressing *ampC*.

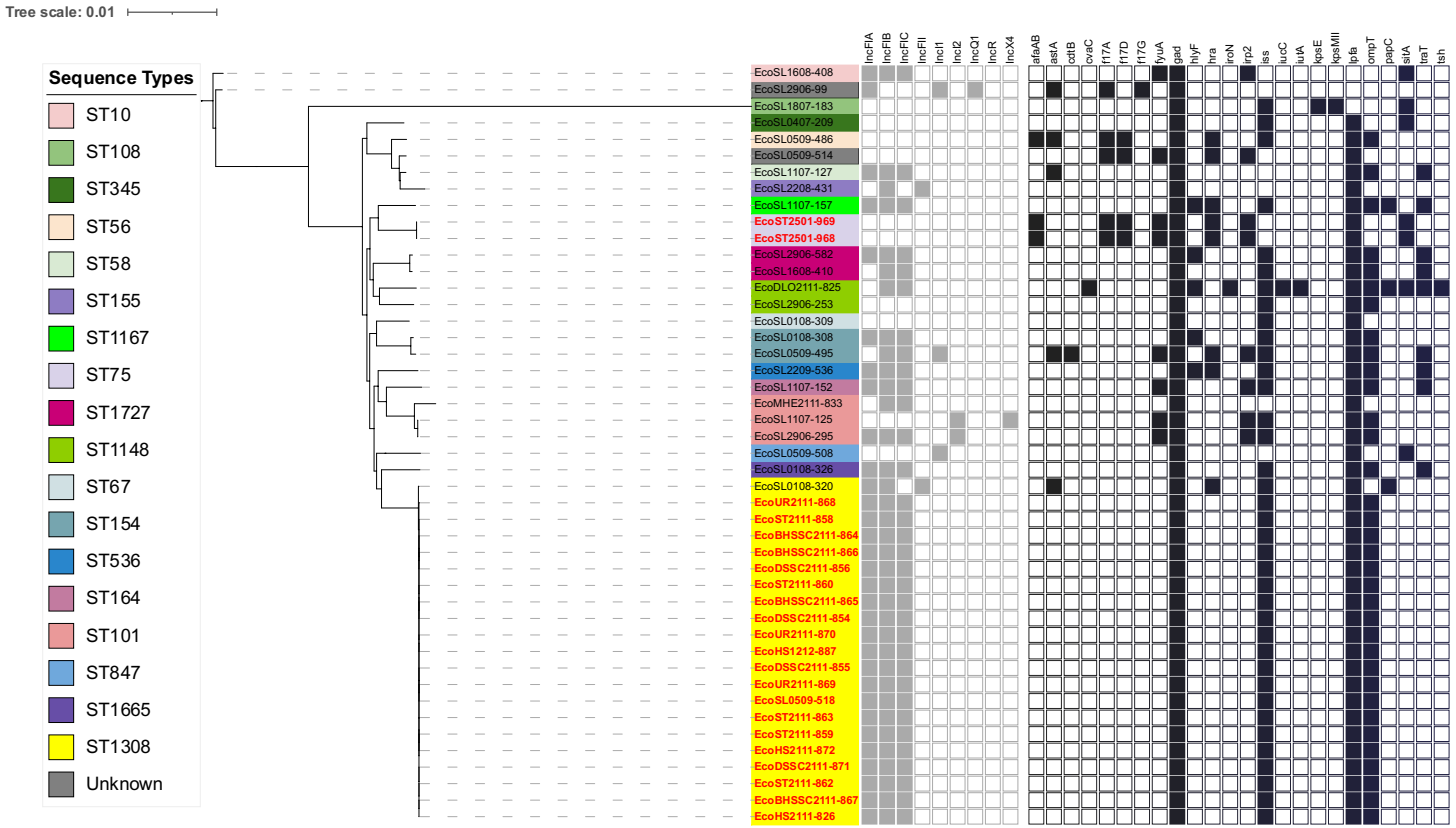


Figure 5.9: Whole genome phylogeny maximum likelihood tree showing the 47 isolates within the *ampC* group. Each ST is annotated as a different shade, which is noted in the key present on the tree and any isolates confirmed as overexpressing of *ampC*, have the isolate name written in red. Also shown to the right of the tree are the Inc groups of the plasmids (grey squares) within each isolate and the virulence genes (black squares), with a white square meaning negative for that specific gene carriage and a grey/black square a positive for that specific gene carriage

5.4.4.1. MLST

The MLST of all isolates placed into the *ampC* group was conducted using the MLST 2.0 from the CGE (**Section 2.8.6.1**). There was a wide variety of STs among the *ampC* group as detailed in the phylogenetic tree shown in **Figure 5.9**. STs with only one isolate represented included ST10, ST108, ST345, ST56, ST58, ST155, ST1167, ST67, ST536 and ST164. STs with more than one isolate represented included ST75, ST1727, ST1148, ST154, ST101 and ST1308. Within the isolates of the same ST, these always clustered together on the tree and a few appeared to be very closely related including ST75 consisting of 968 and 969. The group of 21 ST1308 isolates which appeared to be closely related were investigated further via SNP distance comparison using Snippy as per **Section 2.8.8**.

5.4.4.2. ST1308 A Small Clonal Expansion

A small number of isolates were found to be all ST1308 (n=21) and overexpressing *ampC* (n=20), therefore it was decided that SNP distance comparison (**Section 2.8.7**) should be conducted using Snippy, to assess whether these isolates were closely related or clonal and whether there was any correlation on this dairy farm between overexpression of *ampC* and relatedness of isolates of the same ST. **Figure 5.10** shows the SNP distance matrix of all 21 ST1308 isolates along with the reference genome EcoBHSSC2111-865, which was the WGS with the best coverage and assembly, selected from the ST1308 isolates (the selection of a reference genome is described in **Section 2.8.7**). **Figure 5.11** shows a SNP distance tree of the 21 ST1308 isolates, that was constructed using IQtree v2.0, with annotation achieved using the iTOL v.5.7.

Isolate 320, which was not over expressing *ampC*, was found to be within 431-561 SNPs of the other 20 isolates as shown in the SNP distance matrix of **Figure 5.10**, suggesting it was not closely related to them. In the remaining group of 20 isolates, which included 518, 866, 872, 859, 855, 856, 826, 860, 854, 868, 867, 871, 862, 887, 858, 865, 863, 864, 870 and 869, 518 was found to be within 8-16 SNPs of the other 19 isolates and the remaining 19 were all within only 5-1 SNPs of each other as shown in the SNP distance matrix in **Figure 5.10**. The SNP distance comparison (as per **Section 2.8.8**) suggested good evidence of clonality between the group of 19 and suggested 518 was very closely related to those 19.

On the SNP distance tree shown in **Figure 5.11**, 320 shares a common ancestor with the remaining isolates, but is not closely related to them and forms a separate clade from the rest of the group, as demonstrated by the first branch and internal node from the root (shown in red on F**igure 5.11**), which splits into the two branches which are green and blue on **Figure 5.11**. The green and blue branches are very long, suggesting a large amount of genetic variation has taken place, which was also demonstrated in the number of SNPs found between 320 and the rest of the group. The blue branch leads to a further small divergence into two clades which encompass isolate 518 (blue) and the remaining 19 isolates (yellow). These 19 are all present on the same branch as 19 separate leaves (yellow) in **Figure 5.11**, suggesting these were likely clonal. The length of the branches between 518 and the remaining 19, are very short suggesting 518 and the remaining 19 are closely related, which again was demonstrated by the low number of SNPs between these isolates. The sampling dates also varied between these isolates, with isolate 320 sampled earlier on 1st August 2017 than 518

which was sampled on 5th September 2017. Equally 320 and 518 were sampled earlier than the remaining 19, with 826, 854, 855, 856, 858, 859, 860, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871 and 872 all sampled on 21st November 2017 and 887 sampled on 12th December 2017. Even though there were a few weeks between the two samplings of the remaining 19, this group appeared to be all related and unaffected by the gap between sampling 887 in December and the earlier sampling of the 18 in November. The earlier sampling gap between 320, 518 and the remaining 19 however did appear to have possibly influenced the relatedness of these 21 isolates. This SNP distance comparison appeared to show there was some correlation between ST1308 and overexpression of *ampC* on this dairy farm and suggested there had been clonal spread.

	Ref	320	518	826	854	855	856	858	859	860	862	863	864	865	866	867	868	869	870	871	872	887
Ref	0	561	16	4	3	6	6	2	3	3	3	6	0	0	3	5	2	3	3	4	3	1
320	561	0	552	556	554	558	555	545	557	554	559	560	431	556	555	557	484	558	559	559	553	552
518	16	552	0	12	11	10	12	10	11	10	10	10	9	10	11	11	8	10	10	10	10	10
826	4	556	12	0	2	2	5	1	3	2	2	1	1	1	2	3	2	1	1	1	3	1
854	3	554	11	2	0	1	4	1	2	2	1	1	1	1	2	2	2	1	1	1	2	1
855	6	558	10	2	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
856	6	555	12	5	4	3	0	3	4	4	3	3	2	3	4	4	2	3	3	3	4	3
858	2	545	10	1	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
859	3	557	11	3	2	1	4	1	0	2	1	1	1	1	2	2	2	1	1	1	2	1
860	3	554	10	2	2	1	4	1	2	0	1	1	1	1	2	2	2	1	1	1	2	1
862	3	559	10	2	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
863	6	560	10	1	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
864	0	431	9	1	1	0	2	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
865	0	556	10	1	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
866	3	555	11	2	2	1	4	1	2	2	1	1	1	1	0	2	2	1	1	1	2	1
867	5	557	11	3	2	1	4	1	2	2	1	1	1	1	2	0	2	1	1	1	2	1
868	2	484	8	2	2	1	2	1	2	2	1	1	1	1	2	2	0	1	1	1	2	1
869	3	558	10	1	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
870	3	559	10	1	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
871	4	559	10	1	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0
872	3	553	10	3	2	1	4	1	2	2	1	1	1	1	2	2	2	1	1	1	0	1
887	1	552	10	1	1	0	3	0	1	1	0	0	0	0	1	1	1	0	0	0	1	0

Figure 5.10: SNP distance matrix for the 21 ST1308 isolates produced with Snippy version 4.6.0

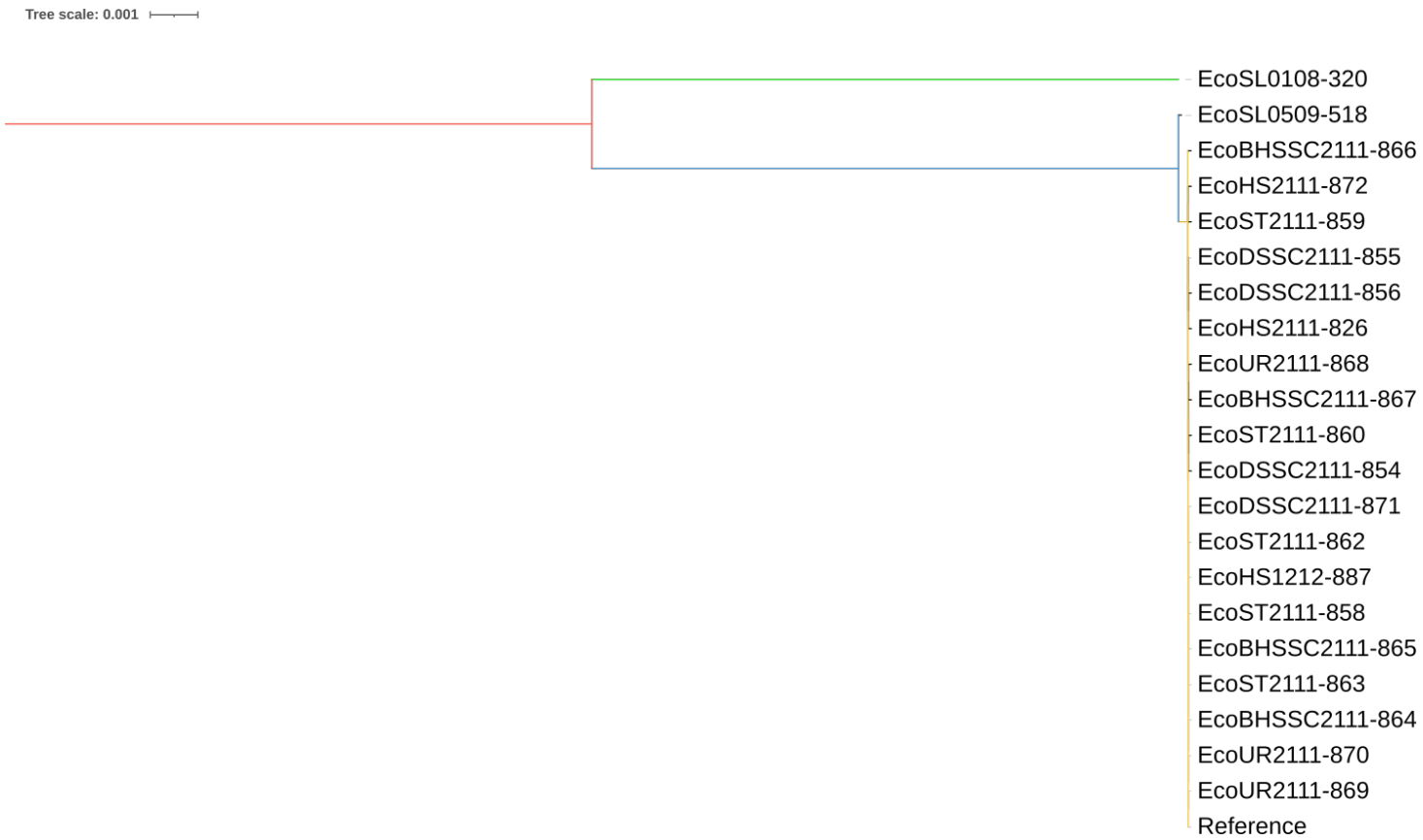


Figure 5.11: SNP distance tree showing the 21 ST1308 isolates with the root and ancestral branch shown in red, 320 on the green branch showing the first divergence from the ancestral line, 518 on the blue branch showing the second divergence from the ancestral line and the clonal group shown as the yellow leaves. Reference genome was EcoBHSSC2111-865

5.4.5. Virulence Genes

The wealth of data provided by the WGS allowed for other areas contributing to pathogenic potential to be investigated, namely the virulence factors. Using VirulenceFinder 2.0 (**Section 2.8.6.3**), any genes in the database identified as being likely virulence factors were collected in the search. As the search returned many genes that are not commonly flagged as being contributors to pathogenicity but may be classed under the virulence gene family, the list was trimmed to include only virulence genes which are frequently associated with pathotypes. The virulence factors of most interest were those encoding adhesins, invasins, toxins, evasion of host defences and capsule genes, although other genes of interest were also included such as those encoding siderophores and iron acquisition systems.

The pathogenicity of an *E. coli* may often be determined by the virulence genes it carries, but this does not necessarily constitute a firm definition as a pathogen, but rather can confer the pathogenic potential of that *E. coli*. Virulence factors may allow *E. coli* to become highly adapted, with an increased ability for new niche adaption and to cause a broad spectrum of different disease types (Kaper et al. 2004). For an *E. coli* to cause pathogenesis, it is a multi-step process involving several different virulence factors that will often work together to colonise and evade host defences. Having a single virulence factor may not be sufficient to create pathogenesis, but rather the combination of virulence factors is what is crucial (Kaper et al. 2004).

Certain pathotypes of *E. coli* owe some or even all of their virulence to the carriage of a virulence plasmid. Therefore, virulence genes associated with these specific pathotypes are generally plasmid-encoded and two examples

include EIEC and EAEC (Kaper et al. 2004; Johnson and Nolan 2009b; Sarowska et al. 2019b). All the EIEC associated virulence factors are encoded on the pINV plasmid (Harris et al. 1982; Sansonnetti et al. 1982; Hale et al. 1983; Pasqua et al. 2017b) and the pAA plasmid of EAEC encodes a large majority of its virulence genes (Nataro et al. 1992; Johnson and Nolan 2009c; Boisen et al. 2014a; Berger et al. 2016b; Jønsson et al. 2017b; Prieto et al. 2021b). However, some pathotypes do have chromosomally-encoded virulence genes. These chromosomally-encoded virulence genes may be present on pathogenicity islands which are common to ExPEC pathotypes and may encode for adhesins, invasins, toxins, autotransporters, iron uptake systems and protectins (Desvaux et al. 2020) or on lysogenic phage with one example being the phage encoded Shiga toxin *stx* in EHEC (Berger et al. 2019b). The association of plasmids with specific virulence pathotypes and chromosomally-encoded virulence factors was explored in detail in **Section 1.4**. Within the 47 *ampC* isolates a large proportion of the virulence genes were found to be chromosomally-encoded with only a few plasmid-encoded. Virulence genes identified by VirulenceFinder 2.0 in the 47 isolates in the *ampC* group are detailed in **Table 5.6**, which details the virulence gene description, function, references to the literature, commonly associated pathotypes and *ampC* group isolates encoding them, with any isolates found with plasmid-encoded virulence genes highlighted by the isolate number in red. Any isolates in **Table 5.4** that weren't written in red text were found to have chromosomally-encoded virulence genes.

Virulence genes identified in the *ampC* isolates were designated to particular pathotypes. This designation was achieved by comparing the virulence factors in the 47 *ampC* isolates with virulence factors which have been

frequently cited in the literature in association with specific pathotypes. However, this designation did not constitute a firm confirmation of that pathotype, rather it concluded with which pathotype that virulence gene has been commonly associated. Therefore, the virulence factors identified in this group of *E. coli* may only serve to suggest the pathogenic potential rather than clearly identifying these isolates as pathogenic. Moreover, it should be remembered that the *E. coli* isolates were not sampled from diseased cattle but rather the environment the cattle were living in and the slurry system in place to deal with waste disposal. The *E. coli* pathotype EHEC, for example, may cause serious disease in humans such as bloody diarrhoea but is found in the intestines of both healthy cattle who are considered a primary reservoir of it (Segura et al. 2021) and other ruminants (Lim et al., 2010; Ferens and Hovde, 2011).

The investigation into the virulence genes in the *ampC* group, provided an insight into the potential stepping point for the *E. coli* towards becoming a pathogen and how spread through the food chain could provide a route to humans and disease occurrence. The virulence genes of interest were also annotated against the phylogenetic tree using iTOL software shown in **Figure 5.9**.

Following the results from VirulenceFinder, the only isolate within than *ampC* group that looked to be close to a specific pathotype through the combination of genes, was isolate 825. A ColV virulence plasmid of 192,986 bp was found in 825 that encoded a virulence region, which was very similar to a 93 kb virulence region of the 180 kb ColV plasmid pAPEC-O2-ColV (accession number NC_007675.1), which has been cited as being an APEC associated plasmid (Johnson et al. 2006a; Johnson et al. 2006b; Skyberg et al. 2006). A

pairwise identity of the two plasmids conducted in Geneious Prime (**Section 2.8.2.1**) resulted in a mean length of the two sequences of 188,743 bp and resulted in a pairwise identity of 81.4% with 64, 323 identical sites. The virulence genes encoding for increased serum survival *iss*, the pore forming avian haemolysin toxin *hlyF, etsC* of the type 1 secretion system, *cvaA* of the Colicin V operon, *sitA* of the iron transport system, the outer membrane omptin *ompT*, *iroN* of the salmochelin siderophore receptor and *iucC* of the aerobactin system have all been cited as a signature of the ColV plasmid virulence region, which has been described in both APEC and ExPEC strains (Johnson et al. 2006b; Peigne et al. 2009; Mariani-Kurkdjian et al. 2014). VirulenceFinder had returned results for the virulence genes *cvaC*, *papC*, *tsh*, *iroN*, *iucC*, *iutA*, *sitA*, *hlyF*, *iss* and *traT* (which are listed in **Table 5.6**) and further manual investigation of the genome sequence identified further virulence genes including *etsC*, the siderophore salmochelin operon *iroBCDEN*, the ColV operon consisting of the genes for ColV export *cvaAB*, ColV synthesis *cvaC* and ColV immunity *cvi*, the iron transport system *sitABCD* and the aerobactin transport system consisting of *iucABCD* and *iutA*, all of which were consistent with the genes located in the virulence region of pAPEC-O2-ColV. These similarities between the pAPEC-O2-ColV plasmid and 825, were suggestive that 825 could be possibly considered an ExPEC type and could therefore have pathogenic potential.

Other virulence genes of note were located in some of the *ampC* group including *astA*, *f17AG*, *gad* and *lpfa*, all of which have been found to be associated with pathogenic strains of *E. coli* that can cause disease in both animals and possibly humans. The EAEC *astA* which produces the EAEC heat-

stable enterotoxin 1 (EAST1), was found chromosomally-encoded in isolates 99, 486, 495 and plasmid-encoded in 127 and 320; *astA* has been reported both chromosomally and plasmid-encoded in a variety of *E. coli* pathotypes including ETEC and EHEC, with some strains encoding multiple copies on both plasmids and the chromosome (McVeigh et al. 2000; Veilleux et al. 2006; Ménard and Dubreuil 2008). The EAST1 enterotoxin is often responsible for postweaning diarrhoea within pigs and diarrhoeal disease in cattle and humans (Osek 2003; Dubreuil 2019). The fimbrial gene *f17A* which encodes the structural major subunit was found in isolates 99, 486, 514, 968 and 969, *f17D* which encodes a chaperone protein for periplasmic transport was found in isolates 486, 514, 968 and 969 and *f17G* that encodes the adhesin minor subunit was found in isolate 99 (Cid et al. 1999; Bihannic et al. 2014). All the *f17* genes were found in the chromosome. The subtypes of each *f17* gene were analysed through a combination of manual investigation of the WGS, BLAST searches of the amino acid sequences and comparison to sequences in the database. All *f17A* were F17d-A type, *f17D* were F17d-D type and *f17G* were F17G2 type and are detailed in **Table 5.6**. The fimbriae genes *f17dA, f17dD* and *f17G2* have been associated with ETEC associated diarrhoea and septicaemia in calves, goat kids and lambs and *f17G2* has also been found expressed within human uropathogenic strains of *E. coli* (Bertin et al. 1996; Cid et al. 1999; Bihannic et al. 2014). The glutamate decarboxylase *gad* which is involved in acid tolerance, was found in all but isolates 826 and 872 and was chromosomally-encoded as per other published data in the literature regarding *gad* most commonly being found chromosomally encoded (Yokoigawa et al. 2003; Tramonti et al. 2006; Bergholz et al. 2007; de Biase and Pennacchietti 2012). Manual investigation of

the WGS amino acid sequence of *gad* and comparison to sequences in the database, found it to be *gad* alpha and that it was identical to a published UPEC strain CFT073, which was cultured from a patient with acute pyelonephritis at University of Maryland Hospital in 1990 (Mobley et al. 1990; Welch et al. 2002).

The long polar fimbriae gene *lpfa* was found in all but isolates 99, 183, 408, 826 and 872. Both *gad* and *lpfa* have been associated with acid tolerance and adhesion respectively of the foodborne pathogen EHEC O157:H7 (with outbreaks of EHEC described in **Section 1.4**). EHEC O157:H7 can be contracted through the consumption of undercooked or cross contaminated raw to cooked bovine meat or other food products such as raw milk (Griffin and Tauxe 1991) or yoghurt (Morgan et al. 1993) but also by direct zoonotic transmission and animal petting zoos are notorious for this (DebRoy and Roberts 2006; Heuvelink" et al. 2007; Schlager et al. 2018). EHEC O157:H7 has a very low infectious dose, due to the acid tolerance associated with *gad* encoded by this pathogen (Griffin and Tauxe 1991; Yokoigawa et al. 2003). Another essential virulence factor associated with EHEC O157:H7 colonisation is the adhesion to the gastrointestinal tract through adhesive factors like the long polar fimbriae encoded by *lpfa* (Torres et al. 2009a), this generally occurs at the terminal ileum and colon within humans and the terminal recto-anal junction within cattle (Naylor et al. 2003; Chong et al. 2007; Mahajan et al. 2009; Pradel et al. 2015). Homologues of *lpf* have also been found in other types of pathogenic *E. coli* such as Locus of Enterocyte Effacement (LEE)-negative STEC strains, with expression of *lpf* thought to be important in the development of severe diarrhoea (Osek et al. 2003; Galli et al. 2010b). In addition, *lpf* has even been found in commensal strains of *E. coli*, as well as in *Shigella* and *Salmonella* (Doughty et

al. 2002; Toma et al. 2004; Torres et al. 2009b; Galli et al. 2010a; Galli et al. 2010b).

It was also interesting to discover that all but isolate 320 of the ST1308 had identical virulence gene carriage, signifying more potential evidence of clonality of 518, 826, 854, 855, 856, 858, 859, 860, 862, 963, 864, 865, 866, 867, 868, 869, 870, 871, 872 and 887.

Pathogenic *E. coli* have evolved to create a large variety of distinct pathotypes, with virulence factors that enable the colonisation of various locations in the host including the gastrointestinal tract, urinary tract and meninges. Through the acquisition of pathogenicity islands, transposons, phage or plasmids an *E. coli* can adapt to better suit the environment or niche which enables the bacteria to cause disease. Efforts to categorise clusters of pathogenic *E. coli* into strict delineated pathotypes, are complicated however by this genome plasticity. The evolutionary process of *E. coli* however still continues, demonstrating it is a highly versatile and adaptable species, capable of colonising a large plethora of environments (Kaper et al. 2004).

Table 5.6: Virulence genes from the 47 isolates in the *ampC* group, with description and functions and commonly associated pathotypes

Virulence Gene	Description	Function	Commonly Associated Pathotypes	Isolate	Percentage Identity
Adhesins and Colonisation Factors					
afaAB	Afimbrial adhesin	Non-fibrous adhesin with haemagglutination capacity that binds the cell surface DAF receptor (Sarowska et al. 2019b)	UPEC	*afaAB* in 486 and *afaB* only in 968 and 969	*afaA* – 100% *afaB* – 99.06% in 486, 98.83% in 968 and 969
cvaC	Colicin V	Colonisation factor (de Carli et al. 2015; Sarowska et al. 2019b)	NMEC, SEPEC, APEC	825	100%
f17ADG	Fimbriae	*f17A* which encodes the structural major subunit, *f17D* which encodes a periplasmic transport chaperone	ETEC, EHEC, APEC, UPEC	99, 486, 514, 968 and 969	*f17A* – 99.82% in 514, 968 and 969,

		protein and *f17G,* that encodes the adhesin minor subunit, have been associated with diarrhoea and septicaemia in both calves, goat kids and lambs and has also been found expressed within human uropathogenic strains of *E. coli* (Bertin et al. 1996; Cid et al. 1999; Bihannic et al. 2014).			97.99% in 99 and 94.87% in 486. *f17D* – 100% in 514, 968 and 969 and 99.9% in 486. *f17G* 99.71% in 99.
lpfa	Long polar fimbriae	Adhesive factor often associated with EHEC O157:H7 important to colonisation of the intestine (Toma et al. 2006; Torres et al. 2009c; Galli et al. 2010c; Dogan et al. 2012)	EHEC, EAEC, EPEC	All but 99, 183, 408, 826, 872.	100% in all but 152 which was 99.83%
hra	Heat-resistant haemagglutinin	Adherence/colonisation factor (Srinivasan et al. 2003; Marrs et al. 2005)	UPEC, EAEC	157, 320, 486, 495, 514, 536, 968, 969	92.71% in 495, 93.71% in 514, 96.79% in 157, 98.61% in 320,

					99.75% in 536 and 100% in 486, 968 and 969
papC	P fimbriae	A colonisation factor in ExPEC infections that stimulates cytokine production (Dale and Woodford 2015; Sarowska et al. 2019).	ExPEC, APEC, UPEC SEPEC	157, 320, 825	90.95% in 320, 95.41% in 157 and 95.45% in 825
tsh	Temperature sensitive haemagglutinin	Has both agglutin and protease activity and can act as both a serine protease and an adhesin. Involved in agglutination of erythrocytes and the deposition of fibrin and the lesion development in the avian air sacs (Stathopoulos et al. 1999; Dozois et al. 2000; Kostakioti and Stathopoulos 2004; Kobayashi et al. 2010)	APEC	825	99.95%

gad	Glutamate decarboxylase	Aids orally acquired bacteria in resisting extreme acid stress of pH <2.5 in transit through the host stomach, for successful colonisation (de Biase and Pennacchietti 2012)	EHEC	Found in all isolates.	98.9-100%
Toxins					
astA	Enteroaggregative *E. coli* heat-stable enterotoxin (EAST1)	Toxin produced by *E. coli* causes mucosa damage and intestinal secretion, which induces diarrhoea in humans but also animals such as calves and piglets (Ménard and Dubreuil 2002; Ménard and Daniel Dubreuil 2002; Maluta et al. 2017b)	EAEC, ETEC, EPEC	99, 127, 320, 486, 495	99.15% in 486 and 100% in all others
cdtB	Cytolethal distending toxin (CDT)	Heat-labile toxin that induces eukaryotic cell death through DNA double-strand breaks in cells (Pandey et al. 2003; Tóth et al. 2009)	EPEC	495	100%

Iron Acquisition					
fyuA	Yersiniabactin Receptor	Important to the formation of biofilms in low iron environments such as human urine (Hancock et al. 2008; Spurbeck et al. 2012)	UPEC	125, 152, 295, 408, 495, 514, 968, 969	100% in all
iroN	Salmochelin receptor	Siderophore involved in iron acquisition (Gao et al. 2012; Sarowska et al. 2019b)	UPEC, NMEC, APEC, SEPEC	825	100%
irp2	Iron repressible protein	Yersiniabactin biosynthesis involved in iron regulation (Tu et al. 2016; Kathayat et al. 2021)	APEC	125, 152, 295, 408, 495, 514, 968, 969	99.97% in 408, 99.98% in 152, 495, 514, 968 and 969 and 100% in 125 and 295.

iucC	Aerobactin synthase	Siderophore iron acquisition, required for bacteria to survive in iron poor environments such as the urinary tract (Ling et al. 2013; Sarowska et al. 2019b)	APEC, UPEC	825	100%
iutA	Aerobactin receptor	Siderophore iron acquisition, required for bacteria to survive in iron poor environments such as the urinary tract (Ling et al. 2013; Sarowska et al. 2019b)	APEC, UPEC	825	100%
sitA	Peri-plasmic iron binding protein	Mediates the transport of iron for iron acquisition (Sabri et al. 2006; Schouler et al. 2012; Ibrahim et al. 2019)	APEC, UPEC	183, 209, 408, 508, 825, 968, 969	99.87% in 209 and 100% in all others
Capsule					
kpsE	Capsular polysaccharide export	A cytoplasmic-membrane-periplasmic auxiliary protein involved in export of components of the capsular polysaccharide across the periplasmic space		183	99.65%

		(Arrecubieta et al. 2001; Silver et al. 2001; McNulty et al. 2006)			
kpsMII	kpsM II group 2 capsule	Capsular polysaccharide protects again hosts defences such as complement-mediated killing and phagocytosis (Johnson and O'Bryan 2004; Zong et al. 2016; Merino et al. 2020)	EXPEC, NMEC, SEPEC	183	100%
hlyF		Haemolysin believed to be involved in outer membrane vesicle over production and in turn virulence factor delivery such as CDT. It is also epidemiological marker for both NMEC and APEC (Murase et al. 2016)	APEC, NMEC	157, 308, 536, 582, 825	100% in all

Host Defence Evasion					
iss	Increased serum survival	Protection from host defence through serum survival and complement resistance. Indicated in the development of septicaemia (Biran et al. 2021)	ExPEC	All but 99, 127, 408, 431, 508, 514, 825 and 833. Found to be plasmid-encoded in 825.	98.9-100%
ompT	Outer membrane protease T	Protectin that can inactivate antimicrobial peptides in the host through OmpT-dependent cleavage (Thomassin et al. 2012), aids in enhancing uroepithelium colonisation (Hui et al. 2010) and outer membrane vesicle biosynthesis (Premjani et al. 2014)	EHEC, EPEC	All but 99, 183, 209, 309, 320, 408, 431, 508, 514, 825, 833, 968 and	96.2% in 127 and 410 and 100% in all others

				969. In all those positive for *ompT*, it was found to be plasmid-encoded.	
traT	Conjugal transfer surface exclusion protein	Complement resistance (Al-Janabi et al. 2018; Sarowska et al. 2019b)	NMEC, SEPEC, APEC	127, 152, 157, 326, 410, 495, 536, 582 and 825.	99.86% in 410 and 100% in all others

***Footnote for Table 5.6**: Virulence genes in specific isolates confirmed as being plasmid-encoded are identified with isolates listed in red text. Any virulence genes which are commonly known to be plasmid associated are written in blue text.*

5.5. CONCLUSIONS

The investigation undertaken in this chapter provided an understanding of other non-ESBL types of beta-lactamase type resistance that were present in the EVAL farms isolates sampled from the dairy farm. It was clear from the phenotypic picture painted initially by the disc assay data provided by EVAL farms (**Figure 5.2**), that another mechanism that was not an ESBL was present, and it was thought highly likely to be an ESC: namely overexpression of chromosomal *ampC*. Susceptibility patterns are a known phenotypic method for identifying the differences between ESBLs and overexpression of *ampC* as was outlined in **Section 5.2.1** and the confident identification of the *ampC* mechanism was made more conclusive with the addition of CFQ in the MIC extended panel of antibiotics. By conducting the MICs in this chapter, the levels of resistance to beta-lactams provided by the overexpression of *ampC* mechanism could be fully understood. What was observed from looking at the two mechanisms of beta-lactamase resistance namely ESBLs and overexpression of *ampC*, was that ESBLs appeared to provide a higher level of resistance to certain beta-lactams than overexpression of *ampC*. When comparing the beta-lactam MICs of the 22 overexpression of *ampC* isolates with the MICs of the $bla_{\text{CTX-15}}$ isolates in **Chapter 3** and detailed in **Section 3.2.2, Table 3.2**, there were some differences to note, especially in the concentrations of the MICs between them. The $bla_{\text{CTX-15}}$ isolates all had consistent and identical resistant MIC results for AMP and CTX at >512 mg L^{-1}, CPD at 512 mg L^{-1}, CAZ at 16 mg L^{-1} and all were also resistant to CFQ with MICs of 128 mg L^{-1}. The 3rd generation cephalosporin MICs for the 22 overexpression *ampC* isolates were much more varied with lower resistance MICs and intermediate and

susceptible MIC results. This was evident with CPD which had a lower MIC of 64 mg L^{-1} and with CTX which returned the majority of susceptible or intermediate results, with only 5 isolates returning a resistant result, which was much lower at 4 mg L^{-1} than the *bla*$_{CTX}$ result. The MICs of CAZ were also quite varied in the *ampC* isolates, whereas the CAZ MICs were consistent throughout the *bla*$_{CTX}$ isolates. There was also very little resistance to ATM in the overexpression of *ampC* isolates compared to ATM resistance in all *bla*$_{CTX\text{-}M\text{-}15}$ isolates. This demonstrated that *bla*$_{CTX\text{-}15}$ not only appears to provide a much higher level of resistance across a broad range of beta-lactams than overexpression of *ampC*, but that *bla*$_{CTX}$ is also more consistent as a resistance mechanism, as was evident with CTX and especially CAZ. However, both mechanisms appear to provide a similar level of resistance to AMP.

A further significant discovery was that the initial disc assays resistance phenotypes had almost all disappeared following MIC assays. What appeared to start as a single group of 39 isolates in the *ampC* group with similar resistances, resulted in more than half having lost the majority of their resistances when tested by MIC. One theory for the reason for loss of resistances could be the loss of MGEs such as plasmids following recovery from frozen culture. Alternatively, as isolates were disc assayed soon after sampling, intrinsic mechanisms such as the down regulation of the porins OmpF and OmpC (Tenover, 2006) and the upregulation of efflux systems such as AcrAB (Thanassi et al., 1997; Rosenberg et al., 2003) could have been present, which were then returned to normal levels following recovery from frozen culture. However, as the carriage of MGEs at time of sampling and the intrinsic mechanisms of efflux and porins were not tested, it is a difficult hypothesis to prove at this late stage.

Therefore, the suggestions for why the disc assay resistances were no longer present are merely theories for why this might have occurred. As was discussed in the conclusion of **Chapter 3**, the disc assays were also only conducted once by EVAL farms and the findings of this Chapter again demonstrated the importance of repeating results.

As was discussed in the conclusion of **Chapter 3**, the selective media utilised during the initial sampling by EVAL farms appeared to play a role in isolating the non-ESBL producing *E. coli* encoding overexpression of *ampC*. The majority of the isolates were grown on TBX supplemented with 2 mg L^{-1} CTX with 2 grown on TBX supplemented with 16 mg L^{-1} AMP and utilising media such as TBX will often allow for a much greater portion of the whole population to be sampled and not just ESBLs for example when using CHROMagar ESBL agar, as demonstrated in **Chapter 3**.

The genotypic analysis of the isolates in the *ampC* group had begun as the finding of overexpression of *ampC* in the single isolate 869. This led to the identification in the *ampC* group of 22 isolates overexpressing *ampC* and all with the same mutation types at -42 and -10 which resulted in the creation of new stronger promoter sequences. The rationale for keeping the PCR results as part of the analysis in this thesis, was to show it was a good screening tool, as the PCR worked well, provided narrative and could be used as a screening tool in the future. Following the WGS, an interesting discovery was that on this particular farm, the majority of the overexpression of *ampC* isolates were all ST1308, with a further two being found to be ST75. With the analysis conducted using SNP distancing, the clonality of the ST1308 isolates was successfully

investigated and interestingly it looked as if there had been a small clonal expansion of a strain encoding the overexpression of *ampC* genotype.

The SNP distance analysis (**Section 5.4.2.2**) and SNP distance tree (**Figure 5.9**) suggested there had been a small clonal expansion of a strain carrying the overexpression of *ampC* mutation within 20 isolates of the *ampC* group. There appeared to have been at least two ancestral divergences of ST1308 however, with the first being 320 which was not overexpressing *ampC*, followed by the proposed second ancestral divergence resulting in the overexpression of *ampC* isolates 518, 866, 872, 859, 855, 856, 826, 860, 854, 868, 867, 871, 862, 887, 858, 865, 863, 864, 870 and 869. This could suggest there was mutation of the non-*ampC* overproducer 320, to become an overproducer of *ampC*, which was likely the result of selective pressure and which the phylogenetic tree in **Figure 5.9** shows, resulted in the group of 20 overexpression of *ampC* isolates that were closely related to 320. Of the 20 ST1308 overexpression of *ampC* isolates, there were some small genetic variations and it would appear the time that passed between the samplings of the earlier isolate 518 and the remaining 19 had possibly played a role in this, showing the changing nature of bacteria over time in the complex environment of a dairy farm. However, the virulence gene carriage was identical in these 19 and different to the virulence carriage in 320, which shows there was definite divergence from the ancestral strain of 320 to the remaining group of ST1308 isolates. The findings in the ST1308 group, suggested that clonality had played some part in the spread of the overexpression of *ampC* resistance type on this specific farm, which could be suggestive of a potential environmental selective pressure maintaining the overexpression of *ampC* ST1308 strains. However, this discovery did not suggest overexpression

of *ampC* is correlated to a specific ST, it did however suggest, that on this particular farm, there had been a small clonal expansion of a specific ST in association with the overexpression of *ampC* genotype. Other possible factors that could influence the differences seen between 320 and 518 could include the limitations of sampling, in that due to the sample size, at each sampling only subpopulations were examined each time. In addition, the size of the slurry tank where samples were taken was vast and it was mixed frequently. The fact that the slurry tank was a huge volume and only a small sample size was taken, it may not be representative of the whole population or the whole farm environment. In addition, the type of bedding the cattle were housed on can influence bacterial growth such as sand and straw, with an example being the contamination of bedding with *Streptococcus uberis* being much higher in straw than with sand or sawdust (Hillerton and Berry 2003; Kabelitz et al. 2021). The cattle on the farm concerned with this study however were housed on sand and ground limestone. The farm management is also an important factor, including the disinfection process and biosecurity on the farm and the farm concerned with this study did employ copper and zinc footbaths for the cattle, formaldehyde disinfectants and did not feed waste mastitic milk to calves. Therefore, with all these points in mind, sampling may not always necessarily be representative of the actual environment and the many interacting factors influencing it on a daily basis. The methods used for sample isolation will therefore bias the representation of the whole population that is picked up.

Surprisingly there was very little plasmid-encoded resistance in this group, and only isolate 99 was found to have all resistance encoded on an IncI1 plasmid containing aminoglycoside, florfenicol, sulphonamide and tetracycline

resistance mediated by *aph3"*, *aph6*, *floR*, *sul2* and *tetA* respectively. All other resistances in the remaining isolates were found to be chromosomal and this could suggest that once in the environment where conditions may be harsh, nutrients low and competition high, plasmid carriage may not be favourable for the fitness cost it confers on the bacteria, resulting in the loss of plasmids. Therefore, the insertion of resistances into the chromosome may have occurred especially if selective pressure in the environment was present. There were additional beta-lactamases present but only within isolates 99 and 486 that were encoding $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$ respectively. With the combination of the phenotypic and genotypic data, it was confirmed that the resistances noted in the MIC assay appeared to mostly match what was found genotypically, with only the florfenicol gene *floR* failing to provide any resistance to CHLOR and in addition the use of the combination SXT in MIC assays was unable to assess the ability of *sul2* to provide sulphonamide resistance. Therefore, it could have been prudent to assess the ability of *floR* and *sul2* to provide resistance by utilising other florfenicol antibiotics and sulphonamides alone respectively.

The investigation of virulence factors in the *ampC* group gave a good indication of the pathogenic potential of some of the isolates. Only a few were encoding virulence genes that may be associated with specific pathotypes and many were chromosomally-encoded. There was a much greater variety of virulence genes in the *ampC* group compared to the bla_{CTX} group in **Chapter 3** and one reason that could be suggested for this, was that the isolation media utilised for the *ampC* group isolates was much less selective than the bla_{CTX} group. The *ampC* group media consisted of TBX, TBX supplemented with 16 or 100 mg L^{-1} AMP or 2 mg L^{-1} CTX and one isolate on CHROMagar ESBL,

whereas the *bla*$_{CTX}$ group utilised MacConkey plus 2 mg L^{-1} CTX for two isolates and CHROMagar ESBL for the remaining isolates. This would support the hypothesis that using different isolation media when sampling *E. coli* gives a much more diverse sector of the whole population.

Isolate 825 which was sampled from the dairy lane outside, gave the most interesting result for virulence, with the finding of the ColV plasmid that has been associated with ExPEC types. This suggested the potential pathogenicity of this isolate and demonstrates how an *E. coli* may adapt by the accumulation of virulence genes to become a pathogen. As isolate 825 was isolated from the environment this was merely an observation, but it does provide an interesting look into an *E. coli* found in the environment of a dairy farm with pathogenic potential.

CHAPTER 6

DISCUSSION AND FUTURE WORK

6.1. THIS STUDY AND THE OBJECTIVES ACHIEVED

This study aimed to elucidate beta-lactamase type resistance mechanisms associated with beta-lactamase resistance phenotypes within a small sample size of 86 isolates, chosen from a collection of over 1,000 *E. coli* isolates, sampled from the environment of a dairy farm by the EVAL farms project (Baker et al. 2022b). Understanding how resistance persists or is disseminated throughout the dairy farm environment was also investigated, by examining clonality and the characterisation and mobility of plasmids, transposons and insertion sequences. Transposition experiments produced an additional 16 transconjugants that were also analysed.

The main objectives outlined at the start of this thesis and successfully achieved were:

i. The phenotypic analysis of 86 dairy farm *E. coli* isolates, sampled from 2017-2018 that had been initially characterised by disc diffusion assay by EVAL farms and through MIC assays as part of this study.

ii. The genotypic characterisation by PCR of targeted individual resistance genes, followed by WGS.

iii. Clonality assessed via WGS, SNP distance comparison and phylogenetic analysis.

iv. Characterisation of the MGEs within all isolates via WGS data.

v. Assessment of $bla_{\text{CTX-M-15}}$ mobility via IS*Ecp1* transposition conjugated to resident plasmids.

vi. Determination of other mechanisms of beta-lactamase type resistance, which was found to be principally via overexpression of *ampC* and in two isolates through bla_{TEM} and bla_{OXA} expression.

As was highlighted in the introduction of this thesis in **Chapter 1**, antibiotic resistance is now a growing global issue and there have been no new antibiotic classes discovered in recent years (explored in **Section 1.1.1)**, resulting in the discovery void as shown in **Figure 1.1**. This thesis highlights the presence of beta-lactamase type resistance within a single dairy farm, the mobile elements associated with its potential dissemination and the potential for clonal expansion of a dominant ST in association with the ESBL $bla_{CTX\text{-}M\text{-}15}$, and the small clonal expansion of another ST encoding an overexpression of *ampC*. This chapter examines the crossover of antibiotic usage between humans and animals and those antibiotics that are listed as critical to human by the WHO and WOAH respectively. The current global usage data for antibiotics in animals and the appearance of antibiotic resistance in animals, will also be discussed.

6.1.1. Antibiotic usage within humans and animals, globally and within the UK and how this compared to the dairy farm in this study

As was described in **Section 1.2.1**, the WHO and WOAH produce priority lists of those antibiotics which are deemed important to either human or animal health, respectively. The WOAH'S list of antimicrobial agents of veterinary importance and the WHO's list of antibiotics critical to human health

are detailed in **Tables 6.1** and **6.2** respectively. There lists are detailed in **Table 1.2**, and demonstrate considerable commonality.

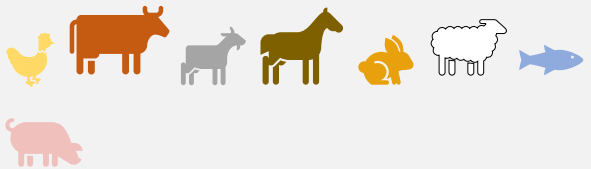
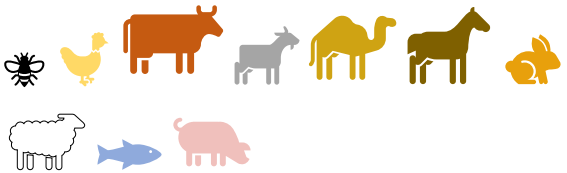
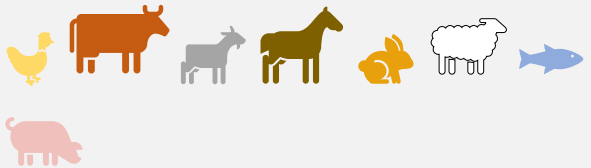
When examining crossover of antibiotics between the two lists (**Table 6.1** and **Table 6.2)**, a total of 10 antibiotics on the WOAH list are classed as critically important human antimicrobials, 9 are classed as highly important human antimicrobials and 3 are classed as important human antimicrobials (WHO 2018; OIE 2021). In addition, there are only a few classes of antibiotics on the WHO's list that are prohibited for use. In 1996, the FDA prohibited unapproved use termed "extra label usage" (which was defined in **Section 1.2.1**) of chloramphenicol, nitroimidazoles, nitrofurans, fluoroquinolones and glycopeptides in food producing animals (FPAs) that included prohibiting use at unapproved dosing levels, frequencies or durations, for disease prevention or for use in unapproved animal species (FDA 1996). In addition, the use of 3rd generation and higher cephalosporins was heavily restricted in 2012 by the FDA in certain FPAs such as cattle, chickens, swine and turkeys (FDA 2012a). Nitrofurans were banned in FPAs by the EU in 1993 due to the potential carcinogenic and mutagenic effects the metabolites from these drugs may have in the human gastric environment. Nitrofurans may occur in animal tissues as protein bound metabolites and are rapidly metabolised, which in turn has led to their banning in several other countries, that recognise their potential toxicity (EFSA 2015; Mologni et al. 2021). Nitroimidazoles are also banned in the US, EU and many other Organisation for Economic Co-operation and Development (OECD) countries due to the potential genotoxic, mutagenic and carcinogenic effects of their residues in animal tissues. As no maximum residue limit, tolerance limit or acceptable daily intake has been established, any detectable

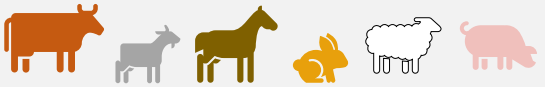
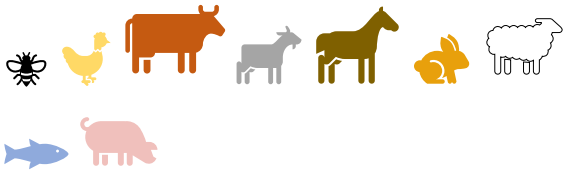
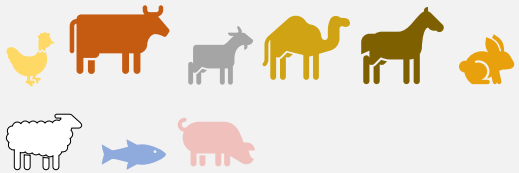
concentration of nitroimidazoles would be considered a violation (Granja et al. 2013; Baynes et al. 2016). The toxic effects of chloramphenicol have been discussed since the 1980s (Schmid 1983; Settepani 1984) and its use in FPAs was banned globally due to the risk of inducing aplastic anaemia in humans and hepatotoxic or reproductive effects in animals (Cowart 2006; Florence et al. 2020; Sathya et al. 2020; Wang et al. 2021). As was outlined in **Section 1.2.1.1**, the ban of certain drugs as growth promoters such as avoparcin and virginiamycin was due to cross resistance (Casewell et al. 2003c; Phillips 2007). However, the banning of chloramphenicol, nitrofurans and nitroimidazoles has been from the risks of toxicity rather than cross resistance. The restrictions on the use of 3rd generation and higher cephalosporins (Sato et al. 2014; Scott et al. 2019b; Speksnijder et al. 2022), glycopeptides (Hayes et al. 2003; Economou and Gousia 2015b; Gousia et al. 2015) and fluoroquinolones (Brierley 2006; Schulz et al. 2019; Yin et al. 2022) however, is due to the associated risks of cross resistance or selection of antimicrobial resistance in humans as a result of the use of these antimicrobials in animals (Marshall and Levy 2011; Wegener 2012; More 2020; Ma et al. 2021).

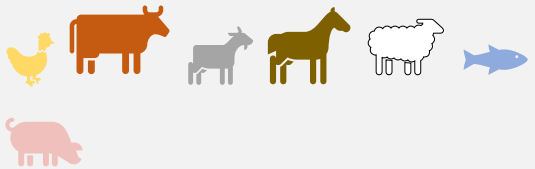
WOAH member countries data for antimicrobial usage in FPAs was available for 2017 and was adapted from the WOAH Fifth Annual Report on Antimicrobial Agents Intended for Use in Animals (OIE 2021a). **Figure 6.1** shows the proportion of antimicrobial classes that were reported for use in animals by the WOAH member states in 2017 and demonstrates that usage of the tetracyclines is by far the highest of all the classes, followed closely by penicillins (especially in Europe). **Figure 6.2** details the FPA species that were included in the quantitative data that were reported by each WOAH member

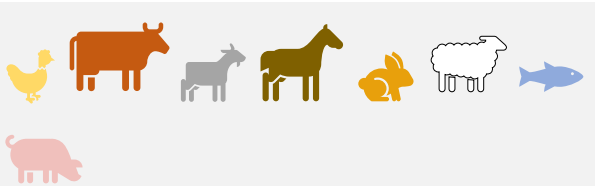
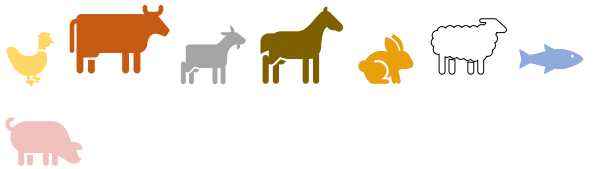
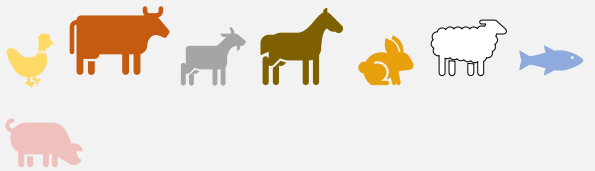
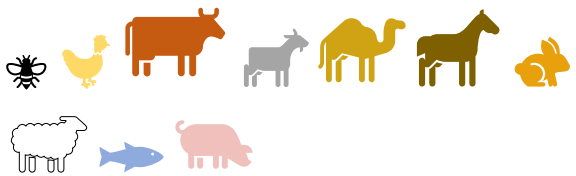
country in 2017, with poultry, bovine, sheep & goat and pigs making up the largest majority. **Figure 6.3** details the sales of antibiotics by class for FPAs in the UK as published by the UK-VARSS Report 2020 (UK-VARSS 2020) and demonstrates that the largest majority were tetracyclines followed by the beta-lactams, as with the WOAH report (OIE 2021a). In **Table 6.1** global usage of antibiotics as reported by WOAH (OIE 2021a), are highlighted against the human CIA listed antibiotics, with high usage (>20 %) highlighted in red, medium (5-20%) highlighted in yellow and low (<5 %) highlighted in blue, with anything that was reported as 0 % by WOAH (OIE 2021a) left unhighlighted. In the highest priority human CIAs in **Table 6.2** macrolides, polymyxins and quinolones and fluoroquinolones had medium global animal usage (5-20%) and the 3rd and 4th generation cephalosporins had low global animal usage (<5 %) as reported by WOAH (OIE 2021a). In addition, the aminopenicillins and aminoglycosides were listed as high priority human CIAs and had high global animal usage (>20%) and medium global animal usage (5-20%) respectively, as reported by WOAH (OIE 2021a). Within the highly important human antimicrobial category in **Table 6.2**, the penicillins (Antistaphylococcal) and (Narrow Spectrum) had high global animal usage (>20%), the amphenicols and lincosamides had medium global animal usage (5-20%) and the 1st and 2nd generation cephalosporins had low global animal usage (<5%) as reported by WOAH (OIE 2021a). These findings show that antibiotics that are critical to human health, are still being heavily used in animals globally and therefore there is a continued risk of cross resistance and selective pressure towards resistance in humans from the usage of these antibiotics in animals.

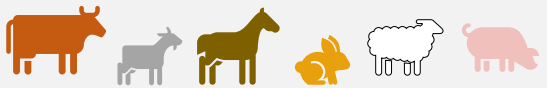
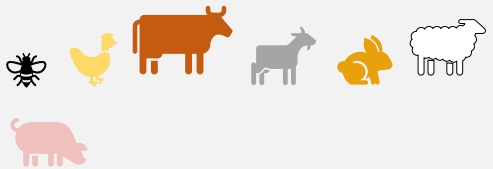
Table 6.1: WOAH list of antimicrobial agents of veterinary importance as of June 2021, with antibiotic examples listed for each antibiotic class and examples of animal species certain antibiotics are commonly used in. Adapted from: (OIE 2021b)

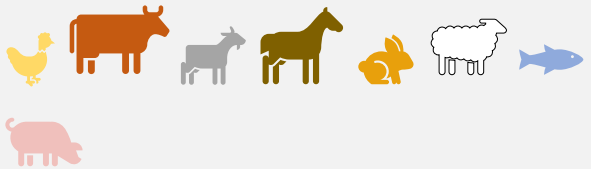
VETERINARY CRITICALLY IMPORTANT ANTIMICROBIAL AGENTS		
Antibiotic Class	**Examples of Antibiotics Within Antibiotic Class**	**Examples of Animal Species in Which Certain Antibiotics in this Class are Commonly Used**
Aminocyclitol	Spectinomycin	
Aminoglycosides	Amikacin, Apramycin, Dihydrostreptomycin, Fortimycin, Framycetin, Gentamicin, Kanamycin, Neomycin, Paromycin, Streptomycin and Tobramycin	
Amphenicols	Florfenicol and Thiamphenicol	

3rd Generation Cephalosporins	Cefoperazone, Ceftiofur and Ceftriaxone	
4th Generation Cephalosporins	Cefquinome	
Macrolides	Carbomycin, Erythromycin, Gamithromycin, Josamycin, Kitasamycin, Mirosamycin, Oleandomycin, Spiramycin, Terdecamycin, Tildipirosin, Tilmicosin, Tulathromycin, Tylosin and Tylvalosin	
Natural Penicillins	Benethamine Penicillin, Benzylpenicillin, Benzylpenicillin procaine/Benzathine Penicillin and Penethamate (hydroiodide)	
Amdinopenicillins	Mecillinam	

Aminopenicillins	Amoxicillin, Ampicillin and Hetacillin	
Carboxypenicillins	Ticarcillin and Tobicillin	
Ureidopenicillin	Aspoxicillin	
Phenoxypenicillins	Phenethicillin and Phenoxymethylpenicillin	
Antistaphylococcal Penicillins	Cloxacillin, Dicloxacillin, Nafcillin and Oxacillin	
Aminopenicillin + Beta-Lactamase Inhibitor	Amoxicillin + Clavulanic Acid and Ampicillin + Sulbactam	

Fluoroquinolones	Ciprofloxacin, Danofloxacin, Difloxacin, Enrofloxacin, Marbofloxacin, Norfloxacin, Ofloxacin, Orbifloxacin and Sarafloxacin	
Sulfonamides	Phthalylsulfathiazole, Sulfacetamide, Sulfachlorpyridazine, Sulfadiazine, Sulfadimerazine, Sulfadimethoxazole, Sulfadimethoxine, Sulfadimidine, Sulfadoxine, Sulfafurazole, Sulfaguanidine, Sulfamerazine, Sulfamethazine, Sulfamethoxine, Sulfamonomethoxine, Sulfanilamide, Sulfapyridine and Sulfaquinoxaline	
Sulfonamides + Diaminopyrimidines	Ormetoprim + Sulfadimethoxine, Sulfamethoxypyridazine and Trimethoprim + Sulfonamide	
Tetracyclines	Chlortetracycline, Doxycycline, Oxytetracycline and Tetracycline	

VETERINARY HIGHLY IMPORTANT ANTIMICROBIAL AGENTS		
Antibiotic Class	**Examples of Antibiotics Within Antibiotic Class**	**Examples of Animal Species in Which Certain Antibiotics in this Class are Commonly Used**
Ansamycin - Rifamycins	Rifampicin and Rifaximin	
1st Generation Cephalosporins	Cefacetrile, Cefalexin, Cefalonium, Cefalotin, Cefapyrin and Cefazolin	
Ionophores	Lasalocid, Maduramycin, Monensin, Narasin, Salinomycin and Semduramicin	
Lincosamides	Lincomycin and Pirlimycin	
Phosphonic Acid Derivatives	Fosfomycin	
Pleuromutilins	Tiamulin and Valnemulin	

Polypeptides	Bacitracin, Enramycin and Gramicidin	
Polymyxins	Polymyxin B and Polymyxin E (Colistin)	
Quinolones	Flumequin, Miloxacin, Nalidixic Acid and Oxolinic Acid	

VETERINARY IMPORTANT ANTIMICROBIAL AGENTS		
Antibiotic Class	**Examples of Antibiotics Within Antibiotic Class**	**Examples of Animal Species in Which Certain Antibiotics in this Class are Commonly Used**
Aminocoumarin	Novobiocin	
Arsenical	Nitarsone and Roxarsone	

Bicyclomycin	Bicozamycin	
Fusidane	Fusidic Acid	
Orthosomycins	Avilamycin	
Quinoxalines	Carbadox and Olaquindox	
Streptogramins	Virginiamycin	
Thiostrepton	Nosiheptide	

Footnote 1 for Table 6.1*: Animals represented by icons include:*

Footnote 2 for Table 6.1*: Any antibiotic classes or antibiotics within an antibiotic class highlighted in green, denoted either the entire class or individual antibiotics within the class are only used in animals. Antibiotic classes from WHO's CIA list highlighted in red are classed as human critically important antimicrobials, blue are classed as human highly important antimicrobials, yellow are classed as human important antimicrobials*

Table 6.2: The WHO's list of critically important antimicrobials for human medicine 6th Revision 2018 with antibiotic examples listed for each antibiotic class. Adapted from (WHO 2018)

CRITICALLY IMPORTANT ANTIMICROBIALS	
Highest Priority Critically Important Antimicrobials	
Antibiotic Class	Examples of Antibiotics Within Antibiotic Class
Cephalosporins (3rd, 4th and 5th Generation)**	Cefcapene, Cefdinir, Cefditoren, Cefepime, Cefetamet, Cefixime, Cefmenoxime, Cefodizime, Cefoperazone, Cefoperazone-Sulbactam, Cefoselis, Cefotaxime, Cefovecin, Cefozopran, Cefpiramide, Cefpirome, Cefpodoxime, Cefsulodin, Ceftaroline, Fosamil, Ceftazidime, Ceftazidime-Avibactam, Ceftibuten, Ceftizoxime, Ceftobiprole, Ceftolozane, Ceftriaxone, Ceftriaxone-Sulbactam, Latamoxef and Tazobactam
Glycopeptides and Lipoglycopeptides	Avoparcin, Dalbavancin, Oritavancin, Ramoplanin, Teicoplanin, Telavancin and Vancomycin
Macrolides and Ketolides	Azithromycin, Cethromycin, Clarithromycin, Dirithromycin, Erythromycin, Fidaxomicin, Flurithromycin, Josamycin, Midecamycin, Miocamycin, Oleandomycin,

	Rokitamycin, Roxithromycin, Spiramycin, Telithromycin, Troleandomycin and Solithromycin
Polymyxins	Colistin And Polymyxin B
Quinolones and Fluoroquinolones**	Besifloxacin, Cinoxacin, Ciprofloxacin, Delafloxacin, Difloxacin, Enoxacin, Enrofloxacin, Fleroxacin, Flumequine, Garenoxacin, Gatifloxacin, Gemifloxacin, Grepafloxacin, Ibafloxacin, Levofloxacin, Lomefloxacin, Marbofloxacin, Moxifloxacin, Nadifloxacin, Nalidixic Acid, Norfloxacin, Ofloxacin, Orbifloxacin, Ozenoxacin, Oxolinic Acid, Pazufloxacin, Pefloxacin, Pipemidic Acid, Piromidic Acid, Pradofloxacin, Prulifloxacin, Rosoxacin, Rufloxacin, Sitafloxacin, Sparfloxacin and Temafloxacin
High Priority Critically Important Antimicrobials	
Antibiotic Class	Examples of Antibiotics Within Antibiotic Class
Aminoglycosides	Amikacin, Apramycin, Arbekacin, Astromicin, Bekanamycin, Dibekacin, Dihydrostreptomycin, Framycetin, Gentamicin, Isepamicin, Kanamycin, Neomycin, Netilmicin, Paromomycin, Plazomicin, Ribostamycin, Streptomycin and Tobramycin

Ansamycins	Rifabutin, Rifampicin, Rifamycin, Rifapentine and Rifaximin
Carbapenems and Other Penems	Biapenem, Doripenem, Ertapenem, Faropenem, Imipenem, Meropenem, Vaborbactam and Panipenem
Glycylcyclines	Tigecycline
Lipopeptides	Daptomycin
Monobactams	Aztreonam And Carumonam
Oxazolidinones	Cadazolid, Linezolid, Radezolid and Tedizolid
Penicillins (Antipseudomonal)	Azlocillin, Carbenicillin, Carindacillin, Mezlocillin, Piperacillin, Piperacillin-Tazobactam, Sulbenicillin, Ticarcillin and Ticarcillin-Clavulanic Acid
Penicillins (Aminopenicillins)	Amoxicillin, Ampicillin, Azidocillin, Bacampicillin, Epicillin, Hetacillin, Metampicillin, Pivampicillin, Sultamicillin, Talampicillin and Temocillin
Penicillins (Aminopenicillins with Beta-Lactamase Inhibitors)	Amoxicillin/Clavulanic Acid and Ampicillin-Sulbactam
Phosphonic Acid Derivatives	Fosfomycin

Drugs used solely to treat tuberculosis or other mycobacterial diseases	Bedaquiline, Calcium Aminosalicylate, Capreomycin, Cycloserine, Delamanid, Ethambutol, Ethionamide, Isoniazid, Morinamide, Para-Aminosalicylic-Acid, Protionamide, Pyrazinamide, Sodium Aminosalicylate, Terizidone and Tiocarlide

HIGHLY IMPORTANT ANTIMICROBIALS

Antibiotic Class	Examples of Antibiotics Within Antibiotic Class
Amphenicols	Chloramphenicol and Thiamphenicol
Cephalosporins (1st and 2nd Generation) and Cephamycins	Cefacetrile, Cefaclor, Cefadroxil, Cefalexin, Cefaloridine, Cefalotin, Cefalotin, Cefamandole, Cefapirin, Cefatrizine, Cefazedone, Cefazolin, Cefbuperazone, Cefmetazole, Cefminox, Cefonicid, Ceforanide, Cefotetan, Cefotiam, Cefoxitin, Cefprozil, Cefradine, Cefroxadine, Ceftezole, Cefuroxime, Flomoxef and Loracarbef
Lincosamides	Clindamycin, Lincomycin and Pirlimycin
Penicillins (Amidinopenicillins)	Mecillinam And Pivmecillinam
Penicillins (Antistaphylococcal)	Cloxacillin, Dicloxacillin, Flucloxacillin, Methicillin, Oxacillin and Nafcillin

Penicillins (Narrow Spectrum)	Benzathine Benzylpenicillin, Benethamine Benzylpenicillin, Benzylpenicillin (=Penicillin G), Clometocillin, Penamecillin, Pheneticillin, Phenoxymethylpenicillin (=Penicillin V), Procaine Benzylpenicillin and Propicillin
Pseudomonic Acids	Mupirocin
Riminofenazines	Clofazimine
Fusidane	Fusidic Acid
Streptogramins	Pristinamycin and Quinupristin-Dalfopristin
Sulfonamides, Dihydrofolate Reductase Inhibitors and Combinations	Brodimoprim, Formosulfathiazole, Iclaprim, Phthalylsulfathiazole, Pyrimethamine, Sulfadiazine, Sulfadimethoxine, Sulfadimidine, Sulfafurazole (=Sulfisoxazole), Sulfaisodimidine, Sulfalene, Sulfamazone, Sulfamerazine, Sulfamethizole, Sulfamethoxazole, Sulfamethoxypyridazine, Sulfametomidine, Sulfametoxydiazine, Sulfametrole, Sulfamoxole, Sulfanilamide, Sulfaperin, Sulfaphenazole, Sulfapyridine, Sulfathiazole, Sulfathiourea, Tetroxoprim and Trimethoprim
Sulfones	Aldesulfone Sodium and Dapsone

Tetracyclines	Chlortetracycline, Clomocycline, Demeclocycline, Doxycycline, Eravacycline, Lymecycline, Metacycline, Minocycline, Omadacycline, Oxytetracycline, Penimepicycline, Rolitetracycline and Tetracycline
IMPORTANT ANTIMICROBIALS	
Antibiotic Class	Examples of Antibiotics Within Antibiotic Class
Aminocyclitols	Spectinomycin
Polypeptides	Bacitracin
Nitrofurans Derivatives	Furaltadone, Furazolidone, Furazidin, Nifurtoinol, Nitrofural and Nitrofurantoin
Nitroimidazoles	Metronidazole, Ornidazole, Secnidazole and Tinidazole
Pleuromutilins	Retapamulin

Footnote for Table 6.2*: Animal uses of antibiotics reported by WOAH* (OIE 2021a) *are highlighted in red (high (>20 %)), highlighted in yellow (medium (5-20%)) and highlighted in blue (low (<5 %)), with anything that was reported as 0 % by WOAH* (OIE 2021a) *left unhighlighted. **Use prohibited or restricted by the FDA in certain major FPAs. Anything highlighted in pink denotes that usage in FPAs is either heavily restricted or prohibited in most countries*

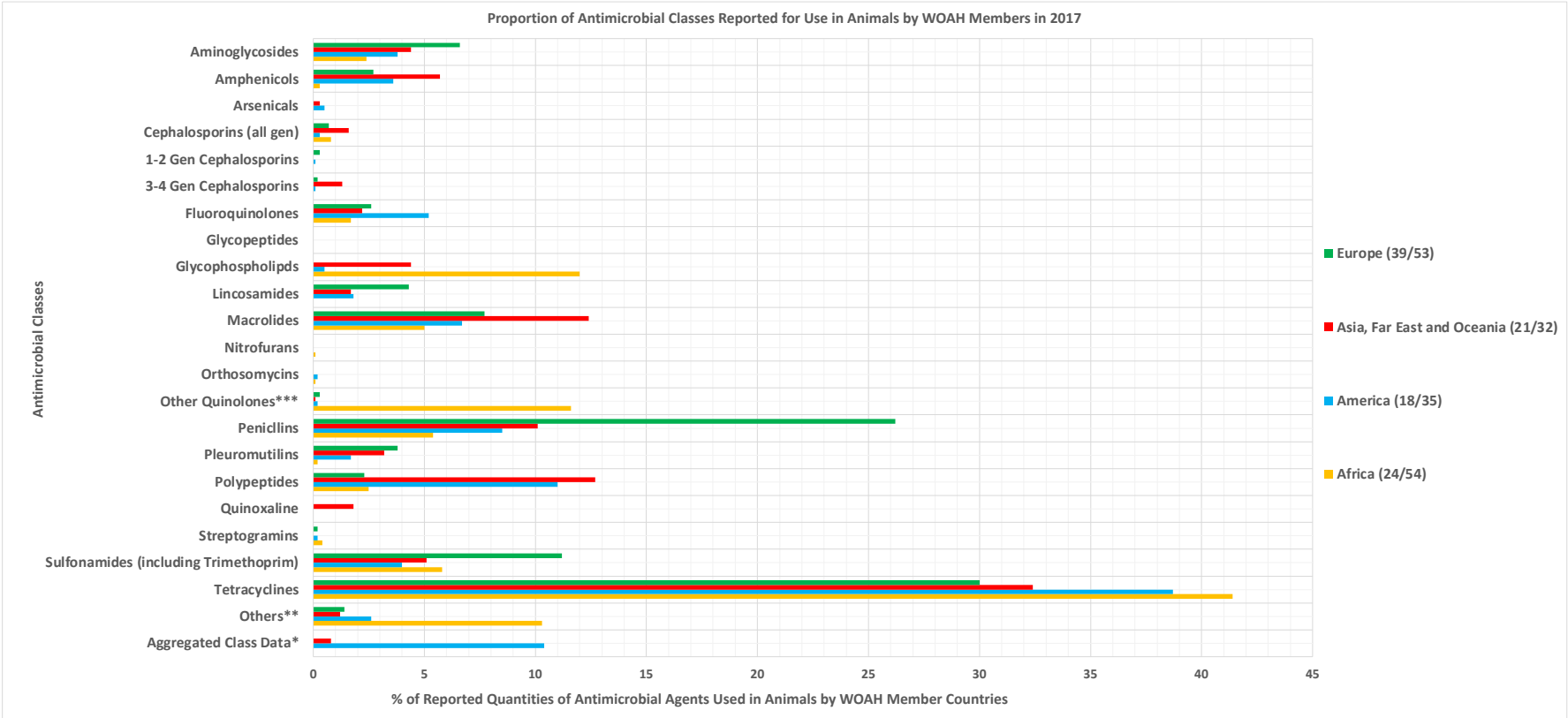


Figure 6.1: The proportion of antimicrobial classes reported for use in animals by the WOAH member states in 2017. Adapted from: (OIE 2021a). Aggregated Class Data* was used when classes could not be disclosed for confidentiality purposes at the national level. Under the others group, most of the countries reported fusidic acid or fosfomycin. Other Quinolones*** included flumequine, nalidixic acid, oxolinic acid and others**

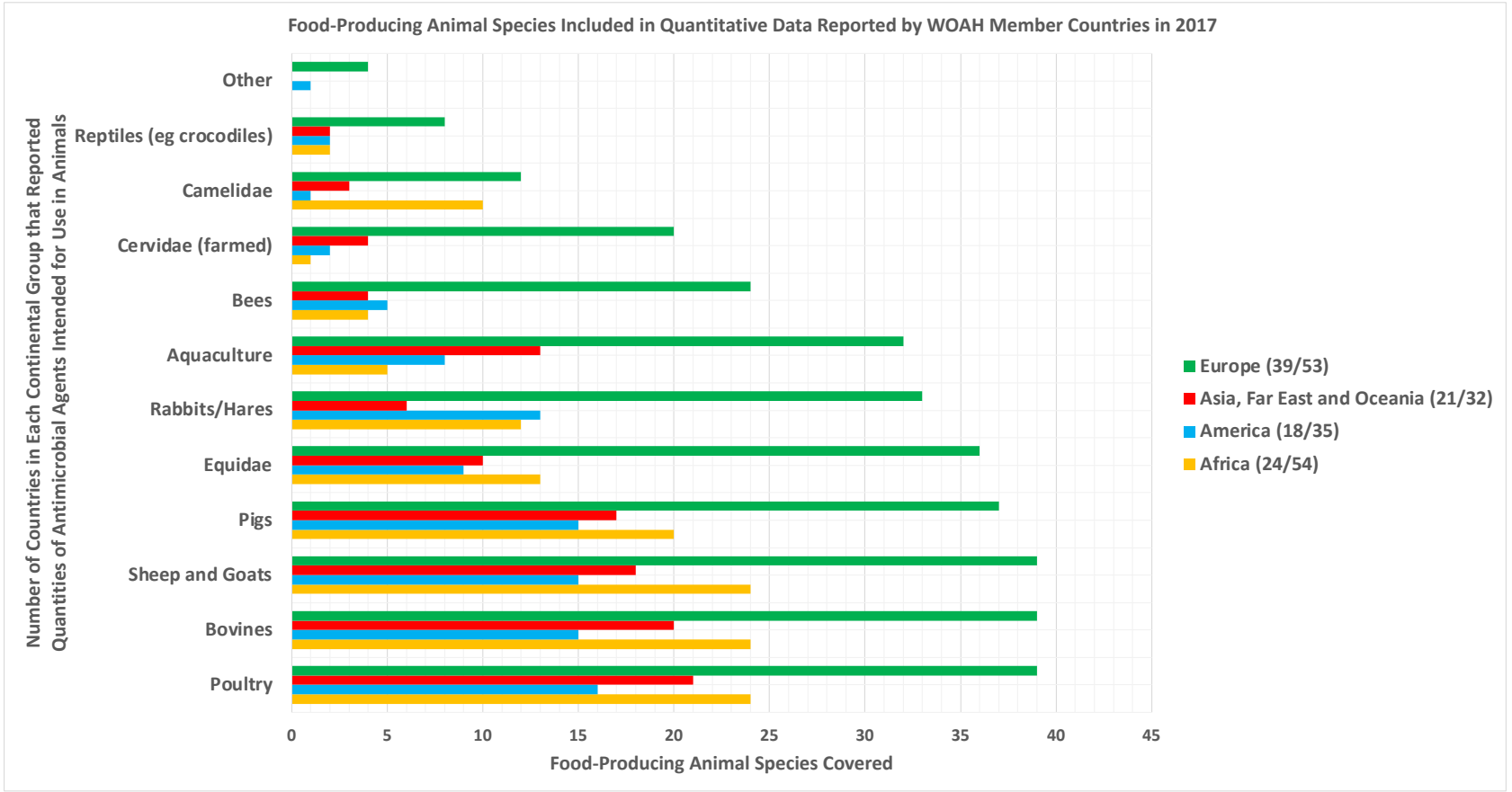


Figure 6.2: The FPA species that were included in the quantitative data that was reported by each WOAH member countries in 2017. Adapted from: (OIE 2021a)

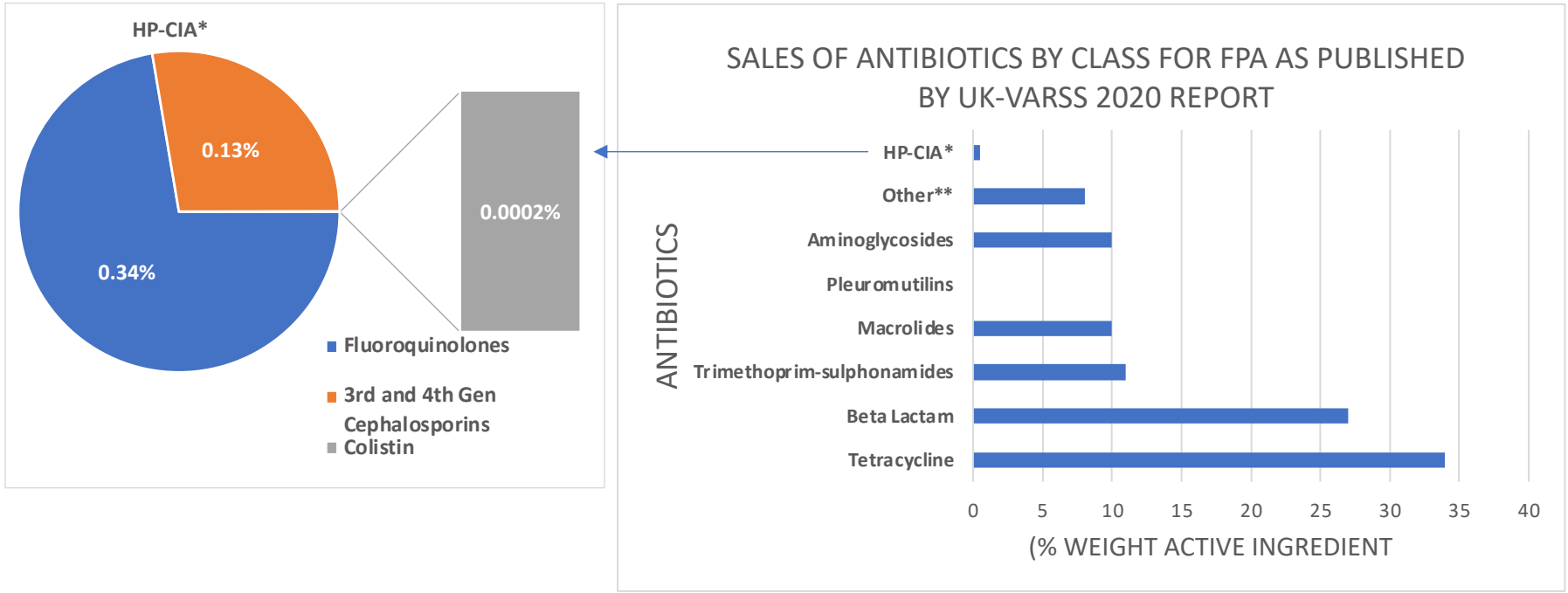


Figure 6.3: The data for sales of antibiotics by class in the UK for use in FPA, as published by the UK-VARSS 2020 report. Adapted from: (UK-VARSS 2020). HP-CIA* highest priority critically important antibiotics. OtherAmphenicols, lincomycins, pleuromutilins, steroidal antibiotics and imidazole derivatives**

6.1.2. How Do the Data from WOAH and WHO Compare to the Data Seen in This Study?

The classes of antibiotics listed as the highest priority CIAs for human health in **Table 6.2** included the quinolones, cephalosporins of 3rd or higher generation, macrolides and ketolides, glycopeptides and the polymyxins. As was explored within this thesis, resistance mechanisms for both 3rd and 4th generation cephalosporins and quinolones, were discovered within *E. coli* isolated from the dairy farm. **Figure 6.4** details the farm antibiotic usage data from June 2015 – December 2016. **Figure 6.5** details the farm antibiotic usage from January 2017 – May 2018 and also identifies the number of isolates sampled each month for this study, (pink dotted line with round markers), which included four in Jun 2017, six in July 2017, seven in August 2017, six in September 2017, three in October 2017, 21 in November 2017, 20 in December 2017 and 19 in January 2018. In **Figure 6.5**, the number of *bla*$_{\text{CTX-M-15}}$ isolates within those samples per month (red dashed line with diamond markers) and the number of confirmed overexpression of *ampC* isolates within those samples per month (black spotted line with square markers) are shown. The two usage graphs in **Figures 6.4** and **6.5**, demonstrate that the last use of 4th generation cephalosporins was in August 2015, the last use of 3rd generation cephalosporins was in January 2016 and the last use of 1st generation cephalosporins was in April 2017. However, the use of penicillins including penicillin G, amoxicillin and cloxacillin were almost consistent every month from July 2015 until May 2018. The isolates from both the *bla*$_{\text{CTX}}$ and *ampC* groups were all sampled from June 2017 – January 2018. The months of March and April 2017 saw the highest usage of penicillin G at 60 and 79 reported doses respectively, May 2017 and September 2017 saw the

highest usage of amoxicillin with 35 and 26 reported doses respectively and September 2017 and December 2017 saw the highest usage of cloxacillin at 13 and 9 reported doses respectively. The average reported number of doses of the three penicillins from January 2017 – May 2018 was 31.24 for penicillin G, 11.59 for amoxicillin and 5.41 for cloxacillin.

It was interesting that in the 86 isolates analysed in this study, the first appearance of overexpression of *ampC* was in September 2017 and the first appearance of *bla*$_{\text{CTX-M-15}}$ was in October 2017 and the highest usage of amoxicillin was reported in September 2017. No overexpression of *ampC* isolates or *bla*$_{\text{CTX-M-15}}$ isolates were seen in the 86 isolates from this study, any earlier than September 2017, despite the usage of penicillin in the months covering when the 86 isolates were sampled. Therefore, the usage of amoxicillin, could possibly indicate a potential selective pressure towards the maintenance of the beta-lactamase type resistance on this farm. The peaks for isolation of *ampC* and *bla*$_{\text{CTX-M-15}}$ were in November 2017 and December 2017 – January 2018 respectively. Interestingly the *ampC* peak came in a month that saw a slight increase in usage of penicillin G, but this did not appear to be significant, as there had been much higher usage of both penicillin G and amoxicillin in the months before and no overexpression of *ampC* was found in isolates from those months. However, what did appear to be significant was that in the months of December 2017 and January 2018 that saw increases in the isolation of *bla*$_{\text{CTX-M-15}}$. There was a large increase in the use of tetracycline in December 2017 and this could indicate co-selection of *bla*$_{\text{CTX-M-15}}$ due to the presence of the *tetAR* genes. In January 2018 tetracycline usage returned to low levels, however *bla*$_{\text{CTX}}$ remained high and it was in this month that the four isolates in the *bla*$_{\text{CTX}}$ group,

that were negative for *tetAR* were isolated. If tetracycline resistance was still the positive selection, this would be a surprising finding. One possible reason could be that tetracycline was an initial selector, but other factors then lead to maintenance of the *bla*$_{CTX}$ in the population and January saw another rise in amoxicillin usage. But of course, this would need to be explored experimentally to thoroughly investigate this hypothesis. Other possible factors that could result in peak being seen in recovery of beta lactamase resistance could be: 1) the dry cow therapy regime, which on this farm was blanket therapy of all cows at the same time but can be staggered in other herds, which could account for sudden increases in beta-lactam resistance, 2) The calving of the cattle, which can lead to increases in metritis, which on this farm was done as block calving and 3) whether the samples were taken from adults or calves, which for this study was only adults.

As was shown with the transposition experiments, the use of the penicillins ampicillin and cloxacillin could possibly contribute to the dissemination of the ESBL *bla*$_{CTX\text{-}M\text{-}15}$ on this dairy farm, showing that selective pressure for ESBL resistance may occur even in the absence of a cephalosporin when earlier penicillins are used. It is therefore important that not only antibiotic class but also related classes are considered when control strategies for resistance spread are implemented. As was demonstrated on this farm, the discontinuation of the use of 3rd and 4th generation cephalosporins, was not enough to prevent the isolation of *bla*$_{CTX\text{-}M}$ and it was highly likely that the continued use of penicillins and possibly the tetracyclines had been enough to maintain the beta-lactamase resistance on this dairy farm. Therefore, if co-selection is also possibly indicated, as was the potential case with tetracycline on this farm with *bla*$_{CTX\text{-}M\text{-}}$

15, additional unrelated classes also need to be considered, if resistance genes are encoded on the same mobile elements. This scenario of potential co-selection has been seen with other resistance genes such as *tetX* and it was believed that the use in agriculture of not only tetracyclines such as oxytetracycline but also the beta lactam ampicillin, has selected for *tetX* (Shen et al. 2018; He et al. 2019b).

When comparing the usage data from the farm in this study to the data produced by WOAH (OIE 2021a), tetracycline use, unlike the global usage, was not the highest used antibiotic on this farm in 2017-2018, although there was still consistent usage of tetracycline almost every month and this may have contributed to the maintenance of the *tetAR* resistance seen in many of the *bla*$_{\text{CTX}}$ group isolates and in isolate 99 in the *ampC* group. In addition, as was explored in **Section 3.5.3**, many of the isolates in the *bla*$_{\text{CTX}}$ group encoded *tetAR*, which was subsequently found to be in the same mobile element IS*Ecp1* as described in **Section 3.7.1**. In addition, other ST2325 isolates downloaded from Enterobase were found to be encoding both *tetAR* and a *bla*$_{\text{CTX-M}}$ variant (as shown in **Figure 3.10**). This could demonstrate that tetracycline selection could be significant in maintaining the *bla*$_{\text{CTX-M}}$ resistance mechanism, from the actions of co-selection due to *bla*$_{\text{CTX-M}}$ and *tetAR* being located on the same MGE, IS*Ecp1*.

This farm also saw high use of aminoglycosides, but as aminoglycoside resistance was not the focus of this study, it was possibly missed when isolates from the 1,000 strains in the EVAL farms collection were selected for each group, as there was bias towards beta-lactamase specific resistance. However, aminoglycoside resistance was noted in a few isolates in the *ampC* group including *aac2'* in 127, *ant3''* in 486 and *aph6* in 99, 486, 968 and 969.

In the months preceding the sampling of 99 at the end of June 2017, 127 at the start of July 2017 and 486 at the start of September, there was high usage of aminoglycoside antibiotics from March 2017 – August 2017, possibly indicating a selective pressure. The months of September and October saw a slight decrease in aminoglycoside usage, but usage began to rise again in November through to March 2018. Both 968 and 969 were sampled towards the end of January 2018 and again the appearance of aminoglycoside resistance, could have been due to the increased usage of aminoglycoside antibiotics at this this time. This was similar to what was seen with the tetracycline usage and the appearance of *tetAR* negative isolates when the usage of tetracyclines was lower. However, as these were only a few isolates, this was merely an observation when usage data was compared to what resistance was seen and when.

What can also be noted from the two usage graphs in **Figure 6.4** and **Figure 6.5** was that antibiotic usage was heavily reduced from 2015/2016 to 2017/2018. A previous study by Ibrahim et al (2016) which sampled 126 isolates from the same dairy farm in two sampling runs in 2014 and 2016, found the highest percentage of resistance was to ampicillin at 56.3 % which was followed by resistance to oxytetracycline, streptomycin, sulphonamides, cefotaxime and amoxicillin/clavulanic acid at 41.2 %, 39.6%, 38.8 %, 38 % and 33.3% respectively. In addition, both *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-32}}$ were discovered in two and six isolates respectively (however an additional six which appeared to be *bla*$_{\text{CTX-M}}$ types remained non-defined), *bla*$_{\text{OXA-1}}$ and *bla*$_{\text{TEM-1}}$ was each found in one isolate and *bla*$_{\text{OXA-1}}$ and *bla*$_{\text{TEM-30}}$ was found in one isolate. No overexpression of *ampC* in the dairy farm isolates was noted by Ibrahim et al (2016). The isolates sampled by EVAL farms and utilised in this study noted no

bla$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-32}}$ or *bla*$_{\text{TEM-30}}$ and there was very little sulphonamide and aminoglycoside (as noted above) resistance found. This could suggest the reduction in antibiotic usage on the farm from 2015/2016, could possibly have impacted the variety of resistance that was subsequently found in 2017/2018. However, a much more thorough investigation of the EVAL farms collection would need to be conducted and compared to the usage data to truly understand how usage may relate to resistance carriage. In addition, there was a large gap in the sampling between the two studies in 2014/2016 (Ibrahim et al. 2016) and 2017/2018 (Baker et al. 2022b). For a more thorough understanding, a survey of regular samplings against in depth usage data would need to be conducted to get a true insight. This could potentially help farmers and veterinarians to understand the long-term implications of antibiotic usage and provide a means of surveillance for monitoring antibiotic resistance.

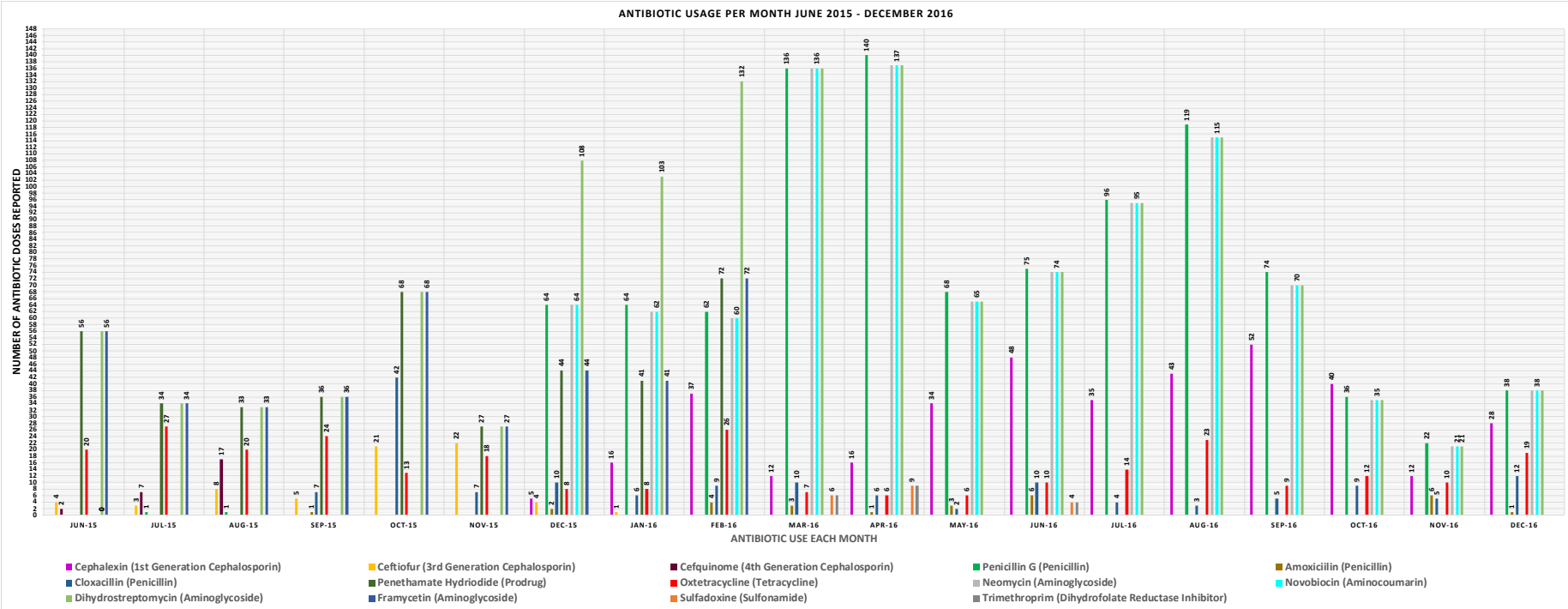


Figure 6.4: Antibiotic usage on the dairy farm within this study each month from June 2015 – December 2016, displayed as number of antibiotic doses reported each month

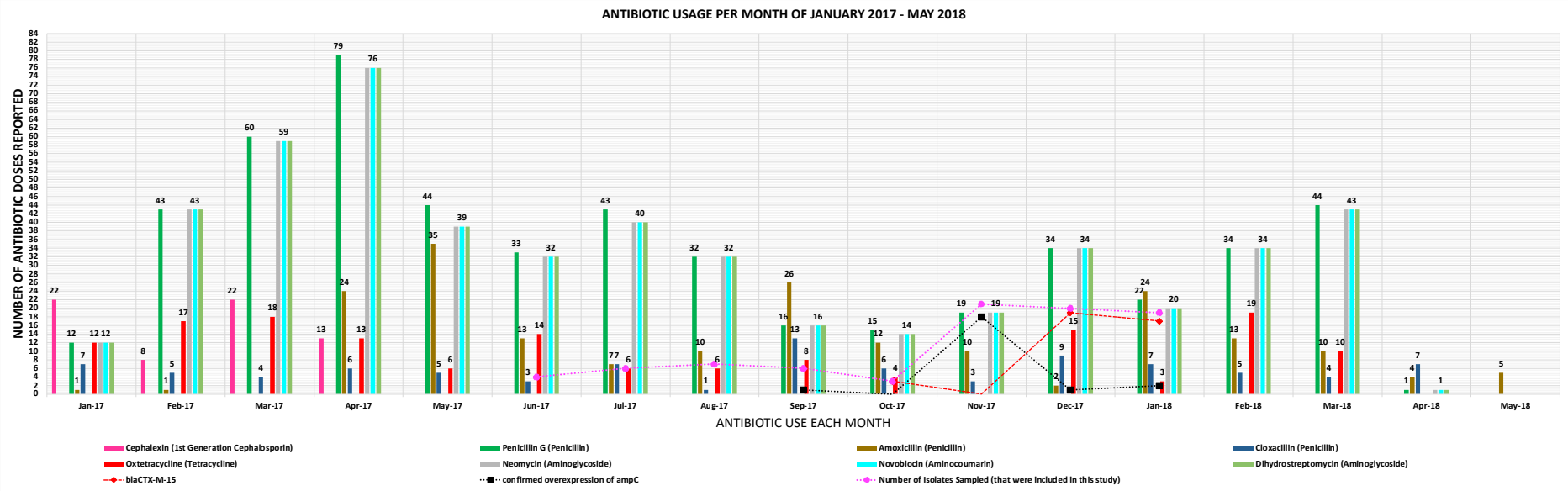


Figure 6.5: Antibiotic usage on the dairy farm within this study from January 2017 – May 2018, displayed as number of antibiotic doses reported each month

6.1.3. Implementation of Restrictions: What is Being Done to Combat the Growing Problem of Antibiotic Resistance Worldwide?

Currently there are many antibiotics and antibiotics of the same class on both the WHO's list of antibiotics critical to human health (WHO 2018; Scott et al. 2019b) and the WOAH's list of antimicrobial agents of veterinary importance (OIE 2021b) which are used in both humans and animals. The only exceptions are the glycopeptides, nitrofurans and nitroimidazoles whose usage is heavily restricted in food producing animals (WHO 2018). It is not surprising therefore that the usage of antibiotics in animals has been demonstrated to result in the appearance of resistance within humans. A good example of how the usage of antibiotics in animals may result in resistance to antibiotics in humans was given in **Section 1.2.1.1**, where avoparcin and VRE were discussed and the appearance of both *mcr-1* and *tetX* was also explored.

China has made a concerted effort in recent years to report on antimicrobial usage in their livestock farms (which was done for the first time in August 2019) and to reduce their antibiotic consumption and administration of antibiotics to animals. The discovery of the transmissible resistance gene *mcr-1* in China in bacterial species in both infected humans and in food production animals in 2015, could well have been the tipping point towards reducing antibiotic usage (Liu et al. 2016c; Wang et al. 2018; Schoenmakers 2020).

As a result of antibiotic usage in animals, the appearance of resistance genes has inevitably occurred, with two examples being *mcr-1* and *tetX* conferring resistance to the last line antibiotics colistin and tigecycline respectively, both of which were described in **Section 1.2.1.1**. The resistance

gene *mcr-1* first came to the attention of scientists when there were increased reports of colistin resistant bacteria in both humans and animals (Liu et al. 2016d; Wang et al. 2017; Wang et al. 2018). Liu et al. (2016d), conducted surveillance of antimicrobial resistance spread and control in animals and put tracking colistin resistance as a priority, when they noted colistin resistance was rapidly increasing in China. The resultant discovery of *mcr-1* was extremely concerning, as previously colistin resistance had been the result of chromosomal mutations; *mcr-1* however was found on a plasmid and as such was capable of HGT. Following the report of this discovery, *mcr-1* was found to be present globally, a devastating blow to the dwindling list of last line antibiotics (Liu et al. 2016c; Wang et al. 2018). The appearance of *mcr-1* may have been the trigger needed for change in antibiotic usage in animals to be implemented in China.

Another discovery that was described in **Section 1.2.1.1**, was *tetX* that confers resistance to the last line antibiotic tigecycline. Worryingly potential pan-resistant scenarios have been emerging with the discovery of resistance to carbapenems via bla_{NDM}, colistin via *mcr* and all tetracyclines via *tetX* and homologs found within the same MDR bacterial isolates (Lu et al. 2022). This one resistance gene *tetX* and its homologs threaten an entire class of antibiotic and with it a last line therapy. This example echoes previously seen scenarios, where consecutive generations of antibiotics have been made vulnerable to enzymatic inactivation, including the beta-lactams, aminoglycosides and amphenicols (Walsh 2000; Gasparrini et al. 2020). TetX is an example of a resistance mechanism that now poses a significant threat to the clinical efficacy of important antibiotics. Tetracyclines have been extensively used over the last 7 decades because they are broad spectrum, low cost and can be taken orally

(Thaker et al. 2010c; Nelson and Levy 2011b). Their widespread usage, and long environmental half-lives provide ideal selective pressures for tetracycline resistance to flourish in the environmental (Knapp et al. 2010), human and animal microbiota (Johnson and Adams 1992) and their carriage by bacterial pathogens (Chopra and Roberts 2001b; Gasparrini et al. 2020). As was shown in **Figure 6.1**, tetracycline was the most commonly used class of antibiotic globally in animals as reported by WOAH (OIE 2021a), demonstrating the maintenance of resistance determinants such as *tetX* and even *mcr-1* and bla_{NDM} (if they are present on the same MGE), is likely to continue. When this is related to the dairy farm in this study, as was discussed in **Section 6.1.2**, the maintenance of $bla_{CTX\text{-}M\text{-}15}$ could have been through the use of tetracycline selecting for *tetAR* located within the same mobile element IS*Ecp1*. This could have potential implications to the continued selection and spread of ESBL type resistance globally, even in the absence of beta-lactam usage and as was seen in **Figure 6.1**, tetracyclines are the most heavily used antibiotic class in farming worldwide.

6.1.4. A few Solutions Currently in Motion to Combat Antibiotic Resistance

Currently there is a continued gap in the discovery of new antibiotic classes, but studies are currently in motion to try and address this shortage. A good example is the INEOS Oxford Institute for Antimicrobial Research (available at: https://www.ineosoxford.ox.ac.uk/) which has been set up with a goal to try and discover new solutions to both animal and human AMR. In addition, the One Health approach that was described in **Section 1.6** aims at a global collaboration towards antibiotic stewardship, global awareness, the

reduction of antimicrobial use in agriculture, global surveillance and the implementation of preventative measures against bacterial infection such as improvements to sanitation (Mackenzie and Jeggo 2019).

However, it should not only be up to governments, medical professionals, pharmaceutical companies, veterinarians and farmers, changes to the relationship the consumer has with meat should also be considered. Currently meat is relatively cheap, can often be available at every meal (Funke et al. 2021) and is an important source of nutrition for many parts of the developed world (Bastian and Loughnan 2017; Hopwood et al. 2021). **Figure 6.6** shows the global meat supply per person as of 2017 (which was adapted from (Clark and Tilman 2017)) as reported by FAO from data available at: https://www.fao.org/faostat/en/#data. In **Figure 6.6** a heatmap against the global map, represents the average meat supply per person in kg. Meat supply per person was highest in high-income countries and the five countries with the highest meat supply per person are the US which was 124.1 kg, Australia (AUS) which was 121.6 kg, Argentina which was 109.38 kg, New Zealand (NZ) which was 100.89 kg and Spain which was 100.25 kg. The lowest meat supply per person was seen in Africa and the Indian Subcontinent.

Meat alternatives are available however and could provide the necessary means to lower the current burden on meat demand, as well as the burden placed on the environment through our current food production practices (Elzerman et al. 2011). In addition, raising animals requires large amounts of land to grow the crops required to feed them (Flachowsky et al. 2017) and with population numbers increasing, as was described in **Section 1.2**, something needs to give for the demand for protein to be addressed, without the need for

more animals to be raised using intensive farming practices and confinement rearing, more land to be utilised for this purpose and as a consequence more antibiotics used to keep animals disease-free. Therefore, one could argue, that the consumer also needs to play a role in moving towards aiding the reduction of antibiotic usage in animals, by reducing the pressure on meat demand. However, even with current meat production practices the elimination of antibiotics used in feed as growth promotors would have an important impact on the level of antibiotic usage.

Antibiotic usage is likely to remain a stable part of modern-day farming, but with greater understanding of how antibiotic usage may influence resistance spread, better management practices can be implemented, with the possible result of an overall reduction of multi-drug resistant bacteria and a positive move towards a successful one health approach outcome. There is no quick fix to AMR but by working together, globally a solution may be found by collaborative efforts.

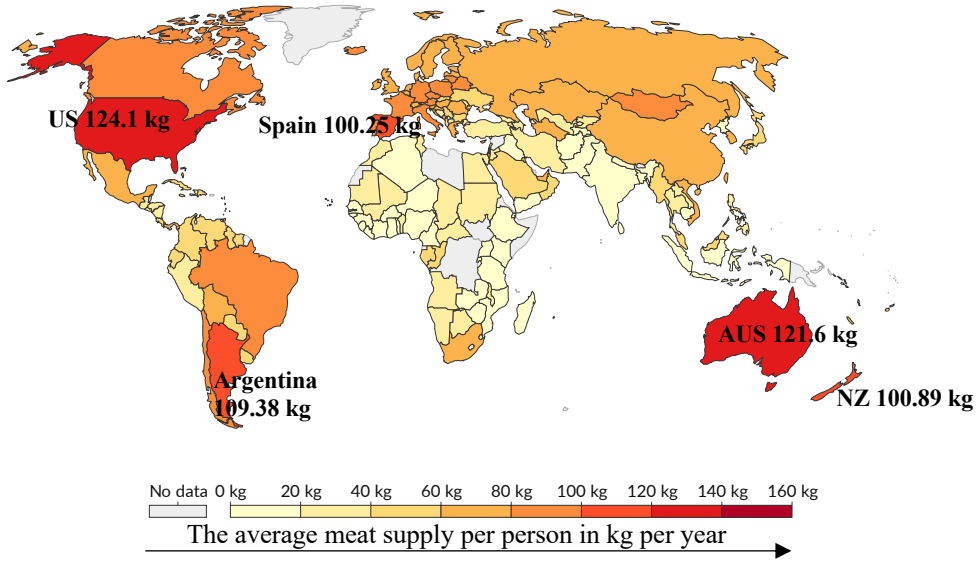


Figure 6.6: The global meat supply per person in kg as of 2017 with the five highest countries identified as US, AUS, Argentina, NZ and Spain. Adapted from: (Clark and Tilman 2017)

6.2. MAJOR NOVEL FINDINGS OF THIS STUDY

Major findings from the data generated as part of this study, was that *bla*$_{\text{CTX-M-15}}$ was chromosomally encoded and that there was the possibility for transmission of it within a dairy farm environment via IS*Ecp1*, even in the absence of antibiotic selective pressure. The ESBL *bla*$_{\text{CTX-M}}$ and its variants are frequently reported to be plasmid encoded (Bonnet 2004b; Cantón and Coque 2006b; Livermore et al. 2007b; Rossolini et al. 2008b; Naseer and Sundsfjord 2011; Cantón et al. 2012c; Zhao and Hu 2012; Tabar et al. 2016; Bevan et al. 2017c; Irrgang et al. 2017b), but there are some references to chromosomally encoded *bla*$_{\text{CTX-M}}$ in the literature (Hirai et al. 2013c; Ferreira et al. 2014; Rodríguez et al. 2014b; Hamamoto et al. 2016; Hamamoto and Hirai 2019; Gomi et al. 2022a). Therefore, the finding of chromosomally encoded *bla*$_{\text{CTX-M}}$ on this dairy farm was unusual but not completely novel, however it did demonstrate a possible reason for the stability of *bla*$_{\text{CTX-M-15}}$ on this dairy farm environment, in the absence of cephalosporin selective pressure. Guenther et al. (2017) found three ST38 *E. coli* isolates sampled from Mongolian birds of prey, that harboured chromosomally encoded *bla*$_{\text{CTX-M}}$ genes, that included *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-24,}}$ flanked by IS*Ecp1*, in the absence of antibiotic selective pressure. This demonstrated maintenance of resistance in environmental isolates, through stable integration into the chromosome, without the fitness costs of plasmid carriage and in the absence of antibiotic selective pressure. Chromosomally encoded *bla*$_{\text{CTX-M}}$ in association with IS*Ecp1* in ST38 has also been noted by Guiral et al (2011) in EAEC isolated from patients with diarrhoea at a hospital in Barcelona, Spain in 2005 and 2006, by Rodriguez et al. (2014c) from *E. coli* obtained in the UK, Germany and The Netherlands between 2005–

2009 and by Gomi et al. (2022b) from *E. coli* isolated from river water in Japan. This association between ST38 and chromosomal *bla*$_{\text{CTX-M}}$, could possibly indicate that chromosomal integration may be promoted by genetic mechanisms present within ST38 (Gomi et al. 2022b).

Chromosomally encoded genes are generally regarded as more stable and it is less costly in energy for the bacteria over maintaining a large resistance plasmid (Hirai et al. 2013d; Rodríguez et al. 2014d; Guenther et al. 2017). However, being chromosomally encoded did not mean there was the inability for HGT of *bla*$_{\text{CTX-M-15}}$ on the dairy farm in this study, as IS*Ecp1* was found upstream of *bla*$_{\text{CTX-M-15.}}$ As was shown in **Chapter 4**, IS*Ecp1* was capable of mobilising the chromosomally located *bla*$_{\text{CTX-M-15}}$, by utilising the resident plasmids as vectors and transposition was enhanced under selective pressure at sub-inhibitory antibiotic levels and even occurred without selective pressure. Enhanced transposition under the selective pressure of sub-lethal levels of AMP, CLOX and CAZ was a worrying finding, as these are similar concentrations to those that might be found within faeces or wastewater. However, beta-lactams are highly susceptible to hydrolysis, which in most surface waters for example occurs over several weeks (Polianciuc et al. 2020). So, any affects sub-lethal levels of these antibiotics might have on the IS*Ecp1* transposition within the dairy farm environment, would likely have occurred *in vivo* within the bovine gut, within the udder itself or during the withdrawal period. In the bovine gut temperature, nutrients and high levels of bacteria provide ideal conditions for HGT and during the withdrawal periods in cattle, there may also be sub-lethal levels of antibiotics present, so this could also be an ideal time for HGT to be stimulated. Alternatively sub-lethal levels of antibiotics may have an affect soon

after excretion from cattle or through mastitic waste milk contaminated with antibiotics entering the slurry tank. However, once in the slurry tank, it is likely the conditions would be less than ideal for HGT, with low temperatures, less nutrients and high dilution levels, as well as competition. However, IS*Ecp1* transposition occurred experimentally within this study without selective pressure, therefore it is likely antibiotics are not essential for IS*Ecp1* transposition to readily occur. However, the presence of sub-lethal levels did enhance IS*Ecp1* transposition, therefore this study should stand as one example of why care should be taken regarding correct disposal of contaminated waste, should that be on a farm or from human waste such as a hospital environment. Other studies have also shown that farm waste (Brooks et al. 2014; Casanova et al. 2020), urban and landfill (Rizzo et al. 2013; Anand et al. 2021) or hospital waste (Hocquet et al. 2016; Chi et al. 2020), may result in resistance dissemination into the environment, creating environmental reservoirs of resistance genes, promoting HGT and the transfer of antimicrobial resistance genes between pathogenic and non-pathogenic bacteria (Kraemer et al. 2019).

Another interesting insight was that most of the plasmids found in the 86 isolates, were cryptic, with only one IncI1 plasmid found to be encoding resistance genes that included *bla*$_{\text{TEM-1}}$, *aph3", aph6, floR, sul2* and the only plasmid encoded tetracycline resistance via *tetAR*. The majority of the multi-drug resistance seen in the isolates of this study, was provided by only three chromosomally located resistance mechanisms namely *bla*$_{\text{CTX-M-15}}$, *tetAR* and overexpression of *ampC*. This absence of plasmid encoded resistance could signify that the reduction in antibiotic usage from 2015-2018 on this particular dairy farm, that was seen in the usage graphs in **Figure 6.4** and **Figure 6.5**, could

have resulted in the retention of chromosomally encoded resistance but not plasmid encoded resistance. This could provide a possible reason for the lack of plasmid encoded resistance seen in this study, although the loss of plasmids following recovery from frozen culture could also have been a factor in this. However, only 86 isolates were analysed as part of this study and this insight into plasmid encoded resistance, would need a more thorough examination of a greater number of *E. coli* from the EVAL farms collection, to thoroughly explore this hypothesis.

One thing that was noticeable between the two groups of strains, was the low diversity of virulence genes from the *bla*$_{CTX}$ group compared to those found in the *ampC* group. This does in part reflect the very clonal nature of the *bla*$_{CTX}$ group in comparison to the greater diversity of the *ampC* group. However, this difference in diversity of virulence genes could again suggest that the use of a range of isolation media influenced the selection of different subpopulations in an environment, as these groups were definitely selective media associated. Therefore, the isolation media should also be considered as a factor when analysing a complex environment such as a dairy farm. No particular pathotypes were associated with virulence factor carriage. However, no specific pathogenicity factors are associated with bovine mastitis and known virulence factors will often be absent from mastitis causing *E. coli* (Burvenich et al. 2003; Fernandes et al. 2011; Blum and Leitner 2013; Suojala et al. 2013; Liu et al. 2014; Nüesch-Inderbinen et al. 2019). So, the lack of specific virulence genes in the *bla*$_{CTX}$ group, would not preclude these *E. coli* as a causative agent of mastitis and in this dairy farm, the cattle were treated with beta-lactam antibiotics. It would have been advantageous to have conducted a more thorough investigation

of virulence from the EVAL farms collection, by analysing isolates that were sampled on a variety of different media, to see how they differed in virulence gene carriage and therefore their potential pathogenesis and potential to cause disease in other hosts such as humans. The media chosen may result in different sub-populations of *E. coli* being selected and additional samplings using, for example, tetracycline as the antibiotic selective agent and a selective medium such as TBX or CHROMagar ESBL, could have been an interesting experiment to conduct. Completely non-selective media, such as nutrient media with or without an antibiotic selection however would not be suitable, as it would be impossible to separate out the *E. coli* from the background level of organisms. Therefore, some form of selection in the form of chromogenic media for example and an antibiotic selection, is required to analyse the fraction of the bacterial population of interest. This of course however does produce a level of bias and therefore the bacteria selected would be representative of only a subpopulation of the population as a whole. Therefore, this study examined only a small portion of a much more complex bacterial population. The study by Ibrahim et al. (2016) that was conducted on the same farm as this study used different media that included non-antibiotic and antibiotic selective media and isolated not only different levels of resistance, but also different subpopulations of *E. coli* were obtained on each medium used. In conclusion, any study will always be limited by the isolation conditions utilised.

6.3. FUTURE WORK

The two groups of isolates analysed within this study demonstrated that at least two major beta-lactamase resistance mechanisms were at play within the dairy farm environment that included *bla*$_{\text{CTX-M-15}}$ and overexpression of *ampC*. A total of 86 isolates were phenotypically analysed via MIC assays, but as isolates were selected from the EVAL farms collection only for their beta-lactamase phenotype, it would have been interesting to conduct further MICs on a greater number of isolates from the EVAL farms study, without the bias of selecting isolates with a beta-lactamase phenotype only. This would have allowed for a greater number of resistance mechanisms to be explored and to discover if there were other dominant types of resistance present on the dairy farm and how this coincided with the antibiotic usage data shown in **Figure 6.5**.

The most significant drawback of this study was the small sample size of the two groups that included *bla*$_{\text{CTX}}$ and *ampC*, although this did allow a much more in-depth analysis to be conducted. In addition, meta-data was not available and factors such as farm practice in relation to disinfection and cleaning and the handling of infections/presence of endemic infection can all influence AMR (Murphy et al. 2018; Moennighoff et al. 2020). In addition, samples were not collected from sick animals or wound sites for example, but from the living environment of healthy dairy cattle and the waste disposal system designed to deal with their faeces and urine. Therefore, it was difficult to allocate strains encoding what might predict a pathotype to a specific disease. What could be deduced was the pathogenic potential of strains on their presenting genotype as was explored in the *ampC* isolates in **Section 5.4.5**. However, it might have been an interesting insight, to explore virulence in a great number of isolates from the

EVAL farms study, to discover the variety of virulence factors in *E. coli* and the possibility on this dairy farm for the appearance of a pathogenic *E. coli* with the potential to cause disease through the food chain into humans.

All 86 isolates were analysed genotypically via WGS and it was noted that all plasmids within the *bla*$_{CTX}$ group were cryptic with the resistances encoded within the IS*Ecp1* element in all but isolate 962, where the Tn*AS1* encoding *tetAR* was found separate and alone in another contig (as described in **Section 3.7.1.2**). As noted in **Section 3.4**, no plasmids were found within 774, so enhanced transposition, using a plasmid introduced into this strain, was considered to discover whether the IS*Ecp1* could still mobilise away from the chromosome of 774. An RP4 plasmid encoding gentamicin resistance was purchased for this purpose and was successfully conjugated into 774 (data not included in this thesis), but no further experimental work was conducted on this. However, what it did demonstrate was 774 could successfully uptake a large plasmid via conjugation and so it would be interesting to complete the remaining work to determine if IS*Ecp1* transposition could be achieved.

It would have been interesting to look at the maintenance of *bla*$_{CTX\text{-}M}$ in the chromosome under serial passage when grown in the presence of sub-lethal levels of either a beta-lactam such as penicillin G, a tetracycline or in a non-selective environment. The *tetAR* negative isolates 950, 953, 955 and 956 and isolate 962, where the *tetAR* was not located in the IS*Ecp1* element region, could also act as a type of control in this experiment, to show whether the presence of tetracycline resistance genes within the IS*Ecp1* element region would select for *bla*$_{CTX\text{-}M\text{-}15}$ under tetracycline selective pressure. This could have answered the potential questions of whether penicillin G or tetracycline can

maintain the *bla*$_{\text{CTX-M}}$ mechanism and by comparing to the non-selective environment, just how stable within the chromosome the IS*Ecp1* element was. In addition, this experiment could have been conducted with some of the TTs to investigate the stability of the plasmid encoded IS*Ecp1* in association with *bla*$_{\text{CTX-M-15}}$ and whether the frequency of transposition into the chromosome was a rare event or not, as was seen in TT 876CLOX64 discussed in **Section 4.2.4.4**.

Valentine et al. (1988), Torres et al. (1991), Showsh and Andrews (1992) and Whittle et al. (2002) have shown that tetracycline may promote MGE element dissemination, and tetracycline is well known to persist in the environment such as aquatic systems (Jeong et al. 2010; Shao and Wu 2020) and is often found within animal manure and wastewater (Zhu et al. 2013; Gasparrini et al. 2020). However, as was discussed in **Section 4.5**, it was believed that IS*Ecp1* mobilisation was enhanced possibly as a result of the induction of the SOS response and tetracycline is not an inducer of the SOS response (Baharoglu and Mazel 2011). Therefore, sub-lethal levels of tetracycline in the environment of the dairy farm where the isolates from this study were sampled, would possibly only promote IS*Ecp1* dissemination through the co-selection of the *tetAR* genes in the +*tetAR* isolates. Whether this could also happen in the Δ*tetAR* isolates would be interesting to investigate, to see whether IS*Ecp1* transposition could also be promoted in the absence of the tetracycline resistance genes *tetAR*. As tetracyclines are heavily used in farming worldwide as was stated in **Section 6.1.1** and shown in **Figure 6.1**, the promotion of IS*Ecp1* transposition in association with *bla*$_{\text{CTX-M-15}}$ through the use of tetracycline, would be an interesting assay to conduct. This would explore just how much influence

tetracycline might have to MGE dissemination in an environment such as a dairy farm.

An important question that remained unanswered within the isolates from the overexpression of *ampC* group, was the transient resistance that was initially seen in the disc assay data provided by EVAL farms. This transient resistance was no longer present following MIC assays and the question of whether other mechanisms were at play remained unanswered in this study. This of course could have been due to errors associated with the disc diffusion method, but other mechanisms could have been responsible for this occurrence and could include overexpression of efflux and under expression of porins. This can be easily elucidated with efflux assays such as the methods outlined by Blair and Piddock (2016) or alternatively through qPCR methods, to detect efflux gene expression levels as described by Chetri et al. (2019). Porin gene expression in response to an external stressor such as tetracycline, for example. which has been shown to decrease porin levels (Viveiros et al. 2007), could also be investigated through methods such as qPCR, to quantify transcription levels of porin associated genes, as outlined by Viveiros et al. (2007). Other studies by Vinson et al. (2010) and Chetri et al. (2019) have also used qPCR to quantify porin gene transcription levels. It was believed that possibly the TBX selective media containing bile salts that the *E. coli* samples were initially isolated on, may have played a role in this. Alternatively, stressors from the environment where isolates were sampled, such as tetracycline could have affected efflux or porin expression. Therefore, it would be advantageous, for this experimental work to have been conducted soon after isolates had been sampled. Alternatively, experiments could be conducted by growing bacteria in the presence of bile salts,

to see if resistances changed when bacteria were grown in the presence and absence of bile salts. Bile salts are known to affect efflux and porin expression levels, so it would be good to test this hypothesis. This could also possibly demonstrate that *E. coli in vivo* may have the potential for transient resistance to antibiotics, when they're exposed to bile salts in their normal environment of the human gut for example. Therefore, it would be interesting to make a comparison study, between isolates freshly sampled from the environment and those isolates that have been frozen down, stored at -80 °C and re-revived as fresh cultures. This would demonstrate the influences transient resistance mechanisms might have been playing and whether the loss of plasmid encoded resistance could have occurred.

Antimicrobial resistance is a global problem and requires a global effort, following the one health approach towards positive change. International cooperation, support for the developing world, surveillance of AMR and novel pathogens, sharing of data and policy implementation all need to continue, if we are to keep this vital treatment option viable for future generations.

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APPENDIX A: DISC DIFFUSION ASSAY METHODS USED BY EVAL FARMS

Disc diffusion assays were conducted according to Clinical & Laboratory Standards Institute (CLSI, 2012; CLSI, 2015). Discs used along with antibiotic concentrations and zone clearing sizes are listed in **APPENDIX B**. Isolates were revived from frozen stocks along with the control ATCC25922, by plating on to LB agar (**Section 2.1.1**) and incubated for between 18-20 hours at 37 °C. MH Broth was prepared to a final concentration of 2 g L^{-1} beef infusion solids, 17.5 g L^{-1} casein hydrolysate and 1.5 g L^{-1} starch. RO water was added to bring to required volumes prior to autoclaving (**Section 2.1**).

A direct colony suspension was made by picking a single colony from the revived isolates and dispersing it in 5 ml of MH broth to achieve a turbidity of 0.5 McFarland standard (Oxoid, UK) when compared to a Wickerham card as was seen in **Figure 2.1**.

The bacterial suspension was then spread evenly onto a MH agar (**Section 2.1.2**) plate using a cotton bud and left to dry at room temperature, for a maximum of 15 minutes, prior to disc application to the plates. Discs were applied to the plates using either a multi disc dispenser (Pro-Lab Diagnostics Inc, UK) or sterile forceps. Plates were then incubated for between 18-20 hours at 37 °C. Results were recorded the following day using a ruler to measure the diameter of the zone of inhibition around the antibiotic discs.

APPENDIX B: DISC CONCENTRATIONS, DISC SUPPLIER AND ZONE CLEARING SIZES

Antibiotic Class	Name	Abbrv.	Disc Conc (µg)	Zone Clearing Sizes (mm)			Disc Supplier
				Res	Interm	Susc	
β-Lactam	Ampicillin	AMP	10	>17	14-16	<13	ProLab
β-Lactam/β-Lactamase Inhibitor	Amoxicillin/Clavulanic Acid	AMC	20/10	>18	14-17	<13	ProLab
2nd Generation Ceph	Cefoxitin	FOX	30	>18	15-17	<14	ProLab
3rd Generation Ceph	Ceftazidime	CAZ	30	>21	18-20	<17	ProLab
3rd Generation Ceph	Cefotaxime	CTX	30	>26	23-25	<22	ProLab
3rd Generation Ceph	Ceftiofur	EFT	30	>23	20-22	<19	Oxoid
3rd Generation Ceph	Cefpodoxime	CPD	10	>21	18-20	<17	ProLab
4th Generation Ceph	Cefquinome	CFQ	30	>23	20-22	<19	Bioconnections
Monobactam	Aztreonam	ATM	30	>21	18-20	<17	ProLab
Carbapenem	Imipenem	IMP	10	>23	20-22	<19	ProLab
Aminoglycoside	Streptomycin	STREP	10	>15	12-14	<11	ProLab

Tetracyclines	Tetracycline	TET	30	>15	12-14	<11	ProLab
Fluoroquinolones	Ciprofloxacin	CIP	5	>21	16-20	<15	ProLab
Fluoroquinolones	Enrofloxacin	ENR	5	>26	19-25	<18	Oxoid
Quinolone	Nalidixic Acid	NAL	30	>19	14-18	<13	ProLab
Folate Pathway Inhibitors	Sulphonamides	SULP	300	>17	13-16	<12	Oxoid
Folate Pathway Inhibitors	Trimethoprim-Sulfamethoxazole	SXT	1.25/23.75	>16	11-15	<10	ProLab
Phenicols	Chloramphenicol	CHLOR	30	>18	13-17	<12	ProLab
Nitrofurans	Nitrofurantoin	NIT	300	>17	13-16	<12	ProLab
Macrolides	Azithromycin	AZM	15	>13	12-14	<11	ProLab

APPENDIX C: FULL META DATA FOR ALL 105 ST2325 E. COLI DOWNLOADED FROM ENTEROBASE

Clonal Groups	Uberstrain on Enterobase	Download Name	Isolate Name	ISEcp1	blaCTX-M Type	Other Beta Lactamase Genes	qnrS1	tetAR	Niche	Sample Type	Sample Details	Simple Pathogen	Isolated	Country	Species	Bio Project ID	Accession Number	Date Entered	Date Released
	ESC_BA0715AA	ESC_CA3639AA	P1a						ND	ND	ND		Apr-14	US	E. coli	PRJNA218110	SAMN03570387	27/08/2015	30/04/2015
	ESC_BA1097AA	ESC_BA1014AA	AZ-TG71259	✓		blaCMY-2			Livestock	Avian	Packaged Turkey		Nov-09	US	E. coli	PRJNA230968	SAMN02463237	27/08/2015	20/04/2015
	ESC_BA7973AA	ESC_CA4118AA	02_24_007_10-sc-2013-10-16T09:20:59Z-1720372						ND	ND	ND	ETEC	Feb-14	UK	E. coli	PRJEB2581	SAMEA2223745	27/08/2015	14/05/2014
	ESC_CA0328AA	ESC_CA1603AA	1120			blaTEM-1			ND	ND	ND		Jun-12	UK	E. coli	PRJEB2879	SAMEA1324945	27/08/2015	04/08/2012
Group 1	ESC_CA2667AA	ESC_FA6543AA	VREC0390			blaTEM-1	✓	✓	Wild Animal	(Wild Boar)	Faeces		Oct-15	UK	E. coli	PRJEB8774	SAMEA3753065	23/10/2015	21/10/2015
Group 1	ESC_CA2671AA	ESC_FA6545AA	VREC0362			blaTEM-1	✓	✓	Wild Animal	Sus scrofa (Wild Boar)	Faeces		Oct-15	UK	E. coli	PRJEB8774	SAMEA3752374	23/10/2015	21/10/2015
Group 1	ESC_CA2672AA	ESC_FA6550AA	VREC0361			blaTEM-1	✓	✓	Wild Animal	Sus scrofa (Wild Boar)	Faeces		Oct-15	UK	E. coli	PRJEB8774	SAMEA3753289	23/10/2015	21/10/2015
	ESC_CA3624AA	ESC_VA5255AA	NCTC9066						ND	ND	ND		Aug-15	UK	E. coli	PRJEB6403	SAMEA3376909	29/10/2015	05/08/2015
Group 2	ESC_CA5117AA	ESC_FA9712AA	VRES0498	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3752611	14/01/2016	13/01/2016
Group 2	ESC_CA5118AA	ESC_FA9713AA	VRES0497	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3753392	14/01/2016	13/01/2016
Group 2	ESC_CA5119AA	ESC_FA9714AA	VRES0495	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3752610	14/01/2016	13/01/2016
Group 2	ESC_CA5185AA	ESC_FA9780AA	VRES0500	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3752311	14/01/2016	13/01/2016
Group 2	ESC_CA5186AA	ESC_FA9781AA	VRES0499	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3751401	14/01/2016	13/01/2016
Group 2	ESC_CA5187AA	ESC_FA9782AA	VRES0496	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3752310	14/01/2016	13/01/2016
Group 2	ESC_CA5188AA	ESC_FA9783AA	VRES0494	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3753391	14/01/2016	13/01/2016
Group 2	ESC_CA5190AA	ESC_FA9785AA	VRES0491	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3753144	14/01/2016	13/01/2016
Group 2	ESC_CA5198AA	ESC_FA9793AA	VRES0490	✓	15	blaTEM-1, blaTEM-102	✓	✓	Livestock	Bovine	Faeces		Jan-16	UK	E. coli	PRJEB8776	SAMEA3753387	14/01/2016	13/01/2016

	ESC_CA9326AA	**ESC_GA5264AA**	VREC0186					✓	ND	ND	ND		Jul-15	UK	E. coli	PRJEB8768	SAMEA3304077	01/04/2016	29/07/2015
	ESC_DA3522AA	**ESC_HA7286AA**	MOD1-EC6903			blaTEM-1			Wild Animal	Deer (Cervidae)	Faeces		Sep-97	US	E. coli	PRJNA230969	SAMN04992269	30/06/2016	01/01/2015
	ESC_DA7410AA	**ESC_HA4055AA**	C70-sc-2235953						Homo sapien	Human (children)	Stool	EAEC	Aug-15	Nigeria	E. coli	PRJEB8667	SAMEA3322052	01/07/2016	25/08/2015
	ESC_DA9011AA	**ESC_HA2283AA**	BCW_3313			blaTEM-1			ND	ND/Others	Culture		Sep-16	US	E. coli	PRJNA203445	SAMN03358875	01/07/2016	24/02/2016
	ESC_EA2302AA	**ESC_HA8256AA**	MOD1-EC6679			blaTEM-101, blaTEM-102, blaTEM-104			Livestock	Bovine	Faeces		May-92	US	E. coli	PRJNA230969	SAMN04992509	30/07/2016	01/01/2015
	ESC_EA3024AA	**ESC_HA9080AA**	MOD1-EC5868			blaTEM-1			Livestock	Bovine	Faeces		May-85	US	E. coli	PRJNA230969	SAMN05468043	02/08/2016	01/01/2015
	ESC_EA3418AA	**ESC_HA9268AA**	1601116	✓	15	blaOXA-1	✓	✓	Livestock	ND/Others	Faeces		2016	LUX	E. coli	NA	NA	09/08/2016	11/02/2017
	ESC_EA6673AA	**ESC_IA5972AA**	1ad1bd70-fa7c-11e5-ae86-3c4a9275d6c8			blaTEM-106	✓	✓	ND	ND	ND		Sep-16	UK	E. coli	PRJEB12887	SAMEA3980700	02/10/2016	30/09/2016
	ESC_EA9865AA	**ESC_IA9637AA**	SCP05-19	✓	15		✓		Homo sapien	Human	Routine Clinical Samples		Oct-16	NTL	E. coli	PRJEB15226	SAMEA4428209	24/10/2016	18/10/2016
	ESC_FA2259AA	**ESC_JA5203AA**	SAMPLE_WTCHG_320308_234190		9				ND	ND	ND		Nov-16	UK	E. coli	PRJEB17631	SAMEA4533609	14/11/2016	11/11/2016
	ESC_FA6578AA	**ESC_LA0878AA**	bbb83b10-fa7c-11e5-a43c-3c4a9275d6c8			blaTEM-1	✓	✓	Livestock	Poultry	Faeces		Jan-17	Vietnam	E. coli	PRJEB12887	SAMEA4061745	15/01/2017	12/01/2017
	ESC_GA2061AA	**ESC_MA4137AA**	0e5c0980-8b0c-11e6-95da-3c4a9275d6c8						Homo sapien	Human (children)	Faeces	ETEC	Apr-17	Nepal	E. coli	PRJEB18106	SAMEA4560397	14/04/2017	14/04/2017
	ESC_IA0439AA	**ESC_RA0679AA**	ERS1912973	✓	15				Livestock	Dairy Cattle	Raw Milk		Nov-17	GER	E. coli	PRJEB22381	SAMEA104287995	30/11/2017	28/11/2017
	ESC_IA4476AA	**ESC_RA5430AA**	CFSAN066364						Livestock	Bovine	Faeces		2016	Chile	E. coli	PRJNA230969	SAMN07446236	23/02/2018	22/02/2018
	ESC_IA7287AA	**ESC_RA9754AA**	PSU-0631						Livestock	Bovine	Faeces		2000	US	E. coli	PRJNA357722	SAMN08978130	26/04/2018	25/04/2018
	ESC_IA8337AA	**ESC_SA1185AA**	FSIS11809767						Livestock	Poultry	Faeces		2018	US	E. coli	PRJNA292667	SAMN09195618	11/05/2018	11/05/2018
	ESC_JA6888AA	**ESC_UA6569AA**	PSU-0827						Livestock	Bovine	Faeces		2018	US	E. coli	PRJNA357722	SAMN09725195	29/07/2018	27/07/2018

	ESC_KA4064AA	**ESC_UA1780AA**	CVM N17EC0674						Livestock	Avian	Ground Turkey		2017	US	E. coli	PRJNA292663	SAMN10221282	23/10/2018	23/10/2018
	ESC_KA4167AA	**ESC_UA1677AA**	CVM N17EC0080						Livestock	Avian	Ground Turkey		2017	US	E. coli	PRJNA292663	SAMN10220997	23/10/2018	23/10/2018
	ESC_KA7394AA	**ESC_VA2816AA**							Homo sapien	Human (children)	Stool	EAEC	Aug-15	Nigeria	E. coli	PRJEB8667	SAMEA104288136	05/12/2018	02/12/2018
	ESC_KA8750AA	**ESC_VA5571AA**	14S02359-2	✓					Livestock	Bovine	Faeces		2014	NTL	E. coli	PRJEB30024	SAMEA5164758	02/01/2019	02/01/2019
	ESC_LA1564AA	**ESC_WA1933AA**	AG19-0026	✓					Livestock	Bovine	Faeces		Dec-18	US	E. coli	PRJNA338676	SAMN10880963	06/02/2019	06/02/2019
Group 4	**ESC_LA5082AA**	**ESC_XA6605AA**	GER_MD11_1505_Eco_023						Companion Animal	Canine (Military dog)	Faeces		May-15	GER	E. coli	PRJNA433857	SAMN08519229	11/02/2019	14/07/2018
	ESC_LA5086AA	**ESC_WA5695AA**	GER_MD11_1505_Eco_027						Companion Animal	Canine (Military dog)	Faeces		May-15	GER	E. coli	PRJNA433857	SAMN08519233	11/02/2019	14/07/2018
	ESC_LA5087AA	**ESC_WA5694AA**	GER_MD11_1505_Eco_029						Companion Animal	Canine (Military dog)	Faeces		May-15	GER	E. coli	PRJNA433857	SAMN08519234	11/02/2019	14/07/2018
	ESC_LA9821AA	**ESC_XA0445AA**	PSU-1067						ND	ND	ND		2003	US	E. coli	PRJNA357722	SAMN09060805	27/02/2019	27/02/2019

Group 5	ESC_MA4516AA	ESC_YA9441AA	US_ESBL051	✓				Livestock	Ovine/Goat (Caprinae)	Carcass swab (Sheep)		Mar-19	US	E. coli	PRJNA293225	SAMN11491431	24/04/2019	24/04/2019
	ESC_MA4519AA	ESC_YA9438AA	US_ESBL048	✓				Livestock	Ovine/Goat (Caprinae)	Carcass swab (Sheep)		Mar-19	US	E. coli	PRJNA293225	SAMN11491433	24/04/2019	24/04/2019
	ESC_MA4521AA	ESC_YA9436AA	US_ESBL021	✓				Livestock	Ovine/Goat (Caprinae)	Feces (Sheep)		Mar-19	US	E. coli	PRJNA293225	SAMN11491429	24/04/2019	24/04/2019
	ESC_MA4523AA	ESC_YA9434AA	US_ESBL060	✓				Livestock	Ovine/Goat (Caprinae)	Carcass swab (Goat)		Mar-19	US	E. coli	PRJNA293225	SAMN11491426	24/04/2019	24/04/2019
	ESC_MA4527AA	ESC_YA9430AA	US_ESBL036	✓				Environment	Water/River	ND		Mar-19	US	E. coli	PRJNA293225	SAMN11491423	24/04/2019	24/04/2019
	ESC_MA4533AA	ESC_YA9424AA	US_ESBL063	✓				Livestock	Ovine/Goat (Caprinae)	Carcass swab (Goat)		Mar-19	US	E. coli	PRJNA293225	SAMN11491417	24/04/2019	24/04/2019
	ESC_MA4537AA	ESC_YA9420AA	US_ESBL066	✓				Livestock	Ovine/Goat (Caprinae)	Carcass swab (Goat)		Mar-19	US	E. coli	PRJNA293225	SAMN11491415	24/04/2019	24/04/2019
	ESC_MA4538AA	ESC_YA9419AA	US_ESBL039	✓				Environment	Water/River	ND		Mar-19	US	E. coli	PRJNA293225	SAMN11491436	24/04/2019	24/04/2019
	ESC_MA4555AA	ESC_YA9402AA	US_ESBL030	✓				Livestock	Ovine/Goat (Caprinae)	Feces (Sheep)		Mar-19	US	E. coli	PRJNA293225	SAMN11491401	24/04/2019	24/04/2019
	ESC_MA4561AA	ESC_YA9396AA	US_ESBL027	✓				Livestock	Ovine/Goat (Caprinae)	Feces (Sheep)		Mar-19	US	E. coli	PRJNA293225	SAMN11491354	24/04/2019	24/04/2019
	ESC_MA4563AA	ESC_YA9394AA	US_ESBL015	✓				Livestock	Ovine/Goat (Caprinae)	Feces (Sheep)		Mar-19	US	E. coli	PRJNA293225	SAMN11491373	24/04/2019	24/04/2019
	ESC_MA7631AA	ESC_ZA5893AA	TMP020445					Livestock	Avian (chicken)	Faeces		Jun-16	Kenya	E. coli	PRJEB32607	SAMEA5611777	30/05/2019	20/05/2019
	ESC_MA8190AA	ESC_ZA5247AA	INT007815					Homo sapien	Human	ND		May-16	Kenya	E. coli	PRJEB32607	SAMEA5611211	30/05/2019	20/05/2019
	ESC_MA8503AA	ESC_ZA4933AA	INT004388					Livestock	Avian	Faeces		Oct-15	Kenya	E. coli	PRJEB32607	SAMEA5610897	30/05/2019	20/05/2019
	ESC_MA8894AA	ESC_ZA4540AA	ALQ018502					Livestock	Avian	ND		Oct-15	Kenya	E. coli	PRJEB32607	SAMEA5610506	30/05/2019	20/05/2019
	ESC_MA9713AA	ESC_ZA9258AA	AG19-0197					Livestock	Dairy Cattle	Milk		2017	US	E. coli	PRJNA338676	SAMN11936914	04/06/2019	03/06/2019

	ESC_NA1543AA	**ESC_BB2471AA**							ND	ND	ND		Jan-19	GER	E. coli	PRJEB23294	SAMEA4607517	16/06/2019	15/06/2019
	ESC_NA6669AA	**ESC_CB8899AA**	76.0186			blaTEM-1			Livestock	Bovine	Faeces		1976	US	E. coli	PRJNA357722	SAMN12438243	07/08/2019	06/08/2019
	ESC_NA8020AA	**ESC_DB1887AA**	CVM N18EC0246			blaHERA-3, blaTEM-150, blaTEM-104, blaTEM-1			Livestock	Avian	Ground Turkey		2018	US	E. coli	PRJNA292663	SAMN12359623	20/08/2019	16/08/2019
	ESC_NA8159AA	**ESC_DB2566AA**	CVM N18EC0256			blaHERA-3, blaTEM-112, blaTEM-104, blaTEM-1			Livestock	Avian	Ground Turkey		2018	US	E. coli	PRJNA292663	SAMN12587161	21/08/2019	20/08/2019
	ESC_NA9774AA	**ESC_FB0845AA**	PSU-1959					✓	Livestock	Bovine	Beef		2016	US	E. coli	PRJNA357722	SAMN12699155	06/09/2019	05/09/2019
	ESC_OA2692AA	**ESC_FB4150AA**	PSU-1986					✓	Livestock	Bovine	Beef		2016	US	E. coli	PRJNA357722	SAMN12799305	21/09/2019	20/09/2019
	ESC_OA7150AA	**ESC_GB2725AA**	19MD07GT 11-EC			blaTEM-1		✓	Livestock	Avian	Ground Turkey		2019	US	E. coli	PRJNA292663	SAMN12684081	10/11/2019	12/09/2019
	ESC_OA7841AA	**ESC_GB3534AA**							Livestock	Avian	Poultry Litter		Feb-19	Nigeria	E. coli	PRJNA293225	SAMN13245768	13/11/2019	09/11/2019
	ESC_PA7252AA	**ESC_HB9080AA**	ARS-CC11289		27			✓	Livestock	Bovine	ND		ND	US	E. coli	NA	NA	02/01/2020	06/07/2020
	ESC_QA1927AA	**ESC_IB8383AA**	PSU-2060						Livestock	Bovine	Faeces		Mar-19	US	E. coli	PRJNA357722	SAMN13941770	14/02/2020	29/01/2020
	ESC_QA5499AA	**ESC_IB6804AA**	FSIS 210069847						Livestock	Bovine	Faeces		Jan-14	US	E. coli	PRJNA292667	SAMN04395980	14/02/2020	23/03/2017
Group 3	**ESC_QA6164AA**	**ESC_JB0045AA**	USECESBL081	✓	15		✓	✓	Environment	Animal-related	Lairage swab		Mar-19	US	E. coli	PRJNA293225	SAMN14147092	23/02/2020	20/02/2020
	ESC_QA6166AA	**ESC_JB0043AA**	USECESBL090	✓	15		✓	✓	Environment	Water/River	Water		Mar-19	US	E. coli	PRJNA293225	SAMN14147097	23/02/2020	20/02/2020
	ESC_QA7360AA	**ESC_JB5131AA**	M30						ND	ND	ND	ExpEC	ND	ND	E. coli	NA	NA	08/03/2020	08/03/2020
	ESC_QA8040AA	**ESC_JB8273AA**	USECESBL1212	✓	27			✓	Livestock	Ovine/Goat (Caprinae)	Faeces (Sheep)		Jan-20	US	E. coli	PRJNA293225	SAMN14400479	20/03/2020	19/03/2020
	ESC_QA8043AA	**ESC_JB8270AA**	USECESBL929					✓	Environment	Water/River	Water		Sep-19	US	E. coli	PRJNA293225	SAMN14400334	20/03/2020	19/03/2020
	ESC_RA0019AA	**ESC_KB3846AA**	0.0334		32				Livestock	Bovine	Faeces		2000	US	E. coli	PRJNA357722	SAMN14503589	08/04/2020	05/04/2020
	ESC_RA3673AA	**ESC_LB2178AA**	LD39-1		14				ND	ND/Others	Faeces		Aug-18	China	E. coli	PRJNA224116	SAMN13829544	07/05/2020	21/01/2020
	ESC_SA4400AA	**ESC_NB6134AA**	ME2L-20-7	✓	32			✓	Livestock	Bovine	Faeces		2017	US	E. coli	PRJNA625290	SAMN14596651	01/10/2020	23/09/2020
	ESC_SA4404AA	**ESC_NB6130AA**	ME2L-20-3		27	blaTEM-1			Livestock	Bovine	Faeces		2017	US	E. coli	PRJNA625290	SAMN14596647	01/10/2020	23/09/2020
	ESC_TA6337AA	**ESC_UB0614AA**	22306838						Livestock	Holstein Friesians Cattle	Dairy Milk		Jul-07	CAN	E. coli	PRJNA612640	SAMN14379595	01/01/2021	16/12/2020
	ESC_TA7388AA	**ESC_UB1096AA**	E121ESBLB						Food	Animal	Raw Meat		Jan-21	SGP	E. coli	PRJEB34067	SAMEA5930154	07/01/2021	06/01/2021

<50 SNPs	**ESC_TA9425AA**	**ESC_UB4127AA**	ZTA17/025 08EB	✓					Livestock	Bovine	Faeces		2017	Spain	E. coli	PRJEB33169	SAMEA5732443	22/01/2021	20/01/2021
	ESC_TA9554AA	**ESC_UB3988AA**	17000123	✓					Livestock	Swine	Faeces		2017	LUX	E. coli	PRJEB33169	SAMEA5732291	22/01/2021	20/01/2021
	ESC_TA9700AA	**ESC_UB3842AA**	35296-214						Livestock	Bovine	Caecal Sample		2017	Croatia	E. coli	PRJEB33169	SAMEA5732124	22/01/2021	20/01/2021
	ESC_UA3483AA	**ESC_VB0481AA**	RKI6290	✓					Companion Animal	Equine	Faeces		2019	GER	E. coli	PRJNA698802	SAMN17756709	01/03/2021	25/02/2021
	ESC_UA4815AA	**ESC_VB1857AA**	PSU-3626						Livestock	Bovine	Faeces		Jan-18	US	E. coli	PRJNA357722	SAMN18267039	13/03/2021	12/03/2021
50-60 SNPs	**ESC_UA8616AA**	**ESC_VB6200AA**	PEROU H19R	✓					ND	ND	ND	ND	2017	ND	E. coli	NA	NA	02/04/2021	06/02/2022
	ESC_UA8818AA	**ESC_WB5107AA**	839-1	✓					Companion Animal	Canine (Domestic Dog)	Anal Swab		2017	China	E. coli	PRJNA650157	SAMN15694438	05/04/2021	02/04/2021
	ESC_VA2292AA	**ESC_WB0932AA**	703560						Homo sapien	Human	Stool		Feb-20	Pakistan	E. coli	PRJNA607273	SAMN14127657	01/05/2021	01/04/2021
	ESC_VA3388AA	**ESC_WB2965AA**	FT652						Companion Animal	Feline	Urine	UPEC	2020	France	E. coli	NA	NA	11/05/2021	11/05/2021
	ESC_VA3831AA	**ESC_WB3275AA**	111804002						Livestock	Bovine (Veal calf)	Veal meat		2015	NTL	E. coli	PRJEB41365	SAMEA7577702	13/05/2021	11/05/2021
	ESC_VA5054AA	**ESC_WB6010AA**	MVC140						Companion Animal	Canine (Domestic Dog)	Faeces/Urine		2010	AUS	E. coli	PRJNA678027	SAMN16787491	24/05/2021	24/05/2021
	ESC_VA5906AA	**ESC_WB7656AA**	508	✓					Companion Animal	Canine (Domestic Dog)	Faeces		Aug-18	US	E. coli	PRJNA671493	SAMN16533515	01/06/2021	01/05/2021
	ESC_VA8844AA	**ESC_XB4559AA**	700460						Homo sapien	Human	Stool		Mar-20	Pakistan	E. coli	PRJNA611810	SAMN14349908	01/07/2021	01/06/2021
	ESC_WA0412AA	**ESC_XB9327AA**	21TX05GT 02-EC						Livestock	Poultry	Ground Turkey		May-21	US	E. coli	PRJNA292663	SAMN20152703	10/07/2021	09/07/2021
	ESC_WA2029AA	**ESC_YB8969AA**	PAWECO_ P36						Livestock	Swine	Stool		Dec-19	SA	E. coli	NA	NA	31/07/2021	31/07/2021
	ESC_WA2049AA	**ESC_YB8989AA**	PAWECO_ P60						Livestock	Swine	Stool		Dec-19	SA	E. coli	NA	NA	31/07/2021	31/07/2021
	ESC_WA7101AA	**ESC_ZB8837AA**	ARS-CC11330						Wild Animal	ND/Others	Faeces		2015	US	E. coli	PRJNA664052	SAMN21542800	23/09/2021	22/09/2021
	ESC_WA7128AA	**ESC_ZB8810AA**	ARS-CC11289						Wild Animal	ND/Others	Faeces		2015	US	E. coli	PRJNA664052	SAMN21542776	23/09/2021	22/09/2021
	ESC_WA9939AA	**ESC_ZB9940AA**	PSU-4005	✓					Livestock	Bovine	Faeces		2020	US	E. coli	PRJNA357722	SAMN18875345	01/10/2021	30/09/2021
	ESC_XA1971AA	**ESC_AC3715AA**	Eco-21-00163			blaTEM-106			ND	ND	ND		ND	GER	E. coli	NA	NA	26/10/2021	26/10/2021
	ESC_XA5006AA	**ESC_AC5677AA**	21NC08GT 08-EC			blaHERA-3, blaTEM-1		✓	Livestock	Poultry	Ground Turkey		Aug-21	US	E. coli	PRJNA292663	SAMN22837437	11/11/2021	11/11/2021
	ESC_XA5229AA	**ESC_AC5898AA**	21OR06GT 03-EC					✓	Livestock	Poultry	Ground Turkey		2021	US	E. coli	PRJNA292663	SAMN23168141	16/11/2021	15/11/2021
	ESC_XA9142AA	**ESC_BC8199AA**	SAMEA464 5141			blaTEM-1	✓	✓	Livestock	Swine	Caecal Content		Apr-15	UK	E. coli	PRJEB26317	SAMEA4645141	04/12/2021	25/11/2021
	ESC_YA1626AA	**ESC_BC7368AA**	BES-1332	✓	15	blaTEM-1	✓	✓	Livestock	Swine	Faeces		Apr-16	France	E. coli	PRJNA795027	SAMN24669410	06/01/2022	06/01/2022

***Footnote for Appendix C**: US – United States, UK – United Kingdom, LUX – Luxembourg, NTL – Netherlands, GER – Germany, CAN – Canada, SGP – Singapore, AUS – Australia, SA – South Africa, ND – Non-Defined, NA – Not Available*

APPENDIX D: THE FULL RESULTS FOR THE RESFINDER SEARCH CONDUCTED ON THE 105 ENTEROBASE ISOLATES

#FILE	SEQUENCE	START	END	GENE	COVERAGE	%COVERAGE	%IDENTITY	ACCESSION
ESC_AC3715AA_AS	NODE_29_length_41806_cov_13.771900	5616	6476	blaTEM-106_1	1-861/861	100	99.88	AY101578
ESC_AC3715AA_AS	NODE_33_length_37396_cov_13.387427	33492	36936	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_AC5677AA_AS	NODE_147_length_1386_cov_55.436855	204	1076	blaHERA-3_1	1-873/873	100	98.86	AF398335
ESC_AC5677AA_AS	NODE_19_length_107768_cov_27.949805	49931	50898	ant(3")-Ia_1	1-972/972	99.59	99.38	X02340
ESC_AC5677AA_AS	NODE_19_length_107768_cov_27.949805	51062	51964	aac(3)-VIa_2	1-903/903	100	100	NC_009838
ESC_AC5677AA_AS	NODE_19_length_107768_cov_27.949805	57623	57907	qacE_1	1-285/333	85.59	99.65	X68232
ESC_AC5677AA_AS	NODE_19_length_107768_cov_27.949805	57937	58803	sul1_5	1-867/867	100	99.89	EU780013
ESC_AC5677AA_AS	NODE_36_length_37691_cov_24.312107	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_AC5677AA_AS	NODE_57_length_12813_cov_34.840533	11332	12192	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_AC5677AA_AS	NODE_91_length_3768_cov_25.967317	919	2165	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_AC5677AA_AS	NODE_96_length_3401_cov_39.842700	127	520	aph(6)-Id_1	1-394/837	47.07	100	M28829
ESC_AC5677AA_AS	NODE_96_length_3401_cov_39.842700	520	1322	aph(3")-Ib_2	2-804/804	99.88	100	AF024602
ESC_AC5898AA_AS	NODE_12_length_125030_cov_42.866769	33866	37310	sitABCD_1	6-3459/3459	99.6	97.48	AY598030

ESC_AC5898AA_AS	NODE_22_length_80923_cov_42.750297	48154	49400	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_AC5898AA_AS	NODE_24_length_69632_cov_42.940450	55658	56625	ant(3")-Ia_1	1-972/972	99.59	99.38	X02340
ESC_AC5898AA_AS	NODE_39_length_37770_cov_41.398507	56789	57691	aac(3)-VIa_2	1-903/903	100	100	NC_009838
ESC_AC5898AA_AS	NODE_9_length_137226_cov_49.789006	63350	63634	qacE_1	1-285/333	85.59	99.65	X68232
ESC_AC5898AA_AS	NODE_9_length_137226_cov_49.789006	63664	64530	sul1_5	1-867/867	100	99.89	EU780013
ESC_AC5898AA_AS	NODE_9_length_137226_cov_49.789006	1034	2224	tet(C)_3	1-1191/1191	100	99.83	AF055345
ESC_BA1014AA_AS	NODE_112_length_2959_cov_15.9961_ID_223	2717	2883	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_BA1014AA_AS	NODE_24_length_69187_cov_8.71222_ID_47	54033	55178	blaCMY-2_1	1-1146/1146	100	100	X91840
ESC_BA1014AA_AS	NODE_3_length_250343_cov_21.3183_ID_5	212820	216264	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_BA1014AA_AS	NODE_59_length_23745_cov_33.0975_ID_117	4205	5410	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_BA1014AA_AS	NODE_81_length_7337_cov_21.9983_ID_161	4950	5816	sul1_5	1-867/867	100	99.89	EU780013
ESC_BA1014AA_AS	NODE_81_length_7337_cov_21.9983_ID_161	5846	6130	qacE_1	1-285/333	85.59	99.65	X68232
ESC_BA1014AA_AS	NODE_81_length_7337_cov_21.9983_ID_161	6294	7261	ant(3")-Ia_1	1-972/972	99.59	99.49	X02340
ESC_BC7368AA_AS	NODE_134_length_3743_cov_11.849834	311	793	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_BC7368AA_AS	NODE_134_length_3743_cov_11.849834	800	967	ant(3")-Ia_1	1-175/972	17.28	96	X02340
ESC_BC7368AA_AS	NODE_53_length_34519_cov_14.648697	461	3903	sitABCD_1	6-3459/3459	99.54	97.42	AY598030

ESC_BC7368AA_AS	NODE_81_length_14508_cov_10.856825	1198	2444	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_BC7368AA_AS	NODE_81_length_14508_cov_10.856825	3780	4640	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_BC7368AA_AS	NODE_81_length_14508_cov_10.856825	7461	8336	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_BC7368AA_AS	NODE_81_length_14508_cov_10.856825	12977	13633	qnrS1_1	1-657/657	100	100	AB187515
ESC_BC8199AA_AS	NODE_25_length_58913_cov_19.229919	1	127	ant(3")-Ia_1	171-297/972	13.07	100	X02340
ESC_BC8199AA_AS	NODE_25_length_58913_cov_19.229919	210	1469	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_BC8199AA_AS	NODE_25_length_58913_cov_19.229919	1731	2532	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_BC8199AA_AS	NODE_25_length_58913_cov_19.229919	2930	3427	dfrA12_8	1-498/498	100	100	AM040708
ESC_BC8199AA_AS	NODE_25_length_58913_cov_19.229919	3424	3599	ant(3")-Ia_1	1-184/972	18.11	94.56	X02340
ESC_BC8199AA_AS	NODE_25_length_58913_cov_19.229919	9857	11103	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_BC8199AA_AS	NODE_31_length_38278_cov_16.102959	34374	37818	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_BC8199AA_AS	NODE_35_length_28768_cov_31.906253	1	170	aadA1_2	623-792/792	21.46	100	FJ591054
ESC_BC8199AA_AS	NODE_35_length_28768_cov_31.906253	334	618	qacE_1	1-285/333	85.59	99.65	X68232
ESC_BC8199AA_AS	NODE_35_length_28768_cov_31.906253	648	1514	sul1_5	1-867/867	100	99.89	EU780013
ESC_BC8199AA_AS	NODE_39_length_22294_cov_32.726124	513	1373	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_BC8199AA_AS	NODE_47_length_4789_cov_36.525526	124	386	mef(B)_1	968-1230/1230	21.38	99.62	FJ196385
ESC_BC8199AA_AS	NODE_47_length_4789_cov_36.525526	2147	2938	sul3_2	1-792/792	100	100	AJ459418

ESC_BC8199AA_AS	NODE_47_length_4789_cov_36.525526	4050	4565	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_BC8199AA_AS	NODE_47_length_4789_cov_36.525526	4620	4789	aadA1_5	623-792/792	21.46	100	JX185132
ESC_BC8199AA_AS	NODE_49_length_4580_cov_30.102613	2501	3526	aph(4)-Ia_1	1-1026/1026	100	100	V01499
ESC_BC8199AA_AS	NODE_49_length_4580_cov_30.102613	3747	4531	aac(3)-IVa_1	1-786/786	99.87	99.87	X01385
ESC_BC8199AA_AS	NODE_58_length_2037_cov_34.139791	878	1534	qnrS1_1	1-657/657	100	100	AB187515
ESC_BC8199AA_AS	NODE_75_length_759_cov_48.113924	1	759	ant(3")-Ia_1	171-929/972	78.09	100	X02340
ESC_BC8199AA_AS	NODE_84_length_507_cov_17.039474	215	507	ant(3")-Ia_1	1-297/972	30.14	98.65	X02340
ESC_CA1603AA_AS	NODE_32_length_38202_cov_9.886_ID_63	34336	37780	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_CA1603AA_AS	NODE_48_length_5222_cov_12.0144_ID_95	1264	2469	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_CA1603AA_AS	NODE_50_length_4564_cov_9.20715_ID_99	1952	2767	sul2_3	1-816/816	100	100	HQ840942
ESC_CA1603AA_AS	NODE_50_length_4564_cov_9.20715_ID_99	2828	3631	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_CA1603AA_AS	NODE_50_length_4564_cov_9.20715_ID_99	3631	4467	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_CA1603AA_AS	NODE_52_length_4374_cov_8.85274_ID_103	3403	4062	catA1_1	1-660/660	100	99.7	V00622
ESC_CA1603AA_AS	NODE_58_length_3078_cov_79.544_ID_115	2281	2925	qnrB19_1	1-645/645	100	100	EU432277
ESC_CA1603AA_AS	NODE_61_length_2208_cov_10.5691_ID_121	681	1541	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_CA3639AA_AS	NODE_36_length_37984_cov_20.0772_ID_71	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_CB8899AA_AS	NODE_36_length_38278_cov_16.616235	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_CB8899AA_AS	NODE_42_length_23226_cov_11.230270	15354	16214	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_CB8899AA_AS	NODE_49_length_6823_cov_17.187724	4286	5491	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_CB8899AA_AS	NODE_57_length_4207_cov_10.997693	3326	4141	aph(3')-Ia_7	1-816/816	100	100	X62115
ESC_CB8899AA_AS	NODE_91_length_1004_cov_10.687571	180	839	catA1_1	1-660/660	100	99.85	V00622

ESC_DB1887AA_AS	NODE_113_length_6820_cov_31.869266	1330	2535	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_DB1887AA_AS	NODE_138_length_3592_cov_10.884560	178	962	aac(3)-IVa_1	1-786/786	99.87	99.87	X01385
ESC_DB1887AA_AS	NODE_138_length_3592_cov_10.884560	1183	2208	aph(4)-Ia_1	1-1026/1026	100	100	V01499
ESC_DB1887AA_AS	NODE_145_length_3107_cov_21.227181	972	2891	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_DB1887AA_AS	NODE_163_length_1917_cov_18.658659	1495	1790	qacH_1	297-592/945	31.32	92.23	FJ172381
ESC_DB1887AA_AS	NODE_175_length_1385_cov_330.035771	204	1076	blaHERA-3_1	1-873/873	100	98.86	AF398335
ESC_DB1887AA_AS	NODE_247_length_489_cov_0.878453	8	489	blaTEM-150_1	80-561/861	55.98	93.15	AM183304
ESC_DB1887AA_AS	NODE_279_length_465_cov_1.020710	320	443	qacH_1	297-420/945	13.12	83.87	FJ172381
ESC_DB1887AA_AS	NODE_376_length_306_cov_28.675978	162	306	blaTEM-104_1	1-145/861	16.84	100	AF516719
ESC_DB1887AA_AS	NODE_44_length_38168_cov_14.876291	36224	37060	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_DB1887AA_AS	NODE_44_length_38168_cov_14.876291	37060	37862	aph(3")-Ib_2	2-804/804	99.88	100	AF024602
ESC_DB1887AA_AS	NODE_77_length_14114_cov_337.540645	9185	10045	blaTEM-1A_1	1-861/861	100	100	HM749966
ESC_DB2566AA_AS	NODE_104_length_3592_cov_32.933333	1385	2410	aph(4)-Ia_1	1-1026/1026	100	100	V01499
ESC_DB2566AA_AS	NODE_104_length_3592_cov_32.933333	2631	3415	aac(3)-IVa_1	1-786/786	99.87	99.87	X01385
ESC_DB2566AA_AS	NODE_107_length_3108_cov_65.646763	217	2136	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_DB2566AA_AS	NODE_121_length_1917_cov_62.852514	1495	1790	qacH_1	297-592/945	31.32	92.23	FJ172381
ESC_DB2566AA_AS	NODE_123_length_1845_cov_67.745634	101	903	aph(3")-Ib_2	2-804/804	99.88	100	AF024602

ESC_DB2566AA_AS	NODE_123_length_1845_cov_67.745634	903	1739	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_DB2566AA_AS	NODE_131_length_1385_cov_135.705087	310	1182	blaHERA-3_1	1-873/873	100	98.86	AF398335
ESC_DB2566AA_AS	NODE_149_length_990_cov_281.594438	1	843	blaTEM-1A_1	19-861/861	97.91	100	HM749966
ESC_DB2566AA_AS	NODE_244_length_306_cov_125.871508	1	145	blaTEM-112_1	1-145/861	16.84	100	AY589493
ESC_DB2566AA_AS	NODE_248_length_253_cov_65.920635	1	92	blaTEM-104_1	1-92/861	10.69	100	AF516719
ESC_DB2566AA_AS	NODE_85_length_6820_cov_72.861796	1330	2535	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_DB2566AA_AS	NODE_99_length_4214_cov_47.021042	4070	4214	blaTEM-104_1	1-145/861	16.84	100	AF516719
ESC_FA6543AA_AS	NODE_103_length_860_cov_36.0281_ID_205	59	860	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA6543AA_AS	NODE_107_length_759_cov_30.5341_ID_213	1	759	ant(3")-Ia_1	171-929/972	78.09	100	X02340
ESC_FA6543AA_AS	NODE_116_length_648_cov_46.2112_ID_231	482	648	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA6543AA_AS	NODE_143_length_222_cov_14.3945_ID_285	1	222	ant(3")-Ia_1	62-283/972	22.84	100	X02340
ESC_FA6543AA_AS	NODE_144_length_222_cov_17.0092_ID_287	1	222	ant(3")-Ia_1	62-283/972	22.84	91.89	X02340
ESC_FA6543AA_AS	NODE_21_length_81470_cov_10.1815_ID_41	34360	37804	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA6543AA_AS	NODE_24_length_55036_cov_12.3607_ID_47	47901	49147	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA6543AA_AS	NODE_39_length_22267_cov_18.0218_ID_77	500	1360	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA6543AA_AS	NODE_48_length_7720_cov_11.0632_ID_95	1	156	aadA1_2	637-792/792	19.7	100	FJ591054
ESC_FA6543AA_AS	NODE_48_length_7720_cov_11.0632_ID_95	320	604	qacE_1	1-285/333	85.59	99.65	X68232

ESC_FA6543AA_AS	NODE_48_length_7720_cov_11.0632_ID_95	634	1500	sul1_5	1-867/867	100	99.89	EU780013
ESC_FA6543AA_AS	NODE_51_length_5274_cov_24.6007_ID_101	2848	3873	aph(4)-Ia_1	1-1026/1026	100	100	V01499
ESC_FA6543AA_AS	NODE_51_length_5274_cov_24.6007_ID_101	4094	4878	aac(3)-IVa_1	1-786/786	99.87	99.87	X01385
ESC_FA6543AA_AS	NODE_54_length_4762_cov_18.9417_ID_107	111	373	mef(B)_1	968-1230/1230	21.38	99.62	FJ196385
ESC_FA6543AA_AS	NODE_54_length_4762_cov_18.9417_ID_107	2134	2925	sul3_2	1-792/792	100	100	AJ459418
ESC_FA6543AA_AS	NODE_54_length_4762_cov_18.9417_ID_107	4037	4552	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA6543AA_AS	NODE_54_length_4762_cov_18.9417_ID_107	4607	4762	aadA1_5	637-792/792	19.7	100	JX185132
ESC_FA6543AA_AS	NODE_64_length_2243_cov_27.7028_ID_127	1098	1754	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA6543AA_AS	NODE_75_length_1771_cov_17.4053_ID_149	1	55	aadA12_1	738-792/792	6.94	100	AY665771
ESC_FA6543AA_AS	NODE_75_length_1771_cov_17.4053_ID_149	317	1576	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA6543AA_AS	NODE_75_length_1771_cov_17.4053_ID_149	1659	1771	ant(3")-Ia_1	171-283/972	11.63	100	X02340
ESC_FA6543AA_AS	NODE_82_length_1293_cov_34.3839_ID_163	1	55	aadA12_1	738-792/792	6.94	100	AY665771
ESC_FA6543AA_AS	NODE_86_length_1126_cov_18.5656_ID_171	1	113	aadA2_1	18-130/819	13.8	99.11	NC_010870
ESC_FA6543AA_AS	NODE_86_length_1126_cov_18.5656_ID_171	511	1008	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA6543AA_AS	NODE_86_length_1126_cov_18.5656_ID_171	1005	1126	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA6545AA_AS	NODE_105_length_860_cov_37.6653_ID_209	59	860	aadA2_1	18-819/819	97.92	99.88	NC_010870

ESC_FA6545AA_AS	NODE_109_length_759_cov_30.2074_ID_217	1	759	ant(3")-Ia_1	171-929/972	78.09	100	X02340
ESC_FA6545AA_AS	NODE_117_length_648_cov_45.4262_ID_233	482	648	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA6545AA_AS	NODE_147_length_222_cov_17.789_ID_293	1	222	ant(3")-Ia_1	62-283/972	22.84	91.89	X02340
ESC_FA6545AA_AS	NODE_148_length_222_cov_13.2202_ID_295	1	222	ant(3")-Ia_1	62-283/972	22.84	100	X02340
ESC_FA6545AA_AS	NODE_21_length_81470_cov_10.164_ID_41	34360	37804	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA6545AA_AS	NODE_24_length_55036_cov_12.3549_ID_47	47901	49147	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA6545AA_AS	NODE_40_length_22267_cov_17.8442_ID_79	500	1360	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA6545AA_AS	NODE_49_length_7720_cov_11.4509_ID_97	1	156	aadA1_2	637-792/792	19.7	100	FJ591054
ESC_FA6545AA_AS	NODE_49_length_7720_cov_11.4509_ID_97	320	604	qacE_1	1-285/333	85.59	99.65	X68232
ESC_FA6545AA_AS	NODE_49_length_7720_cov_11.4509_ID_97	634	1500	sul1_5	1-867/867	100	99.89	EU780013
ESC_FA6545AA_AS	NODE_52_length_5274_cov_24.3625_ID_103	397	1181	aac(3)-IVa_1	1-786/786	99.87	99.87	X01385
ESC_FA6545AA_AS	NODE_52_length_5274_cov_24.3625_ID_103	1402	2427	aph(4)-Ia_1	1-1026/1026	100	100	V01499
ESC_FA6545AA_AS	NODE_55_length_4762_cov_18.8182_ID_109	111	373	mef(B)_1	968-1230/1230	21.38	99.62	FJ196385
ESC_FA6545AA_AS	NODE_55_length_4762_cov_18.8182_ID_109	2134	2925	sul3_2	1-792/792	100	100	AJ459418
ESC_FA6545AA_AS	NODE_55_length_4762_cov_18.8182_ID_109	4037	4552	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA6545AA_AS	NODE_55_length_4762_cov_18.8182_ID_109	4607	4762	aadA1_5	637-792/792	19.7	100	JX185132

ESC_FA6545AA_AS	NODE_65_length_2243_cov_27.2047_ID_129	490	1146	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA6545AA_AS	NODE_77_length_1771_cov_17.6713_ID_153	1	55	aadA12_1	738-792/792	6.94	100	AY665771
ESC_FA6545AA_AS	NODE_77_length_1771_cov_17.6713_ID_153	317	1576	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA6545AA_AS	NODE_77_length_1771_cov_17.6713_ID_153	1659	1771	ant(3")-Ia_1	171-283/972	11.63	100	X02340
ESC_FA6545AA_AS	NODE_83_length_1293_cov_35.1636_ID_165	1	55	aadA12_1	738-792/792	6.94	100	AY665771
ESC_FA6545AA_AS	NODE_89_length_1126_cov_18.6367_ID_177	1	113	aadA2_1	18-130/819	13.8	99.11	NC_010870
ESC_FA6545AA_AS	NODE_89_length_1126_cov_18.6367_ID_177	511	1008	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA6545AA_AS	NODE_89_length_1126_cov_18.6367_ID_177	1005	1126	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA6550AA_AS	NODE_103_length_860_cov_36.2169_ID_205	59	860	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA6550AA_AS	NODE_107_length_759_cov_29.5402_ID_213	1	759	ant(3")-Ia_1	171-929/972	78.09	100	X02340
ESC_FA6550AA_AS	NODE_115_length_648_cov_47.0879_ID_229	482	648	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA6550AA_AS	NODE_142_length_222_cov_12.8716_ID_283	1	222	ant(3")-Ia_1	62-283/972	22.84	100	X02340
ESC_FA6550AA_AS	NODE_143_length_222_cov_17.2385_ID_285	1	222	ant(3")-Ia_1	62-283/972	22.84	91.89	X02340
ESC_FA6550AA_AS	NODE_20_length_81471_cov_10.1369_ID_39	34361	37805	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA6550AA_AS	NODE_24_length_55036_cov_12.4339_ID_47	47901	49147	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA6550AA_AS	NODE_40_length_22267_cov_18.3216_ID_79	500	1360	blaTEM-1B_1	1-861/861	100	100	AY458016

ESC_FA6550AA_AS	NODE_49_length_7720_cov_11.2886_ID_97	1	156	aadA1_2	637-792/792	19.7	100	FJ591054
ESC_FA6550AA_AS	NODE_49_length_7720_cov_11.2886_ID_97	320	604	qacE_1	1-285/333	85.59	99.65	X68232
ESC_FA6550AA_AS	NODE_49_length_7720_cov_11.2886_ID_97	634	1500	sul1_5	1-867/867	100	99.89	EU780013
ESC_FA6550AA_AS	NODE_52_length_5274_cov_24.1996_ID_103	2848	3873	aph(4)-Ia_1	1-1026/1026	100	100	V01499
ESC_FA6550AA_AS	NODE_52_length_5274_cov_24.1996_ID_103	4094	4878	aac(3)-IVa_1	1-786/786	99.87	99.87	X01385
ESC_FA6550AA_AS	NODE_55_length_4762_cov_18.1936_ID_109	111	373	mef(B)_1	968-1230/1230	21.38	99.62	FJ196385
ESC_FA6550AA_AS	NODE_55_length_4762_cov_18.1936_ID_109	2134	2925	sul3_2	1-792/792	100	100	AJ459418
ESC_FA6550AA_AS	NODE_55_length_4762_cov_18.1936_ID_109	4037	4552	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA6550AA_AS	NODE_55_length_4762_cov_18.1936_ID_109	4607	4762	aadA1_5	637-792/792	19.7	100	JX185132
ESC_FA6550AA_AS	NODE_66_length_2243_cov_28.2573_ID_131	1098	1754	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA6550AA_AS	NODE_77_length_1771_cov_17.4204_ID_153	1	55	aadA12_1	738-792/792	6.94	100	AY665771
ESC_FA6550AA_AS	NODE_77_length_1771_cov_17.4204_ID_153	317	1576	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA6550AA_AS	NODE_77_length_1771_cov_17.4204_ID_153	1659	1771	ant(3")-Ia_1	171-283/972	11.63	100	X02340
ESC_FA6550AA_AS	NODE_83_length_1293_cov_35.0169_ID_165	1	55	aadA12_1	738-792/792	6.94	100	AY665771
ESC_FA6550AA_AS	NODE_87_length_1126_cov_19.8006_ID_173	1	113	aadA2_1	18-130/819	13.8	99.11	NC_010870
ESC_FA6550AA_AS	NODE_87_length_1126_cov_19.8006_ID_173	511	1008	dfrA12_8	1-498/498	100	100	AM040708

ESC_FA6550AA_AS	NODE_87_length_1126_cov_19.8006_ID_173	1005	1126	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9712AA_AS	NODE_128_length_310_cov_47.6091_ID_255	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9712AA_AS	NODE_3_length_359769_cov_9.05949_ID_5	34066	37510	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9712AA_AS	NODE_41_length_9108_cov_5.91606_ID_81	1	122	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9712AA_AS	NODE_41_length_9108_cov_5.91606_ID_81	119	616	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9712AA_AS	NODE_41_length_9108_cov_5.91606_ID_81	1014	1815	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9712AA_AS	NODE_41_length_9108_cov_5.91606_ID_81	2077	3336	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9712AA_AS	NODE_41_length_9108_cov_5.91606_ID_81	3419	4220	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9712AA_AS	NODE_41_length_9108_cov_5.91606_ID_81	4275	4790	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9712AA_AS	NODE_41_length_9108_cov_5.91606_ID_81	6148	8067	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9712AA_AS	NODE_44_length_7695_cov_6.94988_ID_87	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9712AA_AS	NODE_44_length_7695_cov_6.94988_ID_87	2878	3753	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9712AA_AS	NODE_47_length_6440_cov_6.82772_ID_93	4684	5340	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9712AA_AS	NODE_50_length_5670_cov_7.59007_ID_99	3303	4516	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9712AA_AS	NODE_51_length_5489_cov_17.0465_ID_101	3263	4509	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9712AA_AS	NODE_55_length_4778_cov_14.7218_ID_109	2059	2874	sul2_2	1-816/816	100	99.88	AY034138

ESC_FA9712AA_AS	NODE_55_length_4778_cov_14.7218_ID_109	2935	3738	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9712AA_AS	NODE_55_length_4778_cov_14.7218_ID_109	3738	4574	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9712AA_AS	NODE_70_length_2150_cov_9.63427_ID_139	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9712AA_AS	NODE_75_length_1987_cov_27.3335_ID_149	334	1149	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9712AA_AS	NODE_88_length_1356_cov_9.07643_ID_175	1	167	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA9712AA_AS	NODE_92_length_1065_cov_14.7122_ID_183	58	918	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9712AA_AS	NODE_98_length_984_cov_14.6418_ID_195	1	114	ant(3")-Ia_1	62-175/972	11.73	100	X02340
ESC_FA9712AA_AS	NODE_98_length_984_cov_14.6418_ID_195	121	603	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9713AA_AS	NODE_135_length_310_cov_44.3147_ID_269	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9713AA_AS	NODE_3_length_359986_cov_9.31287_ID_5	34067	37511	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9713AA_AS	NODE_42_length_9108_cov_5.60345_ID_83	1	122	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9713AA_AS	NODE_42_length_9108_cov_5.60345_ID_83	119	616	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9713AA_AS	NODE_42_length_9108_cov_5.60345_ID_83	1014	1815	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9713AA_AS	NODE_42_length_9108_cov_5.60345_ID_83	2077	3336	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9713AA_AS	NODE_42_length_9108_cov_5.60345_ID_83	3419	4220	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9713AA_AS	NODE_42_length_9108_cov_5.60345_ID_83	4275	4790	qacH_1	77-592/945	54.6	92.64	FJ172381

ESC_FA9713AA_AS	NODE_42_length_9108_cov_5.60345_ID_83	6148	8067	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9713AA_AS	NODE_45_length_7695_cov_6.47837_ID_89	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9713AA_AS	NODE_45_length_7695_cov_6.47837_ID_89	2878	3753	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9713AA_AS	NODE_49_length_6160_cov_6.43013_ID_97	1101	1757	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9713AA_AS	NODE_50_length_5670_cov_7.04805_ID_99	3303	4516	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9713AA_AS	NODE_51_length_5489_cov_16.6791_ID_101	3263	4509	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9713AA_AS	NODE_57_length_4778_cov_13.5213_ID_113	205	1041	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9713AA_AS	NODE_57_length_4778_cov_13.5213_ID_113	1041	1844	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9713AA_AS	NODE_57_length_4778_cov_13.5213_ID_113	1905	2720	sul2_2	1-816/816	100	100	AY034138
ESC_FA9713AA_AS	NODE_79_length_1497_cov_22.9827_ID_157	349	1164	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9713AA_AS	NODE_84_length_1393_cov_5.43281_ID_167	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9713AA_AS	NODE_85_length_1356_cov_8.54787_ID_169	1	167	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA9713AA_AS	NODE_91_length_1065_cov_13.5798_ID_181	58	918	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9713AA_AS	NODE_94_length_984_cov_14.0402_ID_187	1	114	ant(3")-Ia_1	62-175/972	11.73	100	X02340
ESC_FA9713AA_AS	NODE_94_length_984_cov_14.0402_ID_187	121	603	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9714AA_AS	NODE_129_length_282_cov_33.4438_ID_257	227	282	blaTEM-102_1	1-56/861	6.5	100	AY040093

ESC_FA9714AA_AS	NODE_39_length_10285_cov_9.66319_ID_77	1073	1729	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9714AA_AS	NODE_3_length_359986_cov_9.24147_ID_5	34067	37511	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9714AA_AS	NODE_40_length_9108_cov_5.18066_ID_79	1	122	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9714AA_AS	NODE_40_length_9108_cov_5.18066_ID_79	119	616	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9714AA_AS	NODE_40_length_9108_cov_5.18066_ID_79	1014	1815	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9714AA_AS	NODE_40_length_9108_cov_5.18066_ID_79	2077	3336	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9714AA_AS	NODE_40_length_9108_cov_5.18066_ID_79	3419	4220	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9714AA_AS	NODE_40_length_9108_cov_5.18066_ID_79	4275	4790	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9714AA_AS	NODE_40_length_9108_cov_5.18066_ID_79	6148	8067	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9714AA_AS	NODE_42_length_7695_cov_6.16355_ID_83	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9714AA_AS	NODE_42_length_7695_cov_6.16355_ID_83	2878	3753	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9714AA_AS	NODE_47_length_5642_cov_6.11521_ID_93	3303	4516	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9714AA_AS	NODE_49_length_5513_cov_14.8367_ID_97	3287	4533	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9714AA_AS	NODE_51_length_4778_cov_12.725_ID_101	2059	2874	sul2_2	1-816/816	100	100	AY034138
ESC_FA9714AA_AS	NODE_51_length_4778_cov_12.725_ID_101	2935	3738	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9714AA_AS	NODE_51_length_4778_cov_12.725_ID_101	3738	4574	aph(6)-Id_1	1-837/837	100	100	M28829

ESC_FA9714AA_AS	NODE_76_length_1586_cov_5.50577_ID_151	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9714AA_AS	NODE_78_length_1480_cov_21.9781_ID_155	334	1149	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9714AA_AS	NODE_80_length_1356_cov_7.39823_ID_159	1	167	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA9714AA_AS	NODE_88_length_1065_cov_13.5683_ID_175	58	918	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9714AA_AS	NODE_93_length_956_cov_10.0297_ID_185	354	836	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9714AA_AS	NODE_93_length_956_cov_10.0297_ID_185	843	956	ant(3")-Ia_1	62-175/972	11.73	100	X02340
ESC_FA9780AA_AS	NODE_103_length_1150_cov_20.3954_ID_205	33	848	sul2_2	1-816/816	100	100	AY034138
ESC_FA9780AA_AS	NODE_109_length_1065_cov_19.8004_ID_217	58	918	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9780AA_AS	NODE_119_length_899_cov_8.69975_ID_237	1	114	ant(3")-Ia_1	62-175/972	11.73	100	X02340
ESC_FA9780AA_AS	NODE_119_length_899_cov_8.69975_ID_237	121	603	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9780AA_AS	NODE_147_length_523_cov_5.74146_ID_293	1	81	sul2_1	736-816/816	9.93	100	AF542061
ESC_FA9780AA_AS	NODE_158_length_309_cov_45.4694_ID_315	205	309	aph(6)-Id_1	733-837/837	12.54	100	M28829
ESC_FA9780AA_AS	NODE_176_length_223_cov_12.1727_ID_351	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9780AA_AS	NODE_3_length_359882_cov_8.87323_ID_5	34066	37510	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9780AA_AS	NODE_42_length_9108_cov_8.20967_ID_83	1	122	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9780AA_AS	NODE_42_length_9108_cov_8.20967_ID_83	119	616	dfrA12_8	1-498/498	100	100	AM040708

ESC_FA9780AA_AS	NODE_42_length_9108_cov_8.20967_ID_83	1014	1815	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9780AA_AS	NODE_42_length_9108_cov_8.20967_ID_83	2077	3336	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9780AA_AS	NODE_42_length_9108_cov_8.20967_ID_83	3419	4220	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9780AA_AS	NODE_42_length_9108_cov_8.20967_ID_83	4275	4790	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9780AA_AS	NODE_42_length_9108_cov_8.20967_ID_83	6148	8067	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9780AA_AS	NODE_48_length_6075_cov_8.14374_ID_95	1016	1672	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9780AA_AS	NODE_49_length_5796_cov_13.8115_ID_97	4648	5463	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9780AA_AS	NODE_51_length_5751_cov_8.92746_ID_101	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9780AA_AS	NODE_51_length_5751_cov_8.92746_ID_101	2878	3753	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9780AA_AS	NODE_61_length_3443_cov_7.05766_ID_121	1221	2434	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9780AA_AS	NODE_68_length_2927_cov_21.9918_ID_135	981	2227	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9780AA_AS	NODE_69_length_2828_cov_15.2368_ID_137	1	81	sul2_1	736-816/816	9.93	100	AF542061
ESC_FA9780AA_AS	NODE_69_length_2828_cov_15.2368_ID_137	142	945	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9780AA_AS	NODE_69_length_2828_cov_15.2368_ID_137	945	1781	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9780AA_AS	NODE_74_length_2125_cov_11.8479_ID_147	1	70	sul2_1	1-70/816	8.58	100	AF542061
ESC_FA9780AA_AS	NODE_93_length_1356_cov_11.9381_ID_185	1	167	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340

ESC_FA9781AA_AS	NODE_136_length_310_cov_46.4975_ID_271	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9781AA_AS	NODE_3_length_359986_cov_9.12305_ID_5	34067	37511	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9781AA_AS	NODE_42_length_9232_cov_8.70896_ID_83	733	1593	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9781AA_AS	NODE_42_length_9232_cov_8.70896_ID_83	4415	5290	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9781AA_AS	NODE_43_length_9108_cov_5.79177_ID_85	1	122	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9781AA_AS	NODE_43_length_9108_cov_5.79177_ID_85	119	616	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9781AA_AS	NODE_43_length_9108_cov_5.79177_ID_85	1014	1815	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9781AA_AS	NODE_43_length_9108_cov_5.79177_ID_85	2077	3336	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9781AA_AS	NODE_43_length_9108_cov_5.79177_ID_85	3419	4220	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9781AA_AS	NODE_43_length_9108_cov_5.79177_ID_85	4275	4790	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9781AA_AS	NODE_43_length_9108_cov_5.79177_ID_85	6148	8067	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9781AA_AS	NODE_50_length_6160_cov_7.08996_ID_99	1101	1757	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9781AA_AS	NODE_51_length_5647_cov_7.6198_ID_101	1155	2368	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9781AA_AS	NODE_52_length_5489_cov_17.6793_ID_103	3263	4509	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9781AA_AS	NODE_57_length_4778_cov_13.9573_ID_113	2059	2874	sul2_2	1-816/816	100	99.88	AY034138
ESC_FA9781AA_AS	NODE_57_length_4778_cov_13.9573_ID_113	2935	3738	aph(3")-Ib_5	1-804/804	100	100	AF321551

ESC_FA9781AA_AS	NODE_57_length_4778_cov_13.9573_ID_113	3738	4574	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9781AA_AS	NODE_67_length_1987_cov_26.3319_ID_133	334	1149	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9781AA_AS	NODE_77_length_1482_cov_5.86413_ID_153	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9781AA_AS	NODE_84_length_1356_cov_9.99195_ID_167	1190	1356	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA9781AA_AS	NODE_96_length_984_cov_14.2997_ID_191	1	114	ant(3")-Ia_1	62-175/972	11.73	100	X02340
ESC_FA9781AA_AS	NODE_96_length_984_cov_14.2997_ID_191	121	603	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9782AA_AS	NODE_124_length_310_cov_44.9036_ID_247	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9782AA_AS	NODE_16_length_100528_cov_8.88583_ID_31	98302	99548	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9782AA_AS	NODE_3_length_359985_cov_9.76246_ID_5	34067	37511	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9782AA_AS	NODE_41_length_9232_cov_7.99528_ID_81	733	1593	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9782AA_AS	NODE_41_length_9232_cov_7.99528_ID_81	4415	5290	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9782AA_AS	NODE_42_length_9101_cov_5.07488_ID_83	1	115	ant(3")-Ia_1	69-184/972	11.83	97.41	X02340
ESC_FA9782AA_AS	NODE_42_length_9101_cov_5.07488_ID_83	112	609	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9782AA_AS	NODE_42_length_9101_cov_5.07488_ID_83	1007	1808	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9782AA_AS	NODE_42_length_9101_cov_5.07488_ID_83	2070	3329	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9782AA_AS	NODE_42_length_9101_cov_5.07488_ID_83	3412	4213	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340

ESC_FA9782AA_AS	NODE_42_length_9101_cov_5.07488_ID_83	4268	4783	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9782AA_AS	NODE_42_length_9101_cov_5.07488_ID_83	6141	8060	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9782AA_AS	NODE_45_length_6160_cov_6.01736_ID_89	1101	1757	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9782AA_AS	NODE_46_length_5670_cov_6.78496_ID_91	3303	4516	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9782AA_AS	NODE_50_length_4778_cov_13.6034_ID_99	205	1041	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9782AA_AS	NODE_50_length_4778_cov_13.6034_ID_99	1041	1844	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9782AA_AS	NODE_50_length_4778_cov_13.6034_ID_99	1905	2720	sul2_2	1-816/816	100	99.88	AY034138
ESC_FA9782AA_AS	NODE_55_length_3674_cov_8.07526_ID_109	2637	2804	ant(3")-Ia_1	1-175/972	17.28	96	X02340
ESC_FA9782AA_AS	NODE_55_length_3674_cov_8.07526_ID_109	2811	3293	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9782AA_AS	NODE_64_length_2150_cov_8.74718_ID_127	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9782AA_AS	NODE_73_length_1497_cov_21.5491_ID_145	334	1149	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9783AA_AS	NODE_108_length_310_cov_44.0711_ID_215	255	310	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9783AA_AS	NODE_41_length_9108_cov_5.45625_ID_81	1	122	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9783AA_AS	NODE_41_length_9108_cov_5.45625_ID_81	119	616	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9783AA_AS	NODE_41_length_9108_cov_5.45625_ID_81	1014	1815	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9783AA_AS	NODE_41_length_9108_cov_5.45625_ID_81	2077	3336	cmlA1_1	1-1260/1260	100	99.92	M64556

ESC_FA9783AA_AS	NODE_41_length_9108_cov_5.45625_ID_81	3419	4220	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9783AA_AS	NODE_41_length_9108_cov_5.45625_ID_81	4275	4790	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9783AA_AS	NODE_41_length_9108_cov_5.45625_ID_81	6148	8067	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9783AA_AS	NODE_43_length_7695_cov_6.63994_ID_85	1	56	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FA9783AA_AS	NODE_43_length_7695_cov_6.63994_ID_85	2878	3753	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9783AA_AS	NODE_46_length_6160_cov_6.4771_ID_91	1101	1757	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9783AA_AS	NODE_47_length_5670_cov_7.01512_ID_93	3303	4516	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9783AA_AS	NODE_48_length_5489_cov_17.0381_ID_95	3263	4509	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9783AA_AS	NODE_4_length_358086_cov_9.28424_ID_7	34067	37511	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9783AA_AS	NODE_56_length_3255_cov_15.1617_ID_111	536	1351	sul2_2	1-816/816	100	99.88	AY034138
ESC_FA9783AA_AS	NODE_56_length_3255_cov_15.1617_ID_111	1412	2215	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9783AA_AS	NODE_56_length_3255_cov_15.1617_ID_111	2215	3051	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9783AA_AS	NODE_69_length_1586_cov_5.37203_ID_137	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9783AA_AS	NODE_71_length_1480_cov_23.4236_ID_141	334	1149	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9783AA_AS	NODE_76_length_1356_cov_8.0716_ID_151	1	167	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA9783AA_AS	NODE_80_length_1065_cov_15.1103_ID_159	58	918	blaTEM-1B_1	1-861/861	100	100	AY458016

ESC_FA9783AA_AS	NODE_84_length_984_cov_13.9288_ID_167	1	114	ant(3")-Ia_1	62-175/972	11.73	100	X02340
ESC_FA9783AA_AS	NODE_84_length_984_cov_13.9288_ID_167	121	603	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9785AA_AS	NODE_109_length_394_cov_5.55516_ID_217	1	81	sul2_1	736-816/816	9.93	100	AF542061
ESC_FA9785AA_AS	NODE_3_length_359986_cov_9.57287_ID_5	34067	37511	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9785AA_AS	NODE_41_length_11153_cov_8.33288_ID_81	1737	2983	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9785AA_AS	NODE_41_length_11153_cov_8.33288_ID_81	4598	5458	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9785AA_AS	NODE_41_length_11153_cov_8.33288_ID_81	8280	9155	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9785AA_AS	NODE_42_length_9108_cov_6.29683_ID_83	1	122	ant(3")-Ia_1	62-184/972	12.55	97.56	X02340
ESC_FA9785AA_AS	NODE_42_length_9108_cov_6.29683_ID_83	119	616	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9785AA_AS	NODE_42_length_9108_cov_6.29683_ID_83	1014	1815	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FA9785AA_AS	NODE_42_length_9108_cov_6.29683_ID_83	2077	3336	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9785AA_AS	NODE_42_length_9108_cov_6.29683_ID_83	3419	4220	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9785AA_AS	NODE_42_length_9108_cov_6.29683_ID_83	4275	4790	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9785AA_AS	NODE_42_length_9108_cov_6.29683_ID_83	6148	8067	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9785AA_AS	NODE_47_length_6160_cov_6.71308_ID_93	1101	1757	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9785AA_AS	NODE_50_length_4948_cov_6.20207_ID_99	3303	4516	floR_2	1-1214/1215	99.92	98.11	AF118107

ESC_FA9785AA_AS	NODE_62_length_2432_cov_8.25054_ID_123	1	81	sul2_1	736-816/816	9.93	100	AF542061
ESC_FA9785AA_AS	NODE_62_length_2432_cov_8.25054_ID_123	142	945	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9785AA_AS	NODE_62_length_2432_cov_8.25054_ID_123	945	1781	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9785AA_AS	NODE_63_length_2153_cov_5.75245_ID_125	2059	2153	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9785AA_AS	NODE_64_length_2150_cov_8.56259_ID_127	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9785AA_AS	NODE_66_length_2015_cov_15.1909_ID_131	334	1149	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9785AA_AS	NODE_77_length_1356_cov_10.0201_ID_153	1190	1356	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_FA9785AA_AS	NODE_88_length_984_cov_12.4363_ID_175	1	114	ant(3")-Ia_1	62-175/972	11.73	100	X02340
ESC_FA9785AA_AS	NODE_88_length_984_cov_12.4363_ID_175	121	603	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9785AA_AS	NODE_96_length_866_cov_12.0611_ID_191	19	834	sul2_2	1-816/816	100	100	AY034138
ESC_FA9793AA_AS	NODE_39_length_9232_cov_7.8842_ID_77	733	1593	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_FA9793AA_AS	NODE_39_length_9232_cov_7.8842_ID_77	4415	5290	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_FA9793AA_AS	NODE_3_length_359986_cov_9.71074_ID_5	34067	37511	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_FA9793AA_AS	NODE_40_length_9091_cov_4.98385_ID_79	1	105	ant(3")-Ia_1	79-184/972	10.8	97.17	X02340
ESC_FA9793AA_AS	NODE_40_length_9091_cov_4.98385_ID_79	102	599	dfrA12_8	1-498/498	100	100	AM040708
ESC_FA9793AA_AS	NODE_40_length_9091_cov_4.98385_ID_79	997	1798	aadA2_1	18-819/819	97.92	99.88	NC_010870

ESC_FA9793AA_AS	NODE_40_length_9091_cov_4.98385_ID_79	2060	3319	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_FA9793AA_AS	NODE_40_length_9091_cov_4.98385_ID_79	3402	4203	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_FA9793AA_AS	NODE_40_length_9091_cov_4.98385_ID_79	4258	4773	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_FA9793AA_AS	NODE_40_length_9091_cov_4.98385_ID_79	6131	8050	tet(M)_8	1-1920/1920	100	96.15	X04388
ESC_FA9793AA_AS	NODE_46_length_5670_cov_6.13766_ID_91	1155	2368	floR_2	1-1214/1215	99.92	98.11	AF118107
ESC_FA9793AA_AS	NODE_47_length_5654_cov_6.01733_ID_93	1101	1757	qnrS1_1	1-657/657	100	100	AB187515
ESC_FA9793AA_AS	NODE_49_length_5489_cov_15.4807_ID_97	3263	4509	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FA9793AA_AS	NODE_52_length_4778_cov_13.5057_ID_103	205	1041	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_FA9793AA_AS	NODE_52_length_4778_cov_13.5057_ID_103	1041	1844	aph(3")-Ib_5	1-804/804	100	100	AF321551
ESC_FA9793AA_AS	NODE_52_length_4778_cov_13.5057_ID_103	1905	2720	sul2_2	1-816/816	100	99.88	AY034138
ESC_FA9793AA_AS	NODE_59_length_3674_cov_7.58214_ID_117	2637	2804	ant(3")-Ia_1	1-175/972	17.28	96	X02340
ESC_FA9793AA_AS	NODE_59_length_3674_cov_7.58214_ID_117	2811	3293	dfrA14_5	1-483/483	100	99.59	DQ388123
ESC_FA9793AA_AS	NODE_69_length_1509_cov_4.92335_ID_137	1	95	sul2_1	1-95/816	11.64	100	AF542061
ESC_FA9793AA_AS	NODE_70_length_1497_cov_22.4913_ID_139	334	1149	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_FA9793AA_AS	NODE_97_length_310_cov_43.2843_ID_193	255	310	blaTEM-102_1	1-56/861	6.5	100	AY040093
ESC_FB0845AA_AS	NODE_3_length_260238_cov_41.431008	34454	37898	sitABCD_1	6-3459/3459	99.6	97.48	AY598030

ESC_FB0845AA_AS	NODE_5_length_200985_cov_42.196502	32053	33299	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FB0845AA_AS	NODE_5_length_200985_cov_42.196502	39557	39732	ant(3")-Ia_1	1-184/972	18.11	94.56	X02340
ESC_FB0845AA_AS	NODE_5_length_200985_cov_42.196502	39729	40226	dfrA12_8	1-498/498	100	100	AM040708
ESC_FB0845AA_AS	NODE_5_length_200985_cov_42.196502	40624	41425	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FB0845AA_AS	NODE_5_length_200985_cov_42.196502	41589	41873	qacE_1	1-285/333	85.59	99.65	X68232
ESC_FB0845AA_AS	NODE_5_length_200985_cov_42.196502	41903	42769	sul1_5	1-867/867	100	99.89	EU780013
ESC_FB0845AA_AS	NODE_5_length_200985_cov_42.196502	47133	47948	sul2_2	1-816/816	100	100	AY034138
ESC_FB4150AA_AS	NODE_1_length_397603_cov_41.852446	349656	350471	sul2_2	1-816/816	100	100	AY034138
ESC_FB4150AA_AS	NODE_1_length_397603_cov_41.852446	354835	355701	sul1_5	1-867/867	100	99.89	EU780013
ESC_FB4150AA_AS	NODE_1_length_397603_cov_41.852446	355731	356015	qacE_1	1-285/333	85.59	99.65	X68232
ESC_FB4150AA_AS	NODE_1_length_397603_cov_41.852446	356179	356980	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_FB4150AA_AS	NODE_1_length_397603_cov_41.852446	357378	357875	dfrA12_8	1-498/498	100	100	AM040708
ESC_FB4150AA_AS	NODE_1_length_397603_cov_41.852446	357872	358047	ant(3")-Ia_1	1-184/972	18.11	94.56	X02340
ESC_FB4150AA_AS	NODE_1_length_397603_cov_41.852446	364305	365551	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_FB4150AA_AS	NODE_23_length_71683_cov_40.568142	34454	37898	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_GA5264AA_AS	NODE_32_length_38202_cov_8.99475_ID_63	34336	37780	sitABCD_1	6-3459/3459	99.6	97.48	AY598030

ESC_GA5264AA_AS	NODE_33_length_34801_cov_9.69982_ID_65	21828	23074	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_GB2725AA_AS	NODE_17_length_111824_cov_34.451194	48966	49832	sul1_5	1-867/867	100	99.89	EU780013
ESC_GB2725AA_AS	NODE_17_length_111824_cov_34.451194	49862	50146	qacE_1	1-285/333	85.59	99.65	X68232
ESC_GB2725AA_AS	NODE_17_length_111824_cov_34.451194	55805	56707	aac(3)-VIa_2	1-903/903	100	100	NC_009838
ESC_GB2725AA_AS	NODE_17_length_111824_cov_34.451194	56871	57838	ant(3")-Ia_1	1-972/972	99.59	99.38	X02340
ESC_GB2725AA_AS	NODE_17_length_111824_cov_34.451194	64096	65342	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_GB2725AA_AS	NODE_36_length_37769_cov_32.450906	33865	37309	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_GB2725AA_AS	NODE_53_length_12560_cov_57.905336	495	1355	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_GB2725AA_AS	NODE_79_length_3148_cov_66.090382	1	394	aph(6)-Id_1	1-394/837	47.07	100	M28829
ESC_GB2725AA_AS	NODE_79_length_3148_cov_66.090382	394	1196	aph(3")-Ib_2	2-804/804	99.88	100	AF024602
ESC_HA2283AA_AS	NODE_103_length_6485_cov_6.18121_ID_205	344	1204	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_HA2283AA_AS	NODE_105_length_6283_cov_5.90491_ID_209	4377	5213	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_HA2283AA_AS	NODE_105_length_6283_cov_5.90491_ID_209	5213	6015	aph(3")-Ib_2	2-804/804	99.88	100	AF024602
ESC_HA2283AA_AS	NODE_116_length_5461_cov_7.17033_ID_231	92	1297	tet(B)_1	1-1206/1206	100	100	AP000342
ESC_HA2283AA_AS	NODE_43_length_37731_cov_5.14306_ID_85	33748	37192	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_HA4055AA_AS	NODE_114_length_8441_cov_4.78149_ID_227	2036	2872	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_HA4055AA_AS	NODE_114_length_8441_cov_4.78149_ID_227	2872	3675	aph(3")-Ib_5	1-804/804	100	100	AF321551

ESC_HA4055AA_AS	NODE_114_length_8441_cov_4.78149_ID_227	3736	4551	sul2_2	1-816/816	100	100	AY034138
ESC_HA7286AA_AS	NODE_38_length_38278_cov_25.7822_ID_75	461	3905	sitABCD_1	6-3459/3459	99.6	97.45	AY598030
ESC_HA7286AA_AS	NODE_58_length_7153_cov_23.1467_ID_115	356	1171	aph(3')-Ia_3	1-816/816	100	99.88	EF015636
ESC_HA7286AA_AS	NODE_59_length_6824_cov_18.9715_ID_117	4286	5491	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_HA7286AA_AS	NODE_60_length_6816_cov_36.9704_ID_119	313	1173	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_HA7286AA_AS	NODE_62_length_6196_cov_551.51_ID_123	3130	3945	sul2_2	1-816/816	100	100	AY034138
ESC_HA7286AA_AS	NODE_74_length_1925_cov_71.8293_ID_147	108	944	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_HA7286AA_AS	NODE_74_length_1925_cov_71.8293_ID_147	944	1746	aph(3")-Ib_2	2-804/804	99.88	100	AF024602
ESC_HA8256AA_AS	NODE_129_length_325_cov_151.523	1	325	blaTEM-104_1	422-746/861	37.75	100	AF516719
ESC_HA8256AA_AS	NODE_133_length_311_cov_225.165	1	311	blaTEM-104_1	221-531/861	36.12	100	AF516719
ESC_HA8256AA_AS	NODE_363_length_117_cov_192.5	1	117	blaTEM-101_1	215-331/861	13.59	100	AF495873
ESC_HA8256AA_AS	NODE_36_length_38163_cov_13.9124	362	3806	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_HA8256AA_AS	NODE_436_length_112_cov_157	1	112	blaTEM-102_1	421-532/861	13.01	100	AY040093
ESC_HA8256AA_AS	NODE_437_length_112_cov_171	1	112	blaTEM-104_1	605-716/861	13.01	100	AF516719
ESC_HA8256AA_AS	NODE_55_length_10562_cov_12.8478	6558	7394	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_HA8256AA_AS	NODE_55_length_10562_cov_12.8478	7394	8196	aph(3")-Ib_2	2-804/804	99.88	100	AF024602
ESC_HA8256AA_AS	NODE_63_length_6651_cov_9.12999	1288	2493	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_HA8256AA_AS	NODE_81_length_1907_cov_115.808	610	1425	aph(3')-Ia_9	1-816/816	100	100	EU722351
ESC_HA8256AA_AS	NODE_81_length_1907_cov_115.808	1652	1907	blaTEM-105_1	606-861/861	29.73	100	AF516720
ESC_HA8256AA_AS	NODE_87_length_1370_cov_119.831	1046	1370	blaTEM-104_1	1-325/861	37.75	100	AF516719
ESC_HA9080AA_AS	NODE_24_length_75868_cov_5.22251	7890	8756	sul1_5	1-867/867	100	99.89	EU780013
ESC_HA9080AA_AS	NODE_24_length_75868_cov_5.22251	8786	9070	qacE_1	1-285/333	85.59	99.65	X68232
ESC_HA9080AA_AS	NODE_24_length_75868_cov_5.22251	14729	15631	aac(3)-VIa_2	1-903/903	100	100	NC_009838
ESC_HA9080AA_AS	NODE_24_length_75868_cov_5.22251	15795	16759	ant(3")-Ia_1	1-972/972	99.28	99.07	X02340

ESC_HA9080AA_AS	NODE_25_length_72633_cov_5.45224	461	3905	sitABCD_1	6-3459/3459	99.6	97.45	AY598030
ESC_HA9080AA_AS	NODE_46_length_12079_cov_2.88688	11653	12079	sitABCD_1	3033-3459/3459	12.34	100	AY598030
ESC_HA9080AA_AS	NODE_49_length_10057_cov_3.2433	4567	5772	tet(B)_1	1-1206/1206	100	99.92	AP000342
ESC_HA9080AA_AS	NODE_55_length_5141_cov_3.21719	1785	2600	sul2_2	1-816/816	100	100	AY034138
ESC_HA9080AA_AS	NODE_91_length_342_cov_4.28837	4	342	sitABCD_1	2602-2940/3459	9.8	99.7	AY598030
ESC_HA9268AA_AS	NODE_102_length_5347_cov_17.3693	434	1093	catA1_1	1-660/660	100	99.85	V00622
ESC_HA9268AA_AS	NODE_131_length_1565_cov_18.1968	557	1372	aph(3')-Ia_7	1-816/816	100	100	X62115
ESC_HA9268AA_AS	NODE_3_length_175073_cov_15.1127	17855	18730	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_HA9268AA_AS	NODE_3_length_175073_cov_15.1127	23371	24027	qnrS1_1	1-657/657	100	100	AB187515
ESC_HA9268AA_AS	NODE_45_length_37984_cov_15.2879	34080	37524	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	12384	13250	sul1_5	1-867/867	100	99.89	EU780013
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	13280	13564	qacE_1	1-285/333	85.59	99.65	X68232
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	13728	14532	ant(3")-Ia_1	168-972/972	82.82	99.63	X02340
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	14632	15462	blaOXA-1_1	1-831/831	100	100	HQ170510
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	15531	15699	ant(3")-Ia_1	1-173/972	17.39	97.69	X02340
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	21957	23203	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	27508	28344	aph(6)-Id_1	1-837/837	100	100	M28829
ESC_HA9268AA_AS	NODE_55_length_32429_cov_16.9247	28344	29146	aph(3")-Ib_2	2-804/804	99.88	100	AF024602
ESC_HB9080AA_AS	NODE_105_length_13857_cov_1.408886	1185	2398	floR_2	1-1214/1215	99.92	98.02	AF118107
ESC_HB9080AA_AS	NODE_105_length_13857_cov_1.408886	10818	10996	ant(3")-Ia_1	1-184/972	18.42	96.2	X02340
ESC_HB9080AA_AS	NODE_105_length_13857_cov_1.408886	10993	11490	dfrA12_8	1-498/498	100	100	AM040708
ESC_HB9080AA_AS	NODE_105_length_13857_cov_1.408886	11888	12689	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_HB9080AA_AS	NODE_174_length_5733_cov_4.244559	544	1419	blaCTX-M-27_1	1-876/876	100	100	AY156923
ESC_HB9080AA_AS	NODE_176_length_5526_cov_2.406001	1189	2435	tet(A)_6	1-1247/1275	97.8	99.92	AF534183
ESC_HB9080AA_AS	NODE_194_length_4195_cov_1.098574	1281	1796	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_HB9080AA_AS	NODE_194_length_4195_cov_1.098574	1851	2652	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_HB9080AA_AS	NODE_194_length_4195_cov_1.098574	2735	3994	cmlA1_1	1-1260/1260	100	99.84	M64556
ESC_HB9080AA_AS	NODE_230_length_2602_cov_0.968889	168	959	sul3_2	1-792/792	100	100	AJ459418

ESC_HB9080AA_AS	NODE_232_length_2538_cov_0.909166	1314	2129	sul2_2	1-816/816	100	100	AY034138
ESC_HB9080AA_AS	NODE_244_length_2188_cov_3.477923	190	1050	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_HB9080AA_AS	NODE_275_length_1070_cov_1.320255	23	838	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_HB9080AA_AS	NODE_305_length_673_cov_0.998168	154	673	tet(M)_3	1-520/1920	27.08	98.46	U08812
ESC_IA5972AA_AS	NODE_36_length_44043_cov_3.66244	3411	4657	tet(A)_6	1-1247/1275	97.8	99.92	AF534183
ESC_IA5972AA_AS	NODE_36_length_44043_cov_3.66244	6905	7765	blaTEM-106_1	1-861/861	100	99.88	AY101578
ESC_IA5972AA_AS	NODE_36_length_44043_cov_3.66244	10623	11483	aac(3)-IId_1	1-861/861	100	99.88	EU022314
ESC_IA5972AA_AS	NODE_82_length_1824_cov_3.46932	663	1319	qnrS2_1	1-657/657	100	100	DQ485530
ESC_IA9637AA_AS	NODE_35_length_38572_cov_23.5128	34668	38112	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_IA9637AA_AS	NODE_4_length_268445_cov_23.7087	258546	259421	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_IA9637AA_AS	NODE_4_length_268445_cov_23.7087	264062	264718	qnrS1_1	1-657/657	100	100	AB187515
ESC_IB6804AA_AS	NODE_40_length_35770_cov_8.511162	32009	35453	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_IB6804AA_AS	NODE_54_length_6704_cov_7.420521	4145	5350	tet(B)_2	1-1206/1206	100	100	AF326777
ESC_IB8383AA_AS	NODE_29_length_38278_cov_44.893922	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_JA5203AA_AS	NODE_105_length_2004_cov_7.8146	260	1877	mcr-9_1	1-1618/1620	99.88	100	NZ_NAAN01000063.1
ESC_JA5203AA_AS	NODE_22_length_85314_cov_5.95959	652	1527	blaCTX-M-9_1	1-876/876	100	100	AF174129
ESC_JA5203AA_AS	NODE_22_length_85314_cov_5.95959	3800	4666	sul1_5	1-867/867	100	99.89	EU780013
ESC_JA5203AA_AS	NODE_22_length_85314_cov_5.95959	4696	4980	qacE_1	1-285/333	85.59	99.65	X68232
ESC_JA5203AA_AS	NODE_22_length_85314_cov_5.95959	5144	5945	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_JA5203AA_AS	NODE_22_length_85314_cov_5.95959	5993	6526	ant(2")-Ia_1	1-534/534	100	100	X04555
ESC_JA5203AA_AS	NODE_22_length_85314_cov_5.95959	6533	6699	ant(3")-Ia_1	1-174/972	17.18	95.98	X02340
ESC_JB0043AA_AS	NODE_10_length_183498_cov_48.186791	917	2163	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_JB0043AA_AS	NODE_29_length_37984_cov_49.081623	34080	37524	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_JB0043AA_AS	NODE_7_length_220891_cov_48.819654	3728	4384	qnrS1_1	1-657/657	100	100	AB187515
ESC_JB0043AA_AS	NODE_7_length_220891_cov_48.819654	9025	9900	blaCTX-M-15_1	1-876/876	100	100	AY044436

ESC_JB0045AA_AS	NODE_10_length_183473_cov_35.168076	181336	182582	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_JB0045AA_AS	NODE_29_length_37984_cov_35.656206	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_JB0045AA_AS	NODE_7_length_220891_cov_35.402896	210992	211867	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_JB0045AA_AS	NODE_7_length_220891_cov_35.402896	216508	217164	qnrS1_1	1-657/657	100	100	AB187515
ESC_JB5131AA_AS	NODE_28_length_38385_cov_15.372033	569	4013	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_JB8270AA_AS	NODE_34_length_38362_cov_42.006434	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	103	262	ant(3")-Ia_1	9-175/972	16.46	95.81	X02340
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	279	752	dfrA1_8	1-474/474	100	100	X00926
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	1456	1740	qacE_1	1-285/333	85.59	99.65	X68232
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	1770	2636	sul1_5	1-867/867	100	99.89	EU780013
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	4914	5570	qnrA1_1	1-657/657	100	100	AY070235
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	5830	6696	blaCARB-2_1	1-867/867	100	100	M69058
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	6804	7605	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	7769	8053	qacE_1	1-285/333	85.59	99.65	X68232
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	8083	8949	sul1_5	1-867/867	100	99.89	EU780013
ESC_JB8270AA_AS	NODE_44_length_15361_cov_23.551267	14244	15165	mph(A)_2	1-921/921	100	99.67	U36578

ESC_JB8270AA_AS	NODE_49_length_8471_cov_21.466802	884	2158	tet(A)_6	1-1275/1275	100	100	AF534183
ESC_JB8270AA_AS	NODE_49_length_8471_cov_21.466802	2759	3972	floR_2	1-1214/1215	99.92	98.19	AF118107
ESC_JB8270AA_AS	NODE_67_length_1660_cov_37.622962	231	1106	blaCTX-M-27_1	1-876/876	100	100	AY156923
ESC_JB8273AA_AS	NODE_113_length_250_cov_18.243902	124	250	sul1_10	203-329/831	15.28	100	DQ143913
ESC_JB8273AA_AS	NODE_2_length_347663_cov_43.882556	346429	347304	blaCTX-M-32_2	1-876/876	100	100	AJ557142
ESC_JB8273AA_AS	NODE_45_length_9061_cov_44.217820	1	629	sul1_15	212-840/840	74.88	100	EF667294
ESC_JB8273AA_AS	NODE_46_length_8458_cov_18.342576	871	2145	tet(A)_6	1-1275/1275	100	100	AF534183
ESC_JB8273AA_AS	NODE_46_length_8458_cov_18.342576	2746	3959	floR_2	1-1214/1215	99.92	98.19	AF118107
ESC_JB8273AA_AS	NODE_50_length_6409_cov_43.569564	2505	5949	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_JB8273AA_AS	NODE_51_length_6233_cov_17.240255	5117	6038	mph(A)_2	1-921/921	100	99.67	U36578
ESC_JB8273AA_AS	NODE_58_length_3528_cov_46.251397	2384	3351	ant(3")-Ia_1	1-972/972	99.59	99.59	X02340
ESC_JB8273AA_AS	NODE_60_length_3198_cov_958.673396	1550	2194	qnrB19_1	1-645/645	100	100	EU432277
ESC_JB8273AA_AS	NODE_67_length_1324_cov_19.055138	1	117	ant(3")-Ia_1	59-175/972	12.04	100	X02340
ESC_JB8273AA_AS	NODE_67_length_1324_cov_19.055138	134	607	dfrA1_8	1-474/474	100	100	X00926
ESC_JB8273AA_AS	NODE_82_length_792_cov_66.287218	114	398	qacE_1	1-285/333	85.59	99.65	X68232
ESC_JB8273AA_AS	NODE_82_length_792_cov_66.287218	428	792	sul1_5	1-365/867	42.1	100	EU780013
ESC_KB3846AA_AS	NODE_20_length_78500_cov_2.784261	78081	78500	sitABCD_1	3040-3459/3459	12.14	92.86	AY598030
ESC_KB3846AA_AS	NODE_24_length_70508_cov_2.910492	1	3109	sitABCD_1	6-3123/3459	89.88	98.14	AY598030
ESC_LA0878AA_AS	NODE_31_length_37690_cov_6.11708	33786	37230	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_LA0878AA_AS	NODE_42_length_12002_cov_6.59756	3620	3795	ant(3")-Ia_1	1-184/972	18.11	94.56	X02340
ESC_LA0878AA_AS	NODE_42_length_12002_cov_6.59756	3792	4289	dfrA12_8	1-498/498	100	100	AM040708
ESC_LA0878AA_AS	NODE_42_length_12002_cov_6.59756	4687	5488	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_LA0878AA_AS	NODE_42_length_12002_cov_6.59756	5750	7009	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_LA0878AA_AS	NODE_42_length_12002_cov_6.59756	7092	7893	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_LA0878AA_AS	NODE_42_length_12002_cov_6.59756	9064	9855	sul3_2	1-792/792	100	100	AJ459418
ESC_LA0878AA_AS	NODE_42_length_12002_cov_6.59756	11616	11878	mef(B)_1	968-1230/1230	21.38	99.62	FJ196385
ESC_LA0878AA_AS	NODE_45_length_6469_cov_9.35352	4501	5714	floR_2	1-1214/1215	99.92	98.19	AF118107
ESC_LA0878AA_AS	NODE_45_length_6469_cov_9.35352	6315	6469	tet(A)_6	1121-1275/1275	12.16	100	AF534183
ESC_LA0878AA_AS	NODE_49_length_4504_cov_7.83916	1	127	tet(A)_1	299-425/1200	10.58	100	AJ313332

ESC_LA0878AA_AS	NODE_49_length_4504_cov_7.83916	275	1135	blaTEM-1A_1	1-861/861	100	100	HM749966
ESC_LA0878AA_AS	NODE_51_length_4424_cov_8.71585	2779	3639	aac(3)-IId_1	1-861/861	100	99.88	EU022314
ESC_LA0878AA_AS	NODE_55_length_2985_cov_9.81735	784	1440	qnrS1_1	1-657/657	100	100	AB187515
ESC_LA0878AA_AS	NODE_62_length_1730_cov_7.23082	1	155	tet(A)_1	1046-1200/1200	12.92	100	AJ313332
ESC_LA0878AA_AS	NODE_63_length_1606_cov_6.77214	1	500	tet(A)_6	1-500/1275	39.22	99.8	AF534183
ESC_LA0878AA_AS	NODE_75_length_874_cov_12.7831	1	874	tet(A)_3	299-1172/1200	72.83	100	AY196695
ESC_LB2178AA_AS	NZ_CP047658.1	3E+06	3E+06	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_LB2178AA_AS	NZ_CP047659.1	58946	60571	mcr-1.1_1	1-1626/1626	100	100	KP347127
ESC_LB2178AA_AS	NZ_CP047659.1	102223	103144	mph(A)_2	1-921/921	100	99.67	U36578
ESC_LB2178AA_AS	NZ_CP047659.1	123216	124091	blaCTX-M-14_1	1-876/876	100	100	AF252622
ESC_LB2178AA_AS	NZ_CP047659.1	124703	125119	fosA3_1	1-417/417	100	100	AB522970
ESC_LB2178AA_AS	NZ_CP047659.1	127212	127996	aac(3)-IVa_1	1-786/786	99.87	99.75	X01385
ESC_LB2178AA_AS	NZ_CP047659.1	128217	129010	aph(4)-Ia_1	1-794/1026	77.39	100	V01499
ESC_LB2178AA_AS	NZ_CP047659.1	129003	129117	aph(4)-Ia_1	912-1026/1026	11.21	100	V01499
ESC_LB2178AA_AS	NZ_CP047659.1	133964	134779	sul2_2	1-816/816	100	100	AY034138
ESC_LB2178AA_AS	NZ_CP047659.1	137283	138496	floR_2	1-1214/1215	99.92	98.02	AF118107
ESC_LB2178AA_AS	NZ_CP047659.1	148227	148402	ant(3")-Ia_1	1-184/972	18.11	94.56	X02340
ESC_LB2178AA_AS	NZ_CP047659.1	148399	148896	dfrA12_8	1-498/498	100	100	AM040708
ESC_LB2178AA_AS	NZ_CP047659.1	149294	150095	aadA2_1	18-819/819	97.92	99.88	NC_010870
ESC_LB2178AA_AS	NZ_CP047659.1	150357	151616	cmlA1_1	1-1260/1260	100	99.92	M64556
ESC_LB2178AA_AS	NZ_CP047659.1	151699	152500	ant(3")-Ia_1	171-972/972	82.51	99.75	X02340
ESC_LB2178AA_AS	NZ_CP047659.1	152555	153070	qacH_1	77-592/945	54.6	92.64	FJ172381
ESC_LB2178AA_AS	NZ_CP047659.1	154182	154973	sul3_2	1-792/792	100	100	AJ459418
ESC_LB2178AA_AS	NZ_CP047659.1	157674	158489	aph(3')-Ia_1	1-816/816	100	100	V00359
ESC_LB2178AA_AS	NZ_CP047659.1	159939	160805	sul1_5	1-867/867	100	99.89	EU780013
ESC_LB2178AA_AS	NZ_CP047659.1	160835	160979	qacE_1	141-285/333	43.54	99.31	X68232
ESC_MA4137AA_AS	NODE_44_length_37956_cov_3.69231	34066	37510	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6064AA_AS	NODE_11_length_142297_cov_20.383752	9025	9900	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6064AA_AS.result.fasta	NODE_16_length_101288_cov_20.678335	3728	4384	qnrS1_1	1-657/657	100	100	AB187515

ESC_MB6064AA_AS.result.fasta	NODE_20_length_82223_cov_21.888155	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6064AA_AS.result.fasta	NODE_30_length_37984_cov_18.883773	100793	102039	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_MB6065AA_AS	NODE_10_length_142297_cov_11.315657	132398	133273	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6065AA_AS	NODE_26_length_70638_cov_12.278439	137914	138570	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6065AA_AS	NODE_29_length_60903_cov_13.467635	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6065AA_AS	NODE_6_length_202725_cov_10.901347	2776	4022	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_MB6066AA_AS	NODE_12_length_142297_cov_7.867574	9025	9900	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6066AA_AS	NODE_24_length_79046_cov_7.810071	3728	4384	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6066AA_AS	NODE_26_length_70638_cov_7.854562	321867	325311	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6066AA_AS	NODE_6_length_202696_cov_7.481500	2776	4022	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_MB6067AA_AS	NODE_19_length_94380_cov_14.911207	132398	133273	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6067AA_AS	NODE_4_length_359851_cov_14.737493	137914	138570	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6067AA_AS	NODE_7_length_249799_cov_15.699826	322328	325772	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6067AA_AS	NODE_7_length_249799_cov_15.699826	147761	149007	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_MB6185AA_AS	NODE_11_length_146206_cov_17.028806	11230	12105	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6185AA_AS	NODE_19_length_104815_cov_18.441407	5933	6589	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6185AA_AS	NODE_1_length_404810_cov_17.392683	34080	37524	sitABCD_1	6-3459/3459	99.6	97.48	AY598030

ESC_MB6185AA_AS	NODE_24_length_82223_cov_16.891469	2776	4022	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_MB6186AA_AS	NODE_11_length_144502_cov_32.285597	11230	12105	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6186AA_AS	NODE_20_length_94173_cov_30.766200	5933	6589	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6186AA_AS	NODE_22_length_82223_cov_29.901006	322602	326046	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6186AA_AS	NODE_4_length_360125_cov_30.061978	2776	4022	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_MB6189AA_AS	NODE_11_length_144502_cov_31.760270	132398	133273	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6189AA_AS	NODE_22_length_82223_cov_30.898716	137914	138570	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6189AA_AS	NODE_2_length_404807_cov_30.459477	34080	37524	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6190AA_AS	NODE_12_length_144502_cov_32.340371	11230	12105	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6190AA_AS	NODE_23_length_82223_cov_32.759209	5933	6589	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6190AA_AS	NODE_39_length_23181_cov_32.294699	34080	37524	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6190AA_AS	NODE_6_length_206850_cov_31.127814	2776	4022	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_MB6191AA_AS	NODE_11_length_144502_cov_32.759148	132398	133273	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6191AA_AS	NODE_22_length_82223_cov_33.624732	137914	138570	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6191AA_AS	NODE_2_length_404807_cov_31.517960	322402	325846	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6191AA_AS	NODE_4_length_359925_cov_30.057099	2776	4022	tet(A)_6	1-1247/1275	97.8	100	AF534183

ESC_MB6442AA_AS	NODE_11_length_144502_cov_33.866895	11230	12105	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_MB6442AA_AS	NODE_20_length_94173_cov_33.146630	5933	6589	qnrS1_1	1-657/657	100	100	AB187515
ESC_MB6442AA_AS	NODE_22_length_82223_cov_30.482338	322556	326000	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_MB6442AA_AS	NODE_4_length_360306_cov_33.246311	2776	4022	tet(A)_6	1-1247/1275	97.8	100	AF534183
ESC_NB6130AA_AS	NODE_3_length_360044_cov_19.132172	322227	325671	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_NB6130AA_AS	NODE_50_length_5876_cov_63.751957	1583	2398	sul2_2	1-816/816	100	100	AY034138
ESC_NB6130AA_AS	NODE_57_length_5103_cov_14.651326	1408	2268	blaTEM-1B_1	1-861/861	100	100	AY458016
ESC_NB6130AA_AS	NODE_64_length_2835_cov_220.857090	1581	2225	qnrB19_1	1-645/645	100	100	EU432277
ESC_NB6130AA_AS	NODE_70_length_1424_cov_13.644564	319	1194	blaCTX-M-27_1	1-876/876	100	100	AY156923
ESC_NB6134AA_AS	NODE_30_length_38278_cov_18.403082	461	3905	sitABCD_1	6-3459/3459	99.6	97.48	AY598030
ESC_NB6134AA_AS	NODE_40_length_17514_cov_10.531201	196	1117	mph(A)_2	1-921/921	100	99.67	U36578
ESC_NB6134AA_AS	NODE_40_length_17514_cov_10.531201	6412	7278	sul1_5	1-867/867	100	99.89	EU780013
ESC_NB6134AA_AS	NODE_40_length_17514_cov_10.531201	7308	7592	qacE_1	1-285/333	85.59	99.65	X68232
ESC_NB6134AA_AS	NODE_40_length_17514_cov_10.531201	8296	8769	dfrA1_8	1-474/474	100	100	X00926
ESC_NB6134AA_AS	NODE_40_length_17514_cov_10.531201	8786	8945	ant(3")-Ia_1	9-175/972	16.46	95.81	X02340
ESC_NB6134AA_AS	NODE_40_length_17514_cov_10.531201	9927	11201	tet(A)_6	1-1275/1275	100	100	AF534183
ESC_NB6134AA_AS	NODE_40_length_17514_cov_10.531201	11802	13015	floR_2	1-1214/1215	99.92	98.19	AF118107
ESC_NB6134AA_AS	NODE_44_length_7331_cov_9.611466	5389	6264	blaCTX-M-32_2	1-876/876	100	100	AJ557142

ESC_NB6134AA_AS	NODE_52_length_3198_cov_400.183328	1633	2277	qnrB19_1	1-645/645	100	100	EU432277
ESC_RA0679AA_AS	NODE_23_length_64127_cov_8.52366	42114	42989	blaCTX-M-15_1	1-876/876	100	100	AY044436
ESC_RA0679AA_AS	NODE_29_length_38278_cov_13.4797	34374	37818	sitABCD_1	6-3459/3459	99.6	97.48	AY598030

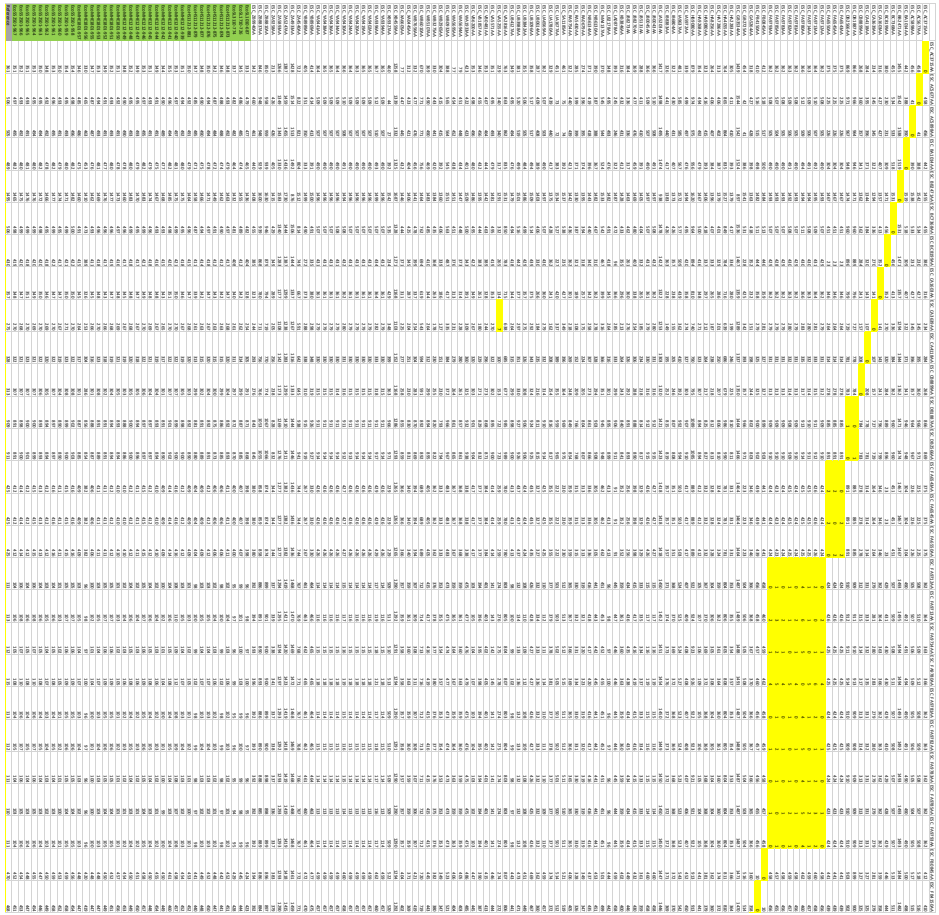
APPENDIX E: THE FULL OUTPUT FROM THE IS*Ecp1* SEARCH OF THE 105 ENTEROBASE ISOLATES

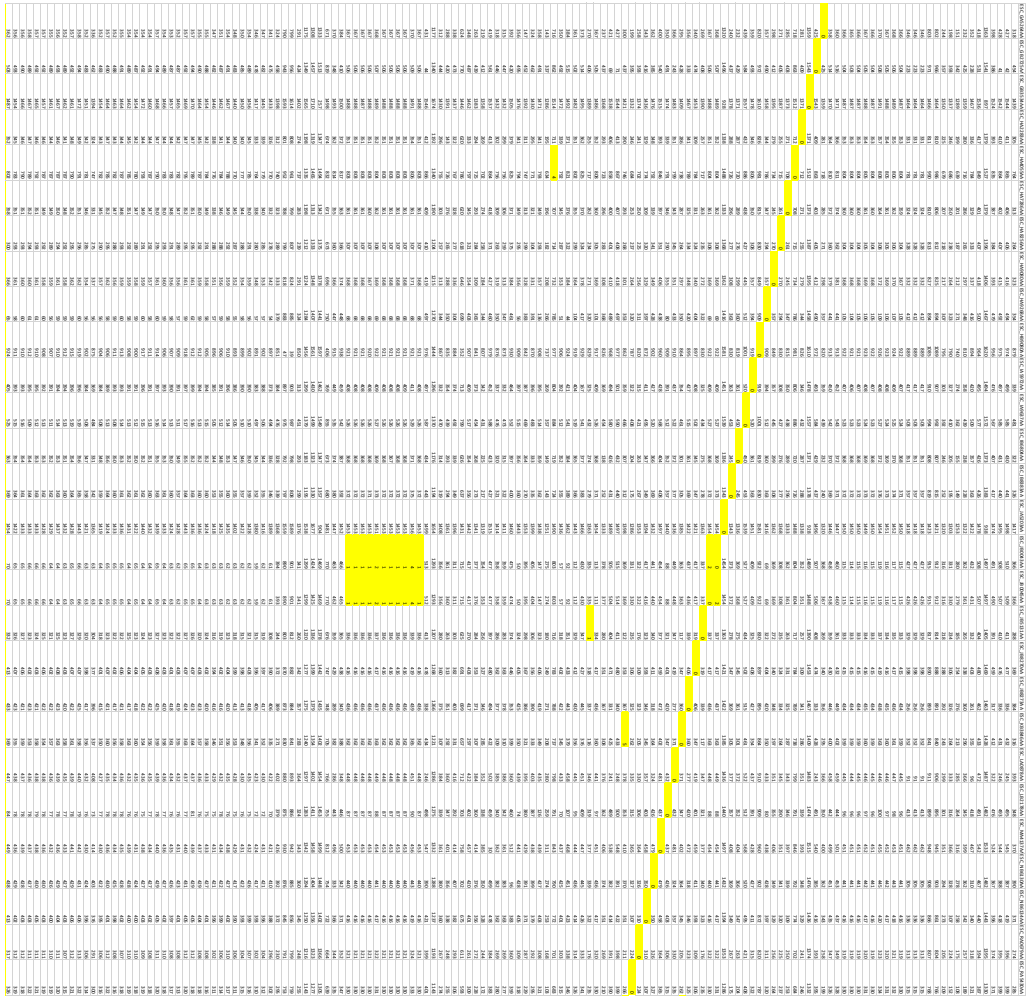
#FILE	SEQUENCE	START	END	STRAND	GENE	COVERAGE	%COVERAGE	%IDENTITY
ESC_BA1014AA_AS	NODE_24_length_69187_cov_8.71222_ID_47	52261	53916	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_BC7368AA_AS	NODE_81_length_14508_cov_10.856825	5758	7412	+	ISEcp1~~~GENE	1-1656/1656	99.94	99.94
ESC_FA9712AA_AS	NODE_44_length_7695_cov_6.94988_ID_87	1174	2829	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9713AA_AS	NODE_45_length_7695_cov_6.47837_ID_89	1174	2829	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9714AA_AS	NODE_42_length_7695_cov_6.16355_ID_83	1174	2829	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9780AA_AS	NODE_51_length_5751_cov_8.92746_ID_101	1174	2829	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9781AA_AS	NODE_42_length_9232_cov_8.70896_ID_83	2711	4366	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9782AA_AS	NODE_41_length_9232_cov_7.99528_ID_81	2711	4366	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9783AA_AS	NODE_43_length_7695_cov_6.63994_ID_85	1174	2829	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9785AA_AS	NODE_41_length_11153_cov_8.33288_ID_81	6576	8231	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_FA9793AA_AS	NODE_39_length_9232_cov_7.8842_ID_77	2711	4366	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_HA9268AA_AS	NODE_3_length_175073_cov_15.1127	16151	17806	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_IA9637AA_AS	NODE_4_length_268445_cov_23.7087	256842	258497	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_JB0043AA_AS	NODE_7_length_220891_cov_48.819654	9949	11604	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_JB0045AA_AS	NODE_7_length_220891_cov_35.402896	209288	210943	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_JB8273AA_AS	NODE_43_length_14907_cov_41.222733	12965	14473	+	ISEcp1~~~GENE	1-1509/1656	91.12	100
ESC_MB6064AA_AS	NODE_11_length_142297_cov_20.383752	9949	11604	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6065AA_AS	NODE_10_length_142297_cov_11.315657	130694	132349	+	ISEcp1~~~GENE	1-1656/1656	100	100

ESC_MB6066AA_AS	NODE_12_length_142297_cov_7.867574	9949	11604	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6067AA_AS	NODE_7_length_249799_cov_15.699826	130694	132349	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6185AA_AS	NODE_12_length_144502_cov_18.161177	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6186AA_AS	NODE_11_length_144502_cov_32.285597	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6189AA_AS	NODE_11_length_144502_cov_31.760270	130694	132349	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6190AA_AS	NODE_12_length_144502_cov_32.340371	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6191AA_AS	NODE_11_length_144502_cov_32.759148	130694	132349	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_MB6442AA_AS	NODE_11_length_144502_cov_33.866895	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_NB6134AA_AS	NODE_44_length_7331_cov_9.611466	2453	3961	+	ISEcp1~~~GENE	1-1509/1656	91.12	100
ESC_RA0679AA_AS	NODE_23_length_64127_cov_8.52366	43038	44693	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_UB3842AA_AS	NODE_43_length_22900_cov_3.362578	9093	10747	+	ISEcp1~~~GENE	1-1656/1656	99.94	99.94
ESC_UB3988AA_AS	NODE_40_length_41731_cov_2.952913	10080	11735	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_UB4127AA_AS	NODE_14_length_124932_cov_11.461840	120341	121996	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_VA5571AA_AS	NODE_53_length_7586_cov_3.200161	2496	4150	+	ISEcp1~~~GENE	1-1656/1656	99.94	99.94
ESC_VB0481AA_AS	NODE_18_length_88840_cov_50.624328	26984	28639	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_VB6200AA_AS	NODE_7_length_223096_cov_26.120990	209288	210943	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_WA1933AA_AS	NODE_102_length_2548_cov_19.163982	123	1450	+	ISEcp1~~~GENE	329-1656/1656	80.19	100
ESC_WB5107AA_AS	NODE_26_length_67502_cov_13.931978	62660	64315	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_WB7656AA_AS	NODE_99_length_6117_cov_5.277629	3597	5251	-	ISEcp1~~~GENE	1-1655/1656	99.94	99.94
ESC_YA9394AA_AS	NODE_7_length_223096_cov_23.785239	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9396AA_AS	NODE_6_length_220891_cov_22.089304	209288	210943	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9402AA_AS	NODE_7_length_223096_cov_47.194206	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9419AA_AS	NODE_7_length_223096_cov_25.335253	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9420AA_AS	NODE_7_length_220891_cov_48.330484	209288	210943	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9424AA_AS	NODE_7_length_220891_cov_18.736470	9949	11604	-	ISEcp1~~~GENE	1-1656/1656	100	100

ESC_YA9430AA_AS	NODE_7_length_223096_cov_18.511529	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9434AA_AS	NODE_31_length_34581_cov_21.481512	9949	11604	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9436AA_AS	NODE_7_length_220890_cov_22.918152	9949	11604	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9438AA_AS	NODE_7_length_223096_cov_22.766909	12154	13809	-	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_YA9441AA_AS	NODE_7_length_220891_cov_33.565550	209288	210943	+	ISEcp1~~~GENE	1-1656/1656	100	100
ESC_ZB9940AA_AS	NODE_23_length_78579_cov_25.416803	29317	30972	+	ISEcp1~~~GENE	1-1656/1656	100	100

APPENDIX F: ENTIRE SNP MATRIX FOR ALL ST2325 EVAL FARMS ISOLATES AND ENTEROBASE ISOLATES





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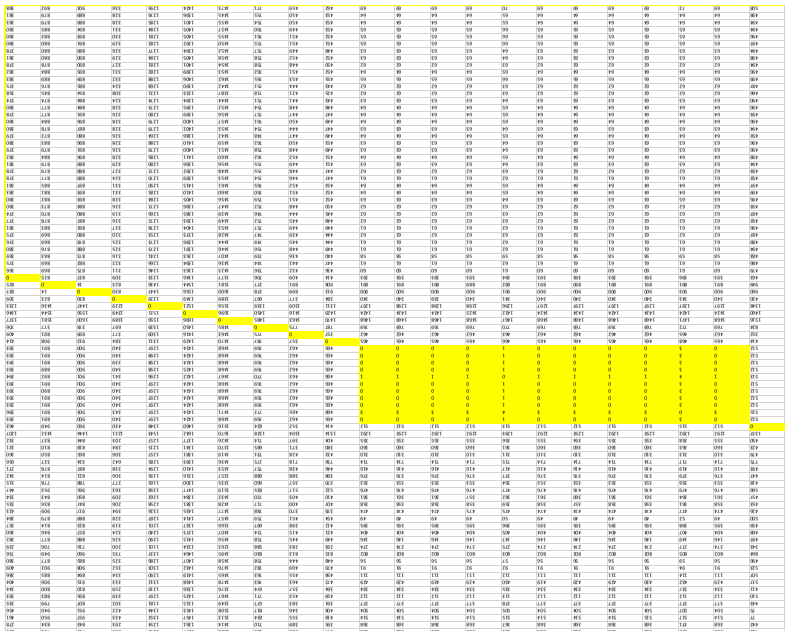

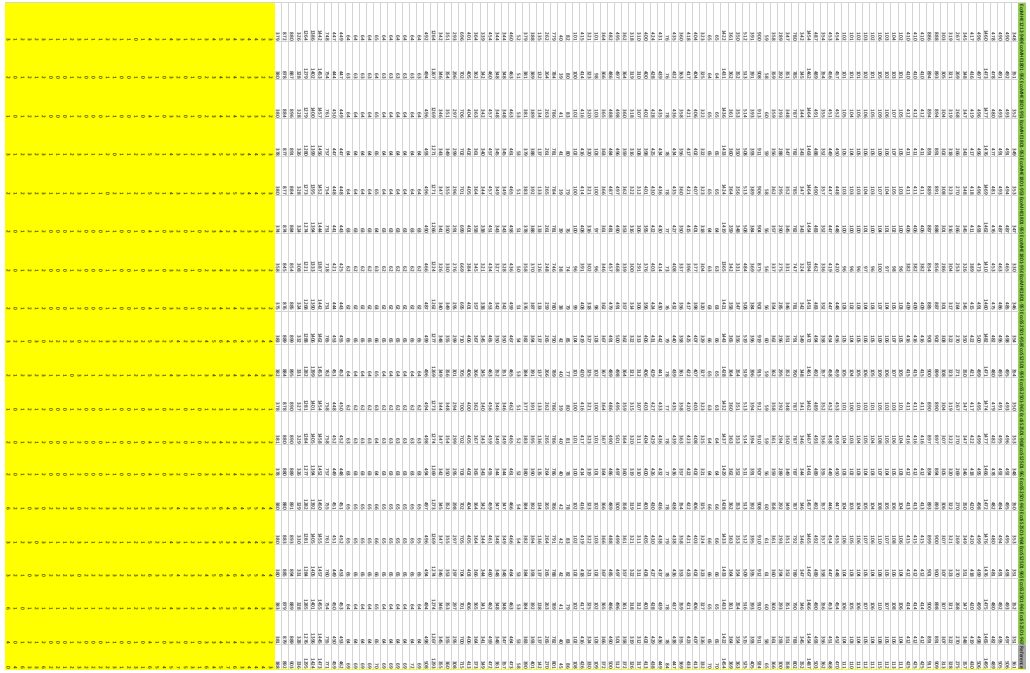

***Footnote for APPENDIX F**: All SNP values <10 are highlighted in yellow. EVAL farms isolates are highlighted in green. Reference is highlighted in grey*